ANTICANCER RESEARCH *25*: 2097-2104 (2005)

The *In Vitro* Effect of Paclitaxel on a LacZ-transfected Malignant Transitional Cell Line

VENDIL VATNE[1], JORUNN LITLEKALSOEY[1],
TORE WENTZEL-LARSEN[2] and JENS HOSTMARK[1]

[1]*Department of Surgery and Department of Pathology and*
[2]*Centre for Clinical Research, Haukeland University Hospital, University of Bergen, Norway*

Abstract. *As bladder cancer is potentially lethal, the development of effective and tolerable therapeutic options is vital. In the present assay, we examined the in vitro effect of paclitaxel (Taxol) on the transitional cell carcinoma (TCC) cell line Hu1703He. Our model has several advantages over other in vitro models. The microenvironment in vivo is mimicked, and the important interaction between benign and malignant cells is consequently preserved in vitro. In addition, the results are not influenced by humoral immune factors. LacZ transfection and exposure to X-gal resulted in blue staining of the tumour cells and made them easy to visualise in sections. Tumour cell aggregates were cultured with continuous paclitaxel exposure to examine the drug's effect on tumour cell migration in monolayer and spheroidal growth in suspension culture. Paclitaxel treatment inhibited both tumour cell migration and spheroidal growth. Invasion was studied by confronting paclitaxel-treated and untreated tumour spheroids with benign bladder fragments in suspension culture. The co-cultures were followed for 4 weeks. Growth of the tumour cells encircling the bladder fragment and cellular infiltration of the bladder stroma were both inhibited by paclitaxel treatment. The expression of MMP-1 in tumour cells was also negatively influenced.*

More than 20% of all bladder tumours are muscle-invasive at diagnosis, either arising *de novo* or progressing from initially superficial tumours. About one-third of the T1 tumours will progress to infiltration of the muscularis propria layer. Surgery may then be insufficient to control the disease, and additional treatment with cytostatica or radiation will be the options. Today, several combinations of cytostatica are available, but more efficacious and less toxic agents are needed (1-3).

During the last decade, agents disturbing the normal dynamics of the microtubules have emerged as important contributions to the available anticancer treatment. One of these, belonging to the taxane-group, is paclitaxel (Taxol). The drug is isolated from the bark of the western yew, *Taxus brevifolia*. Its antitumour activity was demonstrated in rodents as early as 1971 (4). Paclitaxel exerts its effect by binding to the β-tubulin subunit of the microtubules, thus inhibiting the normal tubulin/microtubule interaction and the assembly of microtubules prior to cell division (mitosis). Abnormal binding of microtubule polymers is seen at high concentrations of the drug, while at lower concentrations blocking of mitosis is due to stabilization of spindle microtubuli (5). In addition to this antimitotic effect, the taxanes probably contribute to cytotoxity both by activation of different immunomodulating mechanisms and by induction of programmed cell death (apoptosis) (6-8). Some scientists conclude that abnormal mitotic exit is required for apoptotic cell death to be induced by microtubule-stabilizing drugs (9). Recent findings, though, suggest that paclitaxel-induced apoptosis might occur independently of a phase-(G2+M) arrest (10). In the treatment of superficial and superficially infiltrating bladder cancer (stages T_{is}, T_a and T_1), intravesical instillation of cytostatica is a therapeutic option. Because of the stability of paclitaxel in urine and adequate uptake by the urothelium, the drug may be a future pharmaceutical alternative for intravesical application (3). However, its actual activity against early disease, optimal doses and treatment schedules have not yet been clarified.

Responses to paclitaxel in the treatment of breast, ovarian, testicular, non-small cell lung cancer and transitional cell carcinoma of the urinary bladder have been reported to be satisfactory in several clinical trials (11-13). Head and neck cancer has also been shown to respond well (14). Equally importantly, toxic adverse events of paclitaxel are rare with the exception of neurotoxicity.

Correspondence to: V. Vatne, Department of Surgery and Department of Pathology, Haukeland University Hospital, University of Bergen, Norway, Tel: (47) 55972888, Fax: (47) 55972793, e-mail: vendil.vatne@helse-bergen.no

Key Words: TCC, paclitaxel, MMP, invasion, *in vitro*.

In the present assay, the effect of paclitaxel on the spheroidal growth, cellular migration and the cell cycle of a transitional cell carcinoma (TCC) cell line was estimated. The effect of paclitaxel on co-cultures containing tumour aggregates and benign bladder fragments were also studied. Further, the expression of three matrix metalloproteinases (MMPs), frequently present in TCC tumours, were studied, comparing their expression in co-cultures exposed to paclitaxel with controls.

Materials and Methods

Cell line and cell culture conditions. Cells initiated from a human invasive TCC WHO grade III (Hu1703He), transfected with the *lacZ* gene, were employed in the study (15). The cells were cultured in 80-cm^2 Nunc flasks (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated newborn bovine serum and four times the prescribed concentration of non-essential amino acids, 2% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (complete DMEM). The culture conditions were 37˚C, 100% relative humidity, 95% air and 5% carbon dioxide.

Drug. Paclitaxel (Taxol, Bristol-Myers Squibs Co., Princeton, NJ, USA) was stored at 4˚C. The drug was diluted in complete DMEM to final concentrations of 0.001 μg/ml, 0.01 μg/ml and 0.1 μg/ml 1 h before use.

Tumour cell aggregates (spheroids). Tumour cell spheroids were grown from the *lacZ*-transfected Hu1703He cell line, using the agar overlay suspension technique described in former publications (16). In summary, spheroids were formed by seeding 5x10^6 cells into 20 ml of complete DMEM in 80-cm^2 tissue culture flasks base-coated with agar (Nunc). After 5-10 days in culture, spheroids with a diameter of 300-500 μm were selected for further experiments.

Tumour cell migration. Tumour cell spheroids were individually selected and transferred to uncoated 16-mm multiwell dishes (Nunc) filled with 1 ml of medium. Four treatment groups were established, each with six spheroids. In three of the groups, the spheroids were continuously exposed to three different concentrations of paclitaxel; 0.001 μg/ml, 0.01 μg/ml and 0.1 μg/ml, respectively. The spheroids in the fourth group were used as controls and consequently grown in complete DMEM only. Cell migration was estimated by means of daily phase-contrast microscopy for five days, measuring two orthogonal diameters of the cellular outgrowth area. The migratory capacity of the tumour cells was defined and estimated as the average of the two orthogonal diameters.

Spheroidal growth. Tumour cell spheroids were individually transferred to 16-multiwell dishes filled with 1 ml of complete DMEM and base-coated with 0.5 ml 0.75% agar (Agar Noble, Difco Laboratories, Detroit, MI, USA) in complete DMEM agar. Three groups of spheroids were continuously exposed to paclitaxel of increasing concentrations (0.001 μg/ml, 0.01 μg/ml and 0.1 μg/ml). A fourth group was used as control and exposed to complete DMEM only. There were twelve spheroids in each group. The dishes were incubated at 37˚C. The spheroid size was defined as the average of the two orthogonal diameters and measured by stereomicroscopy every third day in culture.

Viability. Tumour spheroids exposed to 0.1 μg/ml paclitaxel for days were kept in culture in complete DMEM for 4 weeks. The viability of the tumour spheroids was then tested using a live-dead kit (Live/Dead, Viability/Cytotoxicity Assay, Molecular Probes Inc., Eugene, OR, USA). The spheroids were incubated in a solution of 2 μM calcein-AM and 4 μM of ethidium homodimer in PBS at room temperature for 45 min. They were then transferred to 16-mm multiwells containing PBS. Subsequently, the fluorescence was measured in a scanning confocal microscope (Biorad MRC-1000, Hemel Hempstead, UK) using a krypton-argon mixed gas laser. Living cells exhibited green, while dead cells exhibited red fluorescence. Spheroids incubated in complete DMEM only were used as negative controls.

Flow cytometry (FCM). Exponentially growing monolayers of tumour cells were exposed to 0.1 μg/ml of paclitaxel in complete DMEM for 0, 1, 2, 6, 12 and 24 h. At each time-point, the cells were trypsinized, washed in PBS, fixed in 96% ethanol at –20˚C and then stored at 4˚C. Subsequently, the cells were washed in PBS, incubated with 0.5 % pepsin (pH 1.5) (Sigma Chemical Co, St.Louis, MO, USA) for 10 min at 37˚C and washed once in PBS. Two drops of RNAse (1 mg/ml) (Sigma) were added, and staining was performed with propidium iodide (PI 25 μg/ml). The cellular DNA content was measured using a FACScan Flow Cytometer (Becton Dickinson, CA, USA). Human lymphocytes were used as standard diploid (2n) control. Each histogram was obtained by counting a total of 10,000 cells. Cell cycle analysis was performed on the Cellfit analysis system with RFIT for the S-phase.

FCM estimation of paclitaxel effect on apoptosis. Tumour cells in exponentially-growing monolayers were exposed to 0.1 μg/ml of paclitaxel in complete DMEM for 1, 2, 6, 12 and 24 h. The cells were trypsinized, washed in PBS, fixed in methanol for 10 min, in 70% ethanol at –20˚C and ultimately stored at 4˚C. Before DNA measurements, the cells were washed in PBS, incubated in PBS at 37˚C for 20 min and washed once more in PBS. Two drops of RNAse (1 mg/ml) were added, and the cells were stained with PI 5 mg/ml in PBS (17, 18). The cellular DNA content was measured using a FACScan Flow cytometer.

Bladder fragments. The processing of normal bladder fragments is described in detail in former publications (19). In short, cold cup biopsies were taken at cystoscopy, cut into fragments with diameters of 300-450 μm and transferred to Nunc flasks base-coated with 0.75% agar with overlay suspension of complete DMEM. After five days, the bladder fragment spheroids were collected and de-epithelialized with EDTA treatment before confrontation with malignant spheroids.

Co-cultures. Two groups of co-cultures were established; one control group with 48 untreated tumour spheroids, and one experimental group in which the 48 tumour spheroids were exposed to 0.1 μg/ml of paclitaxel for 2 days before being washed five times in complete DMEM. Tumour spheroids with 300-500 μm diameters were collected and individually transferred to 96-multiwell dishes base-coated with agar. Equal-sized, five-day-old and de-epithelialized bladder fragments were added, and each

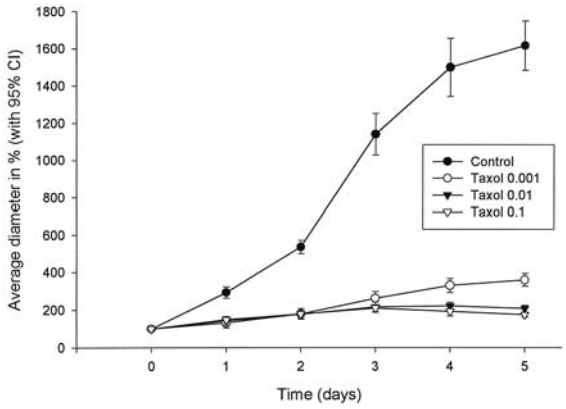

Figure 1. *Migration of tumour cells from spheroids in monolayer, followed for 5 days. Untreated monolayer compared to monolayer continuously exposed to three concentrations of paclitaxel (μg/ml).*

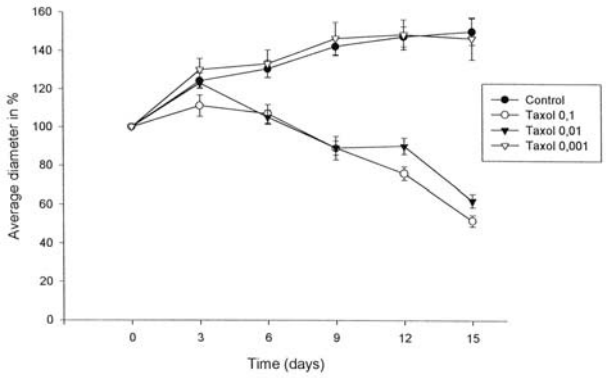

Figure 2. *Growth of tumour spheroids in suspension culture, followed for 15 days. Spheroids cultured in complete DMEM compared to spheroids continuously exposed to three concentrations of paclitaxel (μg/ml).*

well thus contained one tumour spheroid and one bladder fragment. The overlay suspension was 200 μl of complete DMEM. The benign and the malignant spheroids were confronted by bringing them into close contact under a stereomicroscope by means of a sterile syringe. The 48 co-cultures with their 48 parallel controls were then followed in a phase-contrast microscope at close intervals for the first two days (*i.e.* following 2, 4, 6, 9, 12, 16, 19, 24, 30, 40 h), and subsequently 3, 5, 7, 14, 21 and 28 days after confrontation. After 40 h and subsequently 3, 5, 7, 14, 21 and 28 days of culture, four co-cultures and four controls were collected and stained for β-galactosidase activity by overnight exposure to an X-gal solution. They were then photographed and fixed in 2% glutaraldehyde in 0.1 M sucrose adjusted sodium cacodylate buffer (300 ± 25 mOsm). After 24 h, the specimens were washed in the same buffer without glutaraldehyde and post-fixed for 1 h in 10% osmium tetroxide before dehydration by serial exposure to increasing gradients of ethanol (to 96%). Embedment of the co-cultures in Epon 812 was performed using graded mixtures of epon propylenoxide, and the co-cultures were then sectioned.

Eight to twelve sections were obtained from each co-culture. Invasion was scored as (0), (1) and (2). Score (0) corresponded to the presence of encircling tumour cell layers without visible effect on the boundary between the tumour cells and the benign stroma. Score (1) corresponded to visible blurring and degrading of the boundary. Any infiltration of tumour cells in the benign bladder stroma, irrespective of the number, was scored as (2). The highest single score from the available sections was chosen as the score for the actual co-culture. The score for each of the four co-cultures were added, and the respective score sums were compared at each time-point.

Immunostaining. At the same time-points, two of the co-cultures containing paclitaxel-treated tumour spheroids and two with untreated parallels were formalin-fixed and paraffin-embedded, cut into sections of 4 μm and mounted on Dako Chem Mate glass slides (Dako A/S, Denmark). For antigen retrieval, the sections were treated with microwaves 4 x 5 min in citrate buffer (0.01 M, pH 6.0). The sections were then incubated with mouse monoclonal antibodies against MMP-1 (Oncogene Research Products, Cambridge, MA, USA), MMP-2 and MMP-9 (R&D Systems Europe, UK). Standard procedures for indirect immunohistochemical staining using the avidin-biotin peroxidase technique were performed on an automatic Dako Tech Mate 500 machine. Expression of immunostaining was scored as (–), (+), or (++) by two of the authors independently. Score (–) corresponded to no staining, (+) to weak staining of most tumour cells or strong staining of a few cells, and score (++) was given when most tumour cells showed strong staining. From each co-culture 18 to 20 sections were obtained, and the highest single score was given to the actual co-culture. Tissue sections from a colon carcinoma positive for all three MMPs were used as positive controls (score ++), and sections from a colon carcinoma in which the matrix metalloproteinases had been inactivated with IgG were negative controls (score –).

Statistical analyses. Data for cell migration and spheroidal growth were analysed by mixed effects repeated measurement models (20) using the stastical program R (21), with average diameter as dependent variable and time (day), treatment group and initial diameter as covariates. Interaction and time curvature were included when needed. Scores for the invasion in the co-cultures were analysed by Wilcoxon's test for paired samples.

Results

Tumour cell migration. There were no significant differences in migration from the spheroids when treated with the three concentrations of paclitaxel (Figure 1). Compared to the control group, migration was inhibited by paclitaxel, as there was a significantly lower diameter on the first day of treatment (day 1, $p=0.0083$) and a significantly lower slope ($p<0.0001$) for the three groups combined. On the first treatment day, the estimated upward slopes were 30.5 mm per day in the control group and 4.1 mm per day in the combined paclitaxel group.

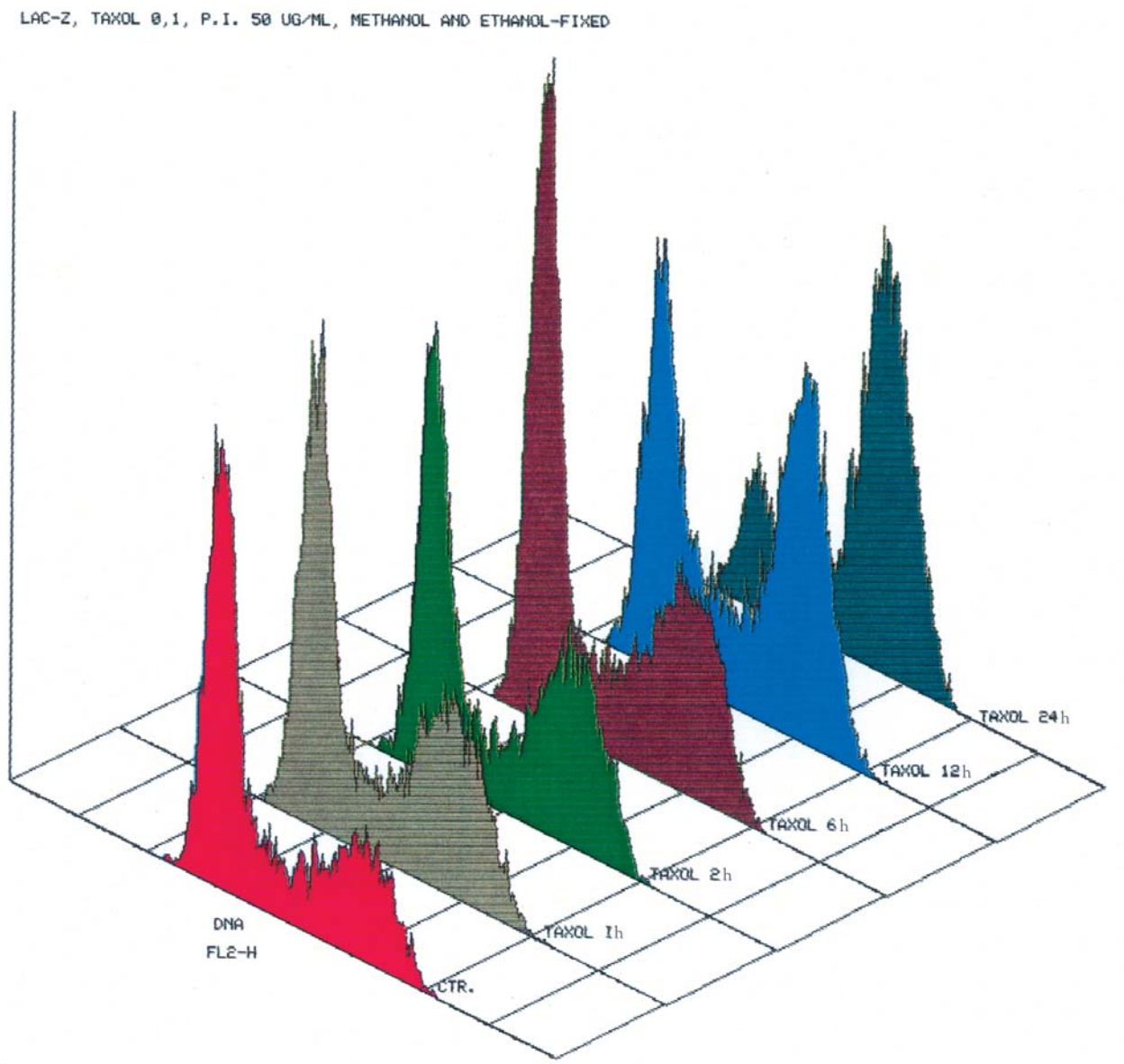

Figure 3. *Flow cytometry of tumour cells showing increasing accumulation of cells in the (G2+M)-phase of the cell cycle due to exposure to paclitaxel.*

Growth of the spheroids. There were no differences in growth between the control spheroids and the spheroids exposed to 0.001 μg/ml paclitaxel, nor between the spheroids exposed to paclitaxel concentrations of 0.01 μg/ml and 0.1 μg/ml (Figure 2). Spheroids exposed to the two higher paclitaxel concentrations seen together had a significantly lower diameter ($p<0.0001$) on the first treatment day (day 3) than the group consisting of controls and spheroids exposed to low paclitaxel concentration. The slopes estimated from the changed spheroidal diameters inclined 0.18 mm per day upwards for the controls and the low paclitaxel concentration-group seen together, and 0.46 mm per day downwards for the high concentration-group, corresponding to its significantly lower gradient ($p<0.0001$).

Viability. After 4 weeks of culture, the paclitaxel-treated spheroids stained with green fluorescence and were consequently viable. Rather few red (and thus dead) cells were detected by confocal microscopy.

Table I. *Percentage of co-cultures (with untreated and paclitaxel-treated tumour spheroids) in which tumour cells enclose the benign bladder fragment – 12, 16, 24 and 40 hours after confrontation.*

	Control co-cultures	Experimental co-cultures
12 h	25% (12/48)	0
16 h	40% (19/48)	2% (1/48)
24 h	83% (40/48)	10% (5/48)
40 h	98% (47/48)	67% (32/48)

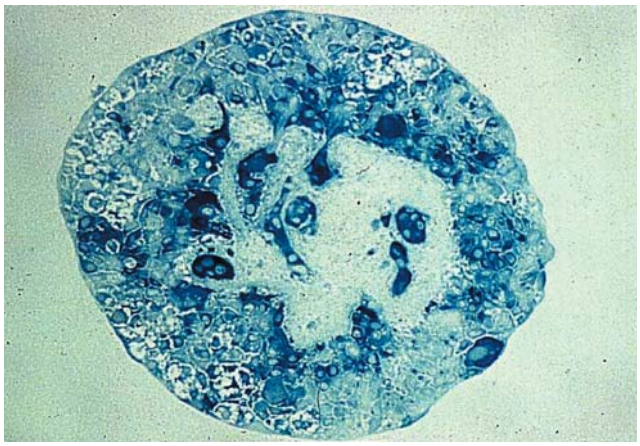

Figure 4. *Co-culture on day 21 after confrontation. Blue tumour cells totally enclose the bladder fragment and infiltration of the benign stroma appears as nests of tumour cells.*

Flow cytometry (FCM). The He1703Hu/*lacZ* cells contained a single near tetraploid (3.7n) cell population. After continuous exposure to 0.1 μg/ml of paclitaxel for 24 h, the FCM DNA histogram showed a steadily increasing accumulation of cells in the (G2+M)-phase. In the control group, the (G2+M)-phase consisted of 20.4% of the cells. After 1 h of exposure to paclitaxel, 26.8% of the cells were demonstrated in the phase, and after 24 h the (G2+M)-phase eventually consisted of 63.5% of the cells (Figure 3).

When stained with PI 5 μg/ml, 99.21% of the cells gathered in one cluster, representing the cycling cells. After 24 h with paclitaxel exposure, the number was reduced to 91.36%, indicating that about 10% of the cells were apoptotic.

Co-cultures. Stereomicroscopy of the control group: All confronted spheroids appeared to be firmly attached to each other two hours after contact was established. After 12 h, tumour cells totally enclosed the benign fragments in 25% (12/48) of the confrontations (Table I). This was the case in 40% (19/48) of the co-cultures after 16 h, in 83% (40/48) following 24 h and in 98% (47/48) after 40 h.

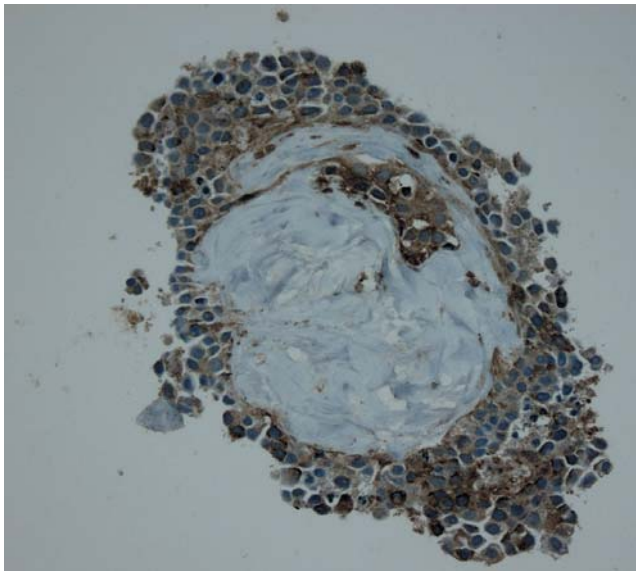

Figure 5. *Co-culture on day 21; untreated tumour spheroid confronted with benign bladder spheroid. Expression of MMP-1 and infiltration of tumour cells are pronounced.*

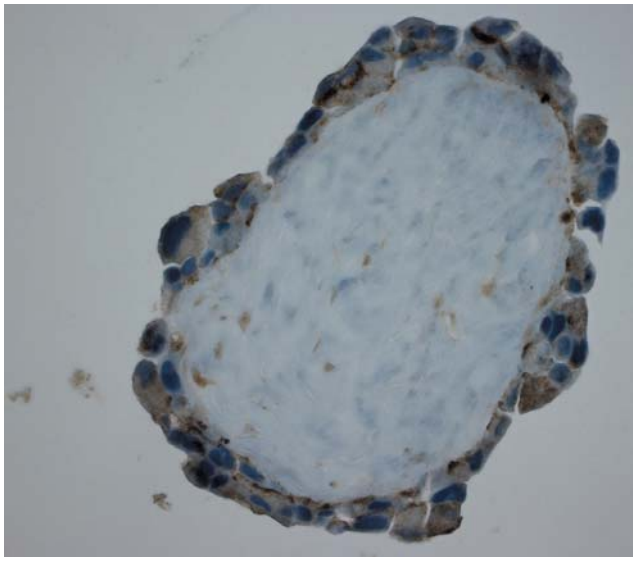

Figure 6. *Co-culture on day 21; tumour spheroid has been exposed to paclitaxel before confrontation with benign bladder fragment. Both MMP-expression and infiltration of tumour cells seem to be inhibited.*

Stereomicroscopy of the co-cultures with paclitaxel-treated tumour spheroids: Paclitaxel treatment of the tumour aggregates before confrontation seemed to inhibit their firm attachment to the bladder fragments. Contact, therefore, had to be re-established in five co-cultures after 2 h. Following 12 h, only scattered migration of tumour cells was

observed in two co-cultures (Table I). Total enclosing of the bladder fragment by tumour tissue seemed to occur in one co-culture after 16 h, in 10% (5/48) of the cases after 24 h and in 67% (32/48) following 40 h.

Invasion scored by light microscope: The score sum for the controls gradually increased from 5 after 40 h to 6, 7, 8, 7, 7 and 8 after 28 days. The course for the group with paclitaxel-treated tumour spheroids showed, in contrast to this, score sum 3 after 40 h, then 1, 5, 7, 0, 0 and eventually 3 after 28 days. In five co-cultures with paclitaxel-treated tumour spheroids there were only a few, thin layers of tumour cells encircling the bladder fragment. After 7, 14, 21 and 28 days, the tumour tissue appeared detached from the bladder fragment in several sections from two of the co-cultures. Invasion was significantly inhibited by paclitaxel treatment ($p<0.001$).

The invasive process was clearly visualised as the *lacZ*-transfected tumour cells stained distinctly blue (Figure 4).

Immunostaining. MMP-1 was strongly expressed (score ++) in 93% (13/14) of the control co-cultures (Figure 5) and weakly expressed (score+) in 100% (14/14) of the paclitaxel-treated (Figure 6). One of the controls showed weak MMP-1 expression 3 days after confrontation. The scores were not influenced by the time which had passed after confrontation. Brown staining was detectable in the cytoplasma of the migrating tumour cells after 40 h, but became distinct after 3 days. After 5, 7, 14, 21 and 28 days, staining was detectable both in tumour cell cytoplasma and benign stroma. MMP-9 and MMP-2 were not expressed in either experimental or control spheroids.

Discussion

Paclitaxel is demonstrated to be both effective and tolerable in treating locally and generally advanced TCC cancer (1, 22, 23). The drug may also turn out to be a pharmacological alternative for intravesical therapy of early stages of the disease, as indicated by our results concerning its inhibition of growth, invasion and migration of TCC cells. In favour of the assumption that intravesical application may be a future option is the finding of Rangel *et al.* (3), that paclitaxel is stable in human urine. It is also reported that paclitaxel readily penetrates the urothelium and is better absorbed than agents such as mitomycin C and doxorubicin, which currently are used in intravesical therapy (24). Besides, only a small fraction of the dose intravesically applied enters the systemic circulation because of its low affinity to proteins in the plasma. This will also add to the tissue concentration of the drug and explains its relatively low toxicity. These qualities all indicate a potential applicability in local therapy of bladder cancer.

The TCC cell lines employed by Rangel *et al.* (3) responded to paclitaxel, even if given over brief periods. In accordance with this, we noticed that the effects of paclitaxel occurred quite rapidly after application, namely inhibition of spheroidal growth and of migration in monolayer. The reduction in growth was mediated by the two higher concentrations of paclitaxel and, as shown by others as well, correlated with the duration of drug exposure. Time-dependent effects were demonstrated by Rowinsky *et al.* (25) when exposing leukemic cell lines to paclitaxel. Similarly, Raymond *et al.* (26) demonstrated correlation between growth inhibition and duration of drug exposure for human tumour colonies originating from ovarian, lung and breast cancer.

The observation that different drug concentrations are needed for growth inhibition in spheroidal culture and in monolayer reflect different culture conditions. A spheroid in suspension culture represents a three-dimensional microenvironment, whereas a monolayer are cells growing as a one-cell-thick layer on a flat surface. Cells in monolayer will, therefore, be exposed to environmental influences without the modulating interaction with surrounding cells, as in the *in vivo* situation. As the cellular architecture of our three-dimensional spheroids mimics the *in vivo* original (the urinary bladder and TCC tumours), the morphological and biochemical conditions of the microenvironment are probably similar. The paclitaxel concentration demonstrated to inhibit spheroidal growth in this *in vitro* study might, thus, correspond to the therapeutic concentration *in vivo*. Another advantage of our model is the lack of humoral immune factors as those interfering *in vivo*. The testing of paclitaxel on tumour spheroids *in vitro* may altogether provide representative information about the therapeutic efficiency of the drug. The tumour cells are *lacZ*-transfected and stain blue with X-gal, which make their identification more accurate.

In the present assay, the rate of invasion appeared to be influenced by exposure of the tumour spheroids to paclitaxel before confrontation with benign bladder fragments. Treatment with paclitaxel seems to inhibit and to postpone the invasion of tumour cells. This may be understood as secondary effects on the cells. Central and early steps of tumour growth are increased proliferation and local growth. For invasion and metastases to occur, the adhesion of tumour cells to each other and to normal tissue will be weakened. Migration of tumour cells through the degraded basement membrane and extracellular tissue, and penetration of capillaries and lymphatic vessels, are also hallmarks of cancer. These events both depend on intrinsic properties of the tumour cells themselves and on their influence on the benign cells. This interaction subsequently results in the production of and responses to specific tissue factors and proteases both in the malignant and benign cells, and eventually in changes of the extracellular tissue, blood and lymphatic tissues. In this study, cellular changes

after paclitaxel exposure were verified by means of FCM and immunostaining. An increased number of cells were demonstrated to accumulate in the (G2+M)-phase of the cell cycle, and this led to lowered proliferation and reduced tumour growth. Recent studies indicate that exposure to paclitaxel stimulates apoptosis, in addition to its other antitumour effects (27). In agreement with this, we observed a reduced tumour cell population of about 10% after paclitaxel treatment. Several anti-neoplastic effects of paclitaxel were thus shown in our assay: arrest of the cell cycle, increased apoptosis, reduced spheroidal growth and tumour cell migration. Additionally, tumour cell invasion was directly inhibited by exposure to the drug.

Enzymes in the matrix metalloproteinase (MMP) family discharge activity that is necessary for tumour invasion and metastases (28). These proteases enhance the spread of malignant cells by degrading components of tumour barriers, such as the basement membrane and the extracellular matrix. Some authors have reported increased serum levels of MMP-1, MMP-2 and MMP-9 in urothelial carcinomas (29, 30). Osdemir *et al.* (31) reported MMP-9 to be overexpressed and correlated to stage and grade in malignant urinary tumours. They further suggested urinary MMP-9 as a promising non-invasive diagnostic marker of bladder cancer. In our study, we demonstrated expression of MMP-1 to follow the infiltration of tumour cells. The expression was weaker after drug exposure, indicating a negative impact on the tumour cells' ability to invade the benign spheroids. This agrees with the finding that paclitaxel treatment directly inhibits invasion. After three days, MMP-1 seemed to be expressed in the benign stroma close to and in front of the infiltrating tumour cells, whereas earlier the enzyme was localized only in the cytoplasma of these cells. This may reflect the progress of tumour cell invasion from one stage to another. In accordance with this observation, it is reported that production of some MMPs are induced in stroma cells. Correlation between tumour stage and the level of MMP expression, and between tumour aggressiveness and expression of MMPs, is also reported (32-34). In the present assay, we did not detect MMP-2 and MMP-9 in any of the co-cultures, although other studies have suggested a positive association of these MMPs to bladder cancer (35, 36).

In our study, we estimated the size of the spheroids by measuring the mean of two orthogonal diameters in the largest section. The volume of the spheroids was considered to correspond to these estimates, and the spheroidal growth was thus negatively influenced by paclitaxel treatment. Terzis *et al.* (37) have reported estimation of spheroidal volumes by means of confocal microscopy. They also used these volumes for subsequent calculation of invasion after confrontation with benign cell aggregates. In their model, though, invasion appeared as diffuse cellular infiltration, while tumour cell encirclement with subsequent infiltration of single cells dominated in our study. This pattern of invasion makes it more difficult to estimate invasion in a reproducible way. The presence of encircling tumour cells around the benign bladder fragment was scored (0) in our study, irrespective of the actual number of encircling cell layers and of the contact between the malignant and benign tissue. Although both factors are influenced by paclitaxel treatment, we chose not to score these variables because of the low number of examined co-cultures. Few and rough parameters used in the scoring enabled and ascertained the stastistical evaluation of our results. The pattern of invasion will probably be determined by the origin of the spheroids employed. Terzis *et al.* confronted benign aggregates originating from rat brain cells with spheroids from human brain tumour cells. In a former publication, we described four alternative ways of invasion, as well as the combination of two of these patterns shown by the spheroids initiated from our *lacZ*-transfected tumour cell line (15). These formerly reported observations are reproduced and established in the present study by using our *in vitro* model on larger numbers of co-cultures.

Exposure to 0.1 μg/ml of paclitaxel was shown to inhibit the spheroidal cell growth and migration of an invasive, *lacZ*-transfected TCC cell line. Paclitaxel treatment was observed to induce accumulation of cells in the (G2+M)-phase of the cell cycle. There are additional indications that paclitaxel stimulates apoptosis. The invasion of *lacZ*-transfected tumour cells is negatively influenced by exposure to the drug.

References

1 Bajorin DF: Paclitaxel in the treatment of advanced urothelial cancer. Oncology *14(1)*: 43-52, 2000.

2 Bokemeyer C, Hartmann JT, Kuczyk MA, Truss MC, Beyer, Jonas U and Kanz L: The role of paclitaxel in chemosensitive urological malignancies: current strategies in bladder cancer and testicular germ-cell tumors. W J Urol *14*: 354-359, 1996.

3 Rangel C, Niell H, Miller A and Cox C: Taxol and taxotere in bladder cancer: *in vitro* activity and urine stability. Cancer Chemother Pharmacol *33*: 460-464, 1994.

4 Wani MC, Taylor HL, Wall ME, Coggon P and McPhail AT: Plant antitumour agents VI. The isolation and strucure of Taxol, a novel antileukemic and antitumour agent from *Taxus brevifolia*. J Am Chem Soc *93*: 2325-2327, 1971.

5 Jordan MA, Toso RJ, Thrower D and Wilson L: Mechanisms of mitotic block and inhibition of cell proliferation by taxol and low concentrations. Proc Natl Acad Sci USA *90*: 9552-9556, 1993.

6 Milross C, Mason K, Hunter N, Chung WK, Peters LJ and Milas L: Relationship of mitotic arrest and apoptosis to antitumor effect of paclitaxel. J Natl Cancer Inst *86*: 1086-1091, 1996.

7 Bhalla K, Ibrado AM, Tourkina E, Tang C, Mahoney ME and Huang Y: Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. Leukemia *7(4)*: 563-568, 1993.

8 Chang YF, Li LL, Wu CW, Liu TY, Lui WY, P'eng FK and Chi CW: Paclitaxel-induced apoptosis in human gastric carcinoma cell lines. Cancer *77*: 14-18, 1995.
9 Chen JG, Yang CP, Cammer M and Horowitz SB: Gene expression and mitotic arrest induced by microtubule-stabilizing drugs. Cancer Res *63(22)*: 7891-7899, 2003.
10 Dziadyk JM, Sui M, Zhu X and Fan W: Paclitaxel-induced apoptosis may occur without a prior G2/M-phase arrest. Anticancer Res *24(1)*: 27-36, 2004.
11 McGuire WP 3rd and Markman M: Primary ovarian cancer chemotherapy: currant standards of care. Br J Cancer *89(suppl 3)*: S3-8, 2003.
12 Murphy WK, Fosella FV, Winn RJ, Shin DM, Hynes HE, Gross HM, Davilla E, Leimert J, Dhingra H, Raber Mn *et al*: Phase II study of taxol in patients with untreated advanced non-small-cell lung cancer. J Natl Cancer Inst *85(5)*: 384-388, 1993.
13 Arbuck SG: Paclitaxel: Current developmental approaches of the National Cancer Institute. Semin Oncol *22[(6) suppl 15]*: 55-63, 1995.
14 Rowinsky EK: Paclitaxel pharmacology and other tumor types. Semin Oncol *24(suppl 19)*:S 19-12, 1997.
15 Vatne V, Litlekalsoey J, Thorsen F, Ness OG, Wentzel-Larsen T and Hoestmark J: Characterization of cell lines originating from an invasive transitional cell carcinoma. Anticancer Res *21*: 3199-3208, 2001.
16 Vatne V, Fjellbirkeland L, Litlekalsoey J and Hoestmark J: A human *in vitro* model of bladder tumour cells invasion. Anticancer Res *18*: 3985-3990, 1998.
17 Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P and Traganos F: Features of apoptotic cells measured by flow cytometry. Cytometry *13*: 795-808, 1992.
18 Stokke T, Solberg K, DeAngelis P and Steen HB: Propidium iodide quenches the fluorescence of TdT-incorporated FITC-labelled dUTP in apoptotic cells. Cytometry *33*: 428-434, 1998.
19 Vatne V, Fjellbirkeland L, Litlekalsoey J and Hoestmark J: Nonadhesive stationary organ culture of normal human urinary bladder mucosa. Anticancer Res *18*: 3979-3984, 1998.
20 Pinheiro JC and Bates DM: Mixed Effects Models in S and S-plus. New York, Springer 2000.
21 Ihaka R and Gemtleman RR: A language for data analysis and graphics. J Comput Graph Stat *5(3)*: 299-314, 1996.
22 Hussain SA and James ND: The systemic treatment of advanced and metastatic bladder cancer. Lancet Oncol *4(8)*: 489-497, 2003.
23 Bamias A, Deliveliotis Ch, Aravantinos G, Kalofonos Ch, Karayami A and Dimipoulos MA: Hellenic Cooperative Oncology Group: Adjuvant chemotherapy with paclitaxel and carboplatin in patients with advanced bladder cancer: a study by the Hellenic Cooperative Oncology Group. J Urol *171(4)*: 1467-1470, 2004.
24 Song D, Wientjes MG and Au JL-S: Bladder tissue pharmacokinetics of intravesical taxol. Cancer Chemother Pharmacol *40*: 285-292, 1997.
25 Rowinsky EK, Donehower RC, Jones RJ and Tucker RW: Microtubule changes and cytotoxicity in leukemic cell lines treated with taxol. Cancer Res *48*: 4093-4100, 1988.
26 Raymond E, Hanauske A, Faivre S, Izbicka E, Clark G, Rowinsky EK and von Hoff DD: Effects of prolonged *versus* short-time exposure paclitaxel (Taxol R) on human tumor colony-forming units. Aniti-Cancer Drugs *8*: 379-385, 1997.
27 Haldar S, Chintapalli J and Croce CM: Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. Cancer Res *56*: 1253-1255, 1996.
28 Johnson M, Lund LR, Romer J, Almholt K and Dano K: Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation. Curr Opin Cell Biol *10*: 667-671, 1998.
29 Davies B, Waxman J, Wasan H, Abel P, Williams G, Krausz T, Neal D, Thomas D, Hanby A and Balkwill F: Levels of matrix metalloproteases in bladder cancer correlate with tumor grade and invasion. Cancer Res *53*: 5365-5369, 1993.
30 Gohji K, Fujimoto N, Komiyama T, Fuji A, Ohkawa J, Kamidono S and Nakajima M: Elevation of serum levels of matrix metalloproteinase-2 and -3 as new predictors of recurrence in patients with urothelial carcinoma. Cancer *78*: 2379-2387, 1996.
31 Osdemir E, Kakehi Y, Okuno H and Yoshida O: Role of matrix metalloproteinase-9 in the basement membrane destruction of superficial urothelial carcinomas. J Urol *161(4)*: 1359-1363, 1999.
32 Nelson AR, Fingleton B, Rothenberg MI and Matrisian LM: Matrix metalloproteinases: biologic activity and clinical implications.J Clin Oncol *18*: 1135-1149, 2000.
33 Curran S and Murray GI: Matrix metalloproteinases: molecular aspects of their roles in tumor invasion and metastasis. Eur J Cancer *36*: 1621-1630, 2000.
34 Kanayama H: Matrix metalloproteinases and bladder cancer. J Med Invest *48(1-2)*: 31-43, 2001.
35 Kanda K, Takahashi M, Murakami Y, Kanayama H and Kagawa S: The role of the activated form of matrix metalloproteinase-2 in urothelial cancer. BJU Int *86*: 553-557, 2000.
36 Papathoma A-S, Petraki C, Grigorakis A, Papakonstantinou H, Karavana V, Stefanakis S, Sotsiou F and Pintzas A: Prognostic significance of matrix metalloproteinases 2 and 9 in bladder cancer. Anticancer Res *20(3B)*: 2009-2013, 2000.
37 Terzis A-JA, Thorsen F, Heese O, Visted T, Bjerkvig R, Dahl O, Arnold H and Gundersen G: Proliferation, migration and invasion of human glioma cells exposed to paclitaxel (Taxol) *in vitro*. Br J Cancer *75(12)*: 1744-1752, 1997

Received June 28, 2004
Revised December 20, 2004
Accepted February 16, 2005