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Synergistic potentiation of interferon activity with maitake mushroom D-fraction on bladder cancer cells

Brandon Louie, Srinivas Rajamahanty, John Won, Muhammad Choudhury and Sensuke Konno

Department of Urology, New York Medical College, Valhalla, NY, USA

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OBJECTIVE

To examine whether the combination of interferon (IFN)-α and maitake mushroom D-fraction (PDF), a bioactive mushroom extract, might potentiate the anticancer activity of IFN-α in bladder cancer T24 cells *in vitro*.

MATERIALS AND METHODS

Effects of recombinant IFN-α_{2b} (0–50 000 IU/mL), PDF (0–700 μg/mL), or their combinations were assessed on T24 cell growth at 72 h. Cell cycle analysis and assays for double-stranded DNA-dependent protein kinase (DNA-PK) were performed to explore possible antiproliferative mechanism of these agents.

RESULTS

IFN-α_{2b} was able to induce a significant (≈50%) growth reduction at 20 000 IU/mL, which further declined to ≈66% at 50 000 IU/mL. PDF had no effects up to 200 μg/mL, but there was an ≈20% and ≈53% growth reduction at 400 and 700 μg/mL, respectively. When the varying concentrations of IFN-α_{2b} and PDF were combined, 10 000 IU/mL of IFN-α_{2b} combined with 200 μg/mL of PDF resulted in an ≈75% growth reduction. This was accompanied by a G_1 cell cycle arrest, shown by cell cycle analysis. Concurrently, DNA-PK activity in IFN-α_{2b}/PDF-treated cells was almost three-fold higher than controls.

CONCLUSIONS

The combination of IFN-α_{2b} (10 000 IU/mL) and PDF (200 μg/mL) reduced growth by ≈75% in T24 cells. This appears to be due to a synergistic potentiation of these two agents, inducing a G_1 arrest with DNA-PK activation. Therefore, the IFN-α_{2b}/PDF combination could trigger DNA-PK activation that may act on the cell cycle to cease cancer cell growth.

KEYWORDS

interferon, D-fraction, combined therapy, synergism, bladder cancer

INTRODUCTION

The bladder is the most common site of cancer in the urinary system and ≈90% of bladder cancers are TCCs. Of these TCCs, ≈80% are diagnosed as superficial bladder tumours [1]. Transurethral resection is the primary method for removal of those superficial bladder tumours; however, nearly 65% of patients will have tumour recurrence in 5 years while 10–20% will have progression to muscle invasion [2,3]. It is thus conceivable that the primary therapeutic aim is to prevent multiple recurrences and progression to a more advanced, invasive disease.

Several cytotoxic and immune modifying agents have been used intravesically for therapeutic purpose. Among them, intravesical administration of BCG is highly effective in reducing the recurrence rate and altering the progression rate of the disease with an increased survival rate [4]. In fact, adjuvant intravesical BCG therapy after surgical resection has become established therapy for superficial bladder cancers, resulting in ≈40% reduction in cancer recurrence [4,5]. However, its benefits are sometimes outweighed by its severe side-effects: cystitis occurs in 90% of patients and other potential adverse effects (fever, allergic reactions, sepsis, etc.) cannot be excluded [6,7]. These drawbacks thus limit its use in clinical practice and request a safer and effective treatment method with few side-effects, promoting the use of unconventional therapies with other various immunomodulators.

Interferons (IFNs) have been widely used as immunotherapy for various human malignancies including prostate, bladder, and RCCs [8–10]. Particularly, IFN-α has been used as an intravesical agent for treating superficial bladder cancer, resulting in an ≈40% response rate in patients [11]. Although this response rate is lower than that of BCG therapy, IFN-α causes only minimal local and systemic toxicity (compared with BCG) [11,12]. To improve the efficacy of IFN-α therapy, combined therapy (e.g. IFN-α/BCG) has been proposed and is being assessed in pilot clinical trials and animal studies, which are showing better and encouraging outcomes [13,14]. This suggests that further exploration of effective treatment methods such as an alternative and/or combined therapy is warranted.

D-fraction (PDF) is a bioactive proteoglucan extracted from maitake mushrooms (*Grifola frondosa*) [15]. The standardized PDF has been commercially available for medical and

scientific research. Several published and unpublished studies have to date suggested the immunomodulatory and antitumour activities of PDF [16,17]. It was shown in an animal model that PDF was capable of activating immune-competent cells such as natural killer cells and cytotoxic T-cells with a concomitant increase in interleukin-1 production [16,17], indicating stimulation of immune responses. Meanwhile, the safety of PDF is supported by the fact that the USA Food and Drug Administration (FDA) has exempted a phase I study of toxicology tests. Additionally, the FDA has approved PDF for the Investigational New Drug application for a phase II pilot study on patients with advanced breast and prostate cancer [18].

Accordingly, we investigated whether IFN-α, PDF or their combination might have a growth inhibitory effect on bladder cancer T24 cells *in vitro*, and the underlying mechanism of such activity was also explored.

MATERIALS AND METHODS

The human bladder cancer T24 cells, derived from a patient with TCC, were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in McCoy's 5a medium containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Routinely, culture medium was changed every 3–4 days and the passage of cells was performed weekly. For experiments, cells were seeded in T-75 flasks or six-well culture plates at the initial cell density of 2×10^5 cells/mL and were cultured with recombinant IFN-α_{2b} (Schering Corp., Kenilworth, NJ, USA), PDF (Maitake Products, Inc., Paramus, NJ, USA) or their combinations. Cell numbers were then assessed at specified times using the Trypan blue exclusion method.

Cell cycle analysis was performed using a FACScan flow cytometer (Becton-Dickinson), equipped with a double discrimination module. About 1×10^6 cells were re-suspended in 500 μL of propidium iodide solution (20 μg/mL propidium iodide, 0.2 mg/mL RNase, 0.2 mg/mL EDTA, 0.5% NP-40) and incubated at room temperature for 1 h. In all, 10 000 nuclei were analysed for each sample, and CellFit software was used to quantify cell cycle compartments and estimate cell cycle phase fractions.

In vitro phosphorylation assay was performed as previously described [19]. Cell lysates were first prepared from control and IFN-α_{2b}/PDF-treated cells by three cycles of freeze-thaw in liquid nitrogen. A 5 μg aliquot of cell lysate preparation was added to the phosphorylation cocktail containing 55.5 kBq of [γ-^{32}P]-ATP (specific activity: 166.5 TBq/mmol) and incubated at 37 °C for 15 min. To activate endogenous double-stranded DNA-dependent protein kinase (DNA-PK), 2 μg/mL of fragmented calf thymus DNA was also included in the reaction mixture. Phosphoproteins were then separated by 10% SDS-PAGE and analysed by autoradiography. Intensities of specific DNA-PK bands were then quantified using a scan densitometer (Silk Scientific, Oregon, UT, USA).

For statistical analysis, all data were presented as the mean (SD), and statistical differences between groups were assessed with the unpaired Student's *t*-test; $P < 0.05$ was considered to indicate statistical significance.

RESULTS

EFFECTS OF IFN-α_{2b} AND PDF ON T24 CELL GROWTH

To examine possible effects of IFN-α_{2b} and PDF on T24 cell proliferation, cells were cultured with the varying concentrations of IFN-α_{2b} (0–50 000 IU/mL) or PDF (0–700 μg/mL) for 72 h. IFN-α_{2b} caused an ≈50% reduction in cell number at 20 000 IU/mL, which further declined to ≈66% at 50 000 IU/mL (Fig. 1A). PDF had no effects up to 200 μg/mL, but there was an ≈20% and ≈53% growth reduction at 400 and 700 μg/mL, respectively (Fig. 1B). Thus, these results show that 20 000 IU/mL of IFN-α_{2b} or 700 μg/mL of PDF is required to significantly inhibit the growth of T24 cells.

SYNERGISTIC GROWTH INHIBITORY EFFECTS OF IFN-α_{2b} AND PDF

We next examined whether the combination of IFN-α_{2b} and PDF might have a better growth inhibitory effect. Cells were cultured with combinations of IFN-α_{2b} and PDF at the varying concentrations and cell growth was assessed at 72 h. The combination of 10 000 IU/mL IFN-α_{2b} and 200 μg/mL PDF resulted in an ≈75% reduction in cell growth (Fig. 2). This suggests that the IFN-α_{2b}/PDF-induced growth reduction is due to a synergistic effect, because the effect of the two agents was greater than the effect of each agent individually (Fig. 1A,B).

*FIG. 1. Effects of IFN-α_{2b} or PDF on T24 cell growth. T24 cells were cultured with varying concentrations of either IFN-α_{2b} (0–50 000 IU/mL) or PDF (0–700 μg/mL), and cell numbers in IFN-α_{2b}-treated (**A**) or PDF-treated (**B**) were determined at 72 h. All data are the mean ± SD from three separate experiments; *P < 0.03; **P < 0.05.*

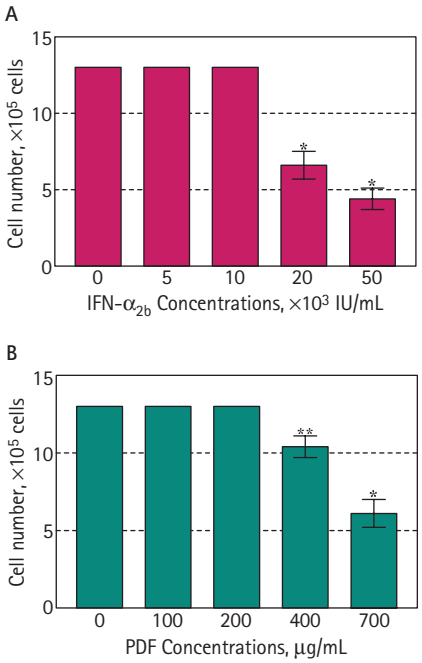


*FIG. 2. Effects of combinations of IFN-α_{2b} and PDF on cell growth. Cells were cultured with varying concentrations of IFN-α_{2b}/PDF combination for 72 h, and cell growth was assessed by the percentage relative to the cell number in control (100%). Cell growth in control, IFN-α_{2b} (10 000 IU/mL)-treated, PDF (200 μg/mL)-treated, or IFN-α_{2b} (10 000 IU/mL)/PDF (200 μg/mL)-treated cells is shown. The data are mean ± SD from three independent experiments; *P < 0.02.*

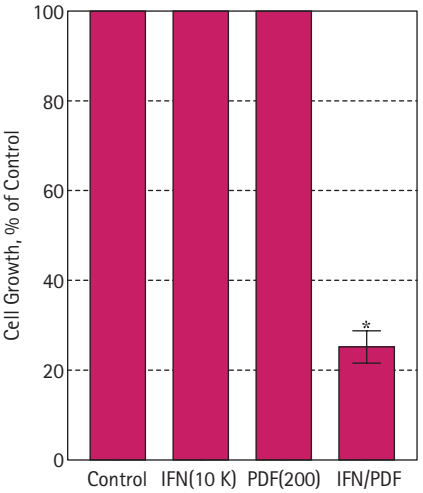
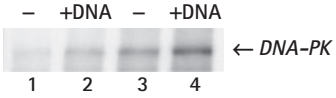


TABLE 1 Cell cycle analysis after treatments with IFN-α_{2b} and PDF

Culture conditions	% of cells in cell cycle phases G_1	S	G_2/M
Mean (SD)†:			
Control	49.1 (4.9)	38.2 (2.6)	12.7 (1.6)
+ IFN-α_{2b} (10 000 IU/mL)	51.4 (4.0)	36.7 (3.5)	11.9 (0.9)
+ PDF (200 μg/mL)	50.2 (4.6)	37.7 (3.0)	12.1 (1.4)
+ IFN-α_{2b}/PDF (10 000 IU/mL)/(200 μg/mL)	76.1 (4.4)*	14.1 (1.6)*	9.8 (1.2)

*†from three separate experiments; *P < 0.05 vs control.*

FIG. 3. In vitro *phosphorylation assays. Cell lysates from control (lanes 1 and 2) and IFN-α_{2b}/PDF-treated cells (lanes 3 and 4) for 72 h were subjected to* in vitro *phosphorylation assays with (lanes 2 and 4) or without (lanes 1 and 3) DNA, as described in the Methods. Autophosphorylated DNA-PK detected on autoradiogram is shown, and the relative intensities of DNA-PK bands were quantified (by a scan densitometer) and expressed by arbitrary values.*



Relative intensities of DNA-PK bands on above autoradiogram

Conditions	Control (Lanes 1 and 2)	+IFN/PDF (Lanes 3 and 4)
No Addition (Lanes 1 and 3)	1.0	**2.8**
+DNA (Lanes 2 and 4)	1.2	**8.6**

EFFECTS OF COMBINED IFN-α_{2b} AND PDF ON THE CELL CYCLE

To explore the underlying mechanism of such a synergistic growth inhibition induced by the IFN-α_{2b}/PDF combination, cells were treated with IFN-α_{2b} (10 000 IU/mL), PDF (200 μg/mL), or their combination for 72 h and subjected to cell cycle analysis. As shown in Table 1, IFN-α_{2b} or PDF alone had little effects similar to cell cycle phase distribution in control cells; in contrast, the IFN-α_{2b}/PDF combination caused an ≈63% decrease in cell number in the S phase with a concomitant 55% increase in the G_1-phase cell population, compared with those in controls. These results thus indicate that the IFN-α_{2b}/PDF combination may cause a blockage of cells entering from the G_1 to the S phase, known as a G_1 cell cycle arrest.

INVOLVEMENT OF DNA-DEPENDENT PROTEIN KINASE (DNA-PK) IN GROWTH INHIBITION

As many proteins and enzymes are known to be modulated by IFNs [20,21], it is feasible that the growth-inhibitory activity of IFN-α_{2b} with PDF may involve specific PK(s) acting on the signal transduction pathway for cell proliferation. Particularly, we are interested in a PK, namely double-stranded DNA-PK, whose activity relies essentially on small double-stranded DNA [22]. Accordingly, cell extracts obtained from control and IFN-α_{2b} (10 000 IU/mL)/PDF (200 μg/mL)-treated cells at 72 h were subjected to *in vitro* phosphorylation assay in the presence or absence of exogenous DNA (served as a DNA-PK activator). The basal phosphorylation state of DNA-PK in IFN-α_{2b}/PDF-treated cells was ≈three-fold higher than controls (compare lanes 1 with 3 in Fig. 3), and such phosphorylation was increased (autophosphorylated) by additional three-fold with DNA (compare lanes 3 with 4 in Fig. 3). Therefore, these studies show that the IFN-α_{2b}/PDF-induced growth inhibition is accompanied by activation of DNA-PK.

DISCUSSION

In an attempt to establish an improved method for bladder cancer therapy, pilot clinical trials using combinations of IFN-α and BCG has been conducted. These trials showed that such combined therapy could lower BCG toxicity against tumours [13]. However, the exact mechanism by which IFN-α potentiates BCG-mediated anti-bladder cancer immunity has not been fully understood. In addition, IFN-α therapy has several drawbacks, such as high cost and repeated administration. A standard intravesical IFN-α instillation (combined with BCG) is carried out with 50–100 x 10^6 IU of IFN-α [23], although such a high dosage appears to be in excess of the actual amount needed for an effective antitumour immunity. Moreover, even a high dose of IFN-α may not be sufficient to induce optimal immunity because of its short retention time inside the bladder [23].

Consequently, we explored an alternative approach for bladder cancer immunotherapy by combining IFN-α_{2b} and PDF, a bioactive proteoglucan of maitake mushroom. We found that IFN-α_{2b} at concentrations of ≥20 000 IU/mL were able to induce a significant (≈50%) growth reduction in T24 cells. PDF could be also effective at a relatively high concentration of 700 μg/mL, leading to an ≈53% growth inhibition. This PDF concentration seems to be yet higher than a physiologically achievable concentration, although such a concentration has not been established at this time.

Nevertheless, the possibility that PDF might be able to enhance or potentiate antiproliferative activity of IFN-α_{2b} was then tested. The combination of 10 000 IU/mL IFN-α_{2b} and 200 μg/mL PDF was capable of inducing an ≈75% growth reduction (Fig. 2). This enhanced growth inhibition probably resulted from a synergistic potentiation of the two agents, because neither IFN-α_{2b} (10 000 IU/mL) nor PDF (200 μg/mL) alone had such growth-inhibitory activity (Fig. 1A,B). It also shows that a relatively low concentration (10 000 IU/mL) of IFN-α_{2b} was required to attain a better growth inhibitory effect (≈75%) when combined with PDF (Fig. 2), compared with the inhibitory effect (≈66%) attained by 50 000 IU/mL IFN-α_{2b} alone (Fig. 1A). In other words, compared with the amount of IFN-α_{2b} needed to be effective as a sole agent, merely a fifth of that IFN-α_{2b} dose would be needed to exhibit the better, enhanced growth-inhibitory activity when combined with PDF. Thus, it is plausible that PDF may not only help potentiate IFN-α_{2b} activity but may also help to reduce the cost of treatment.

We next examined the effects of IFN-α_{2b}/PDF combination on cell cycle regulation to explore the growth inhibitory mechanism. Cell cycle analysis showed an ≈63% decrease in the S-phase cell number with a concomitant 55% increase in the G_1-phase cell number after treatment with the IFN-α_{2b}/PDF combination (Table 1). Thus, these results

confirm that the IFN-α_{2b}/PDF-induced growth inhibition was mediated through a blockage of the G_1–S phase transition (i.e. a G_1 arrest). This further suggests that the growth inhibitory action of the IFN-α_{2b}/PDF combination may target primarily the G_1–S phase progression in the cell cycle. To date there is very little information relevant to such INF-α combined therapy, but IFN-α has been shown to block the S–G_2 phase transition in some cancers including bladder cancer cells [24]. Yet, this difference in cell cycle arrest (from the present study) is most probably due to the different bladder cancer cells that were studied.

As mentioned earlier, IFNs can regulate cell proliferation via signal transduction pathways involving specific PKs [20,21]. Also, it has been reported that IFNs can induce DNA fragmentation, leading to an accumulation of small or low-molecular weight DNA [25]. Accordingly, we focused on one of such PKs, DNA-PK, which requires small double-stranded DNA for its activation [22] and plays an important role in cell cycle regulation [26]. It should be mentioned that our pilot DNA analysis verified DNA fragmentation detected in IFN-α_{2b}/PDF-treated cells (data not shown). The possible involvement of DNA-PK in such an IFN-α_{2b}/PDF-induced growth inhibition was then examined using *in vitro* phosphorylation assays. The basal phosphorylation state of DNA-PK was significantly (about three-fold) higher in IFN-α_{2b}/PDF-treated cells and was further increased in response to exogenously added DNA. This DNA-PK autophosphorylation (self-phosphorylation) with DNA is indicative of its enzymatic activation [26]. Thus, these results confirm that endogenous DNA-PK is indeed activated by the IFN-α_{2b}/PDF combination in T24 cells, although the exact role of DNA-PK in this IFN-α_{2b}/PDF-induced growth inhibition yet remains to be fully defined.

In conclusion, the present study shows that PDF synergistically potentiates the anticancer/antiproliferative activity of IFN-α_{2b} on bladder cancer T24 cells. This enhanced growth inhibition results from a G_1 cell cycle arrest together with activation of DNA-PK. It is conceivable that such an antiproliferative mechanism is associated with an accumulation of low-molecular weight DNA, triggering DNA-PK activation that might act primarily on the cell cycle to cease cancer cell growth. Therefore, the low-dose IFN-α_{2b}/PDF combination may provide an alternative, improved immunotherapy for superficial bladder cancer and thus clinical studies/trials are warranted.

CONFLICT OF INTEREST

None declared. Source of funding: departmental.

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Correspondence: Sensuke Konno, Department of Urology, New York Medical College, Munger Pavilion 4th Floor, Valhalla, NY 10595, USA.
e-mail: sensuke_konno@nymc.edu

Abbreviations: **IFN**, interferon; **PDF**, maitake mushroom D-fraction; **FDA**, USA Food and Drug Administration; **DNA-(PK)**, double-stranded DNA-dependent (protein kinase).