INTRODUCTION

Our phase I study of intravesical Ad-IFNα/Syn3 treatment in patients with non-muscle invasive bladder cancer previously failing BCG (bacillus Calmette-Guérin) therapy has been completed. The Syn3 is an excipient Syn3 that facilitates effective gene transfer and expression of interferon-α (IFNα) within the urothelium and tumor with subsequent secretion into the urine. In the phase I study, only a single instillation of Ad-IFNα/Syn3 was given. A complete remission (CR) as defined by no evidence of tumor and negative biopsies by cystoscopy 90 days after treatment was found in 6 of the 14 patients treated who produced high levels of interferon-α in their urine.

Three distinct mechanisms of cancer cell death caused by Ad-IFNα have been identified by us. The IFNα produces a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated cancer cell kill, whereas cancer cells which are resistant to the interferon α protein are killed by Ad-IFNα in two additional ways, one indirect (apoptotic) and the other direct (necrotic). The indirect mechanism involves a potent soluble bystander factor(s) secreted by both Ad-IFNα-infected tumor and normal urothelial cells, which is strongly cytotoxic to all tumor types tested to date, including bladder cancer, but is not cytotoxic to normal cells. The direct cancer cell kill occurs from the high level IFNα accumulation found perinuclearly after Ad-IFNα treatment, which causes ER stress-related cancer cell death.

Levels of cytokeratins, especially those which detect both caspase-cleaved and intact cytokeratin 18 (CK18), have been used as biomarkers of tumor cell kill measured in the blood after chemotherapeutic treatment. The CK18 fragment that is produced following caspase cleavage of the CK18 protein and is thought to be a selective biomarker for apoptotic cell death, whereas the M65 ELISA uses the M5 antibody thought to detect both the uncleaved and cleaved CK18 protein fragments. By subtracting the concentration of cleaved CK18 obtained using the M30 ELISA (M30 increase) from the level obtained in the M65 ELISA, the necrotic component of cell death is determined (M65 increase).

Both the TRAIL- and bystander-related cancer cell apoptosis produced by Ad-IFNα can be measured by increases in M30, whereas the endoplasmic stress-related direct cancer cell death is mostly necrotic and can be measured by an increase in M65.

In this report, patients from the phase I trial were studied. Changes in urine M30 and M65 levels as well as IFN and TRAIL levels were examined at various times after intravesical Ad-IFNα/Syn3 treatment. We hypothesized that increases in M30 and M65 level would reflect the mechanisms of tumor cell death produced by the Ad-IFNα and that changes in M30 and M65 levels might be predictive of the CRs obtained.

MATERIALS AND METHODS

Urine samples

The urine samples were collected on ice after treatment and were stabilized by the addition of a buffer containing 10% bovine serum albumin and 50 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic. A volume of 1 ml of buffer was added to 10 ml of urine. After centrifugation, urine was then stored at −80°C until it was thawed to conduct the various assays. Both early morning samples brought by the patients with bacillus Calmette-Guérin-resistant superficial bladder cancer showed a complete remission (CR) of 43% at 90 days after treatment with high levels of interferon-α (IFNα) being produced. Ad-IFNα kills bladder cancer cells by two apoptotic and one necrotic mechanism that can be measured by soluble forms of cytokeratin 18 (CK18) using M30 and M65 ELISAs, assays for caspase-cleaved (apoptotic) and uncleaved (necrotic) cell death, respectively. Therefore, we determined whether M30 and M65 levels in the urine after treatment could document all three mechanisms of cancer cell kill and also predict having a CR. High levels of both M30 and M65 were found in all patients within 24 h after treatment with all three types of cancer cell death occurring. Moreover, the return of both M30 and M65 levels in the urine to normal levels within 5 days or more after treatment was strongly associated with obtaining a CR (P = 0.003). This is the first time that such assays have been used to study response to therapy in the urine of patients with bladder cancer and in the future may prove valuable in predicting clinical outcome.

Keywords: Adenoviral-mediated interferon α treatment; urine cytokeratin 18 levels; bladder cancer; phase I study
Correlation between M30 and M65 returning to normal levels in the urine after Ad-IFNz treatment and achieving a CR at 90 days. In this phase 1 study, 6 out of 14 patients (43%) achieved a CR. All six patients with a CR (100%) had M30 levels in their urine, which returned to normal (<175 U l⁻¹) by day 5 or later, whereas two out of eight (25.0%) patients who did not achieve a CR had M30 levels, which returned to normal levels (P = 0.010). In addition, five of six patients with a CR (83.3%) had M65 levels, which returned to normal (<175 U l⁻¹) at day 5 or later, whereas none of the eight patients without a CR (0%) had M65 levels, which returned to normal (P = 0.003). Similarly, five of six patients with a CR (83.3%) had both M30 and M65 levels, which returned to normal levels at day 5 or later, whereas none of the eight patients without a CR (0%) had both M65 and M30 levels return to normal (P = 0.003).

DISCUSSION
Our phase 1 study using intravesical Ad-IFNz/Syn3 for the treatment of patients with BCG refractory nonmuscle invasive bladder cancer resulted in high and prolonged urine IFNz levels. In addition, 43% of the patients with measurable urine IFNz levels achieved a CR at 90 days after treatment.1 Using ELISAs to examine the levels of both caspase-cleaved (M30) and intact (M65) CK18, strong evidence was provided that significant apoptosis and necrosis occurred in each of the patient’s tumors. We believe that this represented all three mechanisms of Ad-IFNz-produced cancer cell death.9 The increases in M65 levels were likely from the direct tumor cell kill caused by the significant gene transfer and expression of the Ad-IFNz in many cancer cells resulting in necrotic cancer cell death, whereas the high levels of M30 observed were the result of apoptosis from both TRAIL- and bystander-mediated mechanisms of Ad-IFNz-produced cancer cell death.9 In those patients in whom significant TRAIL urine levels were seen, much of the increase in M30 levels likely was indicative of TRAIL-mediated cancer cell death caused by the high and prolonged IFNz levels produced in the urine. This mechanism of tumor cell kill was likely reflected by the M30 levels in which the TRAIL-related tumor cell kill cannot be distinguished from the bystander-produced tumor cell kill at both mechanisms cause apoptotic cell death. The initial large increase in M65 levels seen in this patient is likely the result of the direct mechanism of Ad-IFNz-produced cell death, namely necrotic cancer cell kill following Ad-IFNz infection and expression in a significant portion of the tumor cells. In contrast, patient 14, who also achieved a CR, now maintained for more than 2 years, produced no TRAIL in his urine. Therefore, his tumor likely was IFN resistant and the increase seen in the urine M30 levels was likely the result of bystander factor caused cancer cell kill and was substantial. This patient had never responded to any prior intravesical therapy and would have been directed to cystectomy, if not for serious co-morbidities. Again both M30 and M65 levels returned to normal by 10 days after treatment.

In contrast, patient 9 did not achieve a CR. This patient had very extensive disease within the bladder at the time of treatment with Ad-IFNz/Syn3. After therapy, significant levels of IFNz, TRAIL, M30 and M65 in the urine were obtained, but the M30 and M65 levels remained high for the urine samples obtained 14 days after treatment. These results suggest that much tumor cell kill occurred in this patient from all three mechanisms of Ad-IFNz-produced cancer cell kill and that M30 and M65 never reached a normal level because of the extensive and persisting tumor burden present.

RESULTS
Urine IFN levels
The phase 1 study design provided for a single intravesical instillation of Ad-IFNz 75 ml in 1 mg ml⁻¹ Syn3) with re-treatment if a CR was achieved at 90 days. High and prolonged dose-dependent urine IFN levels were observed in all dose levels of 1 x 10¹⁰ particles per ml and above with the top concentration of 3 x 10¹¹ particles per ml producing the most increase in urine IFN levels, which in certain patients were measurable up to 7 days or more (Figure 1). The patients who achieved a CR are shown by arrows. The difference in scale of IFN levels at each dose level should be noted. In addition, a significant IFN urine level was measured in patient 14 retreated 90 days after his initial treatment at the 24-h time point (6082 pg ml⁻¹). Six of fourteen patients (43%) receiving doses who produced measurable IFNz in the urine achieved a CR at 3 months after treatment.

M30, M65 and TRAIL levels indicate that all mechanisms of Ad-IFNz-produced cell death occur in a clinical setting
Increases in both M30 and M65 levels were found following all 14 patients in whom increases in IFN were also seen (all patients shown in Figure 1). Representative examples of the changes found in M30 an M65 levels over time after treatment as well as TRAIL and IFNz levels are shown in Table 1. Patient 7, who achieved a CR, had high M30 and M65 levels in his urine one day after treatment, which returned to normal levels by day 4. He also had high TRAIL levels indicating that the tumor was IFNz sensitive with considerable tumor cell kill occurring because of the high and prolonged IFNz levels produced in the urine. This mechanism of tumor cell kill was likely reflected by the M30 levels in which the TRAIL-related tumor cell kill cannot be distinguished from the bystander-produced tumor cell kill as both mechanisms cause apoptotic cell death. The initial large increase in M65 levels seen in this patient is likely the result of the direct mechanism of Ad-IFNz-produced cell death, namely necrotic cancer cell kill following Ad-IFNz infection and expression in a significant portion of the tumor cells.

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prolonged IFNα levels produced in IFNα-sensitive tumors such as in patient 7 (Table 1). In contrast, the tumors in patients showing little or no TRAIL levels in their urine after Ad-IFNα treatment but high M30 levels most likely were IFNα resistant and the M30 increase seen reflected primarily bystander tumor cell death such as that seen in patient 14 (Table 1).

As high M30 and M65 levels in the urine determined within 24 h after treatment appeared to indicate that significant tumor kill occurred in all patients, we thought it would be prudent to examine whether return to normal levels in the urine over time might correlate with a better prognosis. This was indeed the case. In all six patients with a CR at day 90, the M30 levels returned to normal by day 5 or later, whereas this occurred in only two of eight patients who did not achieve a CR (P = 0.010). Moreover, five of six patients with a CR had M65 levels, which returned to normal, whereas none of the eight patients without a CR had M65 levels, which returned to normal (P = 0.003). To our knowledge, this study is the first to suggest that examining M30 and M65 levels in the urine might be used as a potential surrogate biomarker of tumor cell kill and prognosis after treatment of non-muscle invasive bladder cancer with any therapeutic agent.

Although this analysis was done retrospectively, it indicates that measurement of M30 and M65 levels in urine after intravesical Ad-IFNα/Syn3 treatment, particularly return to normal levels, might be useful as a prognostic indicator of attaining a CR at 90 days. Such studies may therefore have clinical value in future trials.

Consequently, these determinations will be done in a phase IB trial planned, in which intravesical Ad-IFNα/Syn3 treatment will be given on day 1 and again on day 4 to examine whether or not even higher and more prolonged urine IFNα levels can be achieved as well as whether an increase in the number of CRs can be obtained. Finally, measurement of M30 and M65 levels in urine might be useful to follow tumor cell kill for other clinical studies in which tumor in the bladder is present.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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