学 位 論 文 の 要 旨

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学 位 論 文 名　Pifithrin-μ, an Inhibitor of Heat-Shock Protein 70, Can Increase the Antitumor Effects of Hyperthermia Against Human Prostate Cancer Cells

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論 文 内 容 の 要 旨

INTRODUCTION

Hyperthermia (HT) is an effective therapy that has low toxicity, mild side-effects, and can improve the efficacy of other types of anti-cancer therapies. However, HT is inevitably associated with heat-shock proteins (HSPs). Among the HSPs, HSP70 is a stress-inducible HSP that has been reported to play a role in therapy-resistance. Reducing HSP70 levels in some cultured tumor cells has been reported to induce cell death and to sensitize them to cytotoxic agents, while having no obvious deleterious effects on non-tumor cells.

Pifithrin (PFT)-μ (2-phenylethynesulfonamide) was initially identified as a small-molecule inhibitor of p53. Thereafter, PFT-μ was revealed to interact selectively with HSP70 and to inhibit its functions. This information led us to test the hypothesis that PFT-μ could enhance HT-induced antitumor effects against cancer cells. In this study, after confirming that HSP70 is constitutively expressed and/or enhanced by HT and plays a pro-survival role in human prostate cancer cells, we determined whether the combination of suboptimal doses of PFT-μ could enhance HT-induced antitumor effects on human prostate cancer *in vitro*, and whether the combination therapy could inhibit tumor growth in a xenograft mouse model.

MATERIALS AND METHODS

Three human prostate cancer cell lines (LNCaP, PC-3, and DU-145) were used. Cell viability was evaluated using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay. To carry out HT, these cell

lines were incubated at 43°C for 2 h. To selectively knock down HSP70, HSP70 siRNA was transfected using Lipofectamine™ RNAiMAX, according to the manufacturer's instructions. Colony-forming ability was determined by counting colonies after fixing with methanol and staining with 0.05% crystal violet. Immunoblot was performed to examine the protein levels of HSP70, HSP90, c-Myc, and cyclinD1. Cell death was assessed by using the Annexin V-FITC Apoptosis Detection Kit, APC-conjugated Annxin V, and PI. To examine cell cycle and proliferation of cancer cells, a BrdU/7AAD Prolifesration Kit was used. To examine the *in vivo* antitumor effect, BALB *nu/nu* male mice were inoculated in the right footpad with PC-3 cells with Matrigel. Thereafter, the mice were pooled and divided into four groups, and treated with PFT-μ and/or HT. To perform HT therapy, these PC-3-bearing mice had only their footpads bathed in 43°C water for 30 min.

RESULTS AND DISCUSSION

We first assessed the HSP70 protein levels in three human prostate cancer cell lines before and after treatment with HT (43°C for 2 h). Although no definite change was observed in PC-3, the expression levels in LNCaP and DU-145 were increased after HT. We next determined whether HSP70 plays a pro-survival role in prostate cancer cells. Selective knockdown of HSP70 in three prostate cancer cell lines decreased the cell viability and the colony-forming ability, suggesting that HSP70 plays a pro-survival role in human prostate cancer cells.

We next compared the antitumor effect of PFT-μ to that of Quercetin, a heat-shock factor (HSF)-1 inhibitor, which inhibits all heat-shock-induced genes, including *HSP70* gene. Both Quercetin and PFT-μ decreased the viability of three prostate cancer cell lines in a dose-dependent manner, but PFT-μ exerted its antitumor effect at almost one-tenth the dose of Quercetin. PFT-μ had no effect on the HSP70 protein expression. In addition, knockdown of HSP70 failed to influence the expression of HSP90, another key HSP of the stress response pathway. Then, we assessed the antitumor effects induced by the combination of HT and PFT-μ. The viability of cancer cells was decreased significantly when HT was combined with a suboptimal dose (5 μM) of PFT-μ. We also determined whether the combination effect could be observed in cancer cells that were pre-transfected with HSP70 siRNA, and found that pre-knockdown of HSP70 abolished the combination effect against PC-3 and DU-145. We next investigated the effect of combination therapy with HT and PFT-μ on the colony-forming ability of prostate cancer cells. The combination therapy significantly decreased the colony-forming ability of PC-3 and DU-145 cells and decreased the viability of LNCaP cells in the long-term (12-day) culture. These results indicate that suboptimal doses of PFT-μ can enhance HT-induced antitumor effects on human prostate cancer cells via HSP70 inhibition.

We evaluated the antitumor effects by measuring cell viability 2 days after treatment with HT and PFT-μ. However, such effects on cell viability may reflect alterations in cell death and/or growth. Therefore, we next assessed the underlying mechanisms of action. HT alone failed to

increase the percentage of Annexin-V^+ cells, whereas treatment with PFT-μ increased it slightly. However, the combination treatment drastically increased the percentage of Annexin V^+ cells, especially in LNCaP cells. Additionally, adding z-VAD, a pan-caspase inhibitor, partially reduced the percentage of Annexin V^+ cells in LNCaP after combination treatment of HT and PFT-μ. These results indicate that, although the efficacy varies among cancer cell lines, combination therapy with HT and PFT-μ can enhance death of prostate cancer cells, and that the combination therapy-induced cell death of LNCaP is partially caspase-dependent. We further investigated whether cell growth arrest was involved in the antitumor effects induced by combination therapy with HT and PFT-μ. We assessed the proliferation and cell cycle of cancer cells by evaluating BrdU uptake and 7AAD staining. We found that combination therapy with HT and PFT-μ decreased the percentage of $BrdU^+$ S-phase cancer cells and increased that of G2/M phase cancer cells in the three cell lines. We also assessed the expression of cell cycle-related molecules and found that the combination therapy resulted in decreased expression of c-Myc in LNCaP and decreased expression of cyclinD1 in PC-3 and DU-145. Additionally, the expression of $p21^{WAF1/Cip}$ was increased in the three cancer cell lines. These data suggest that G2/M growth arrest contributes to the antitumor effects induced by combination therapy with HT and PFT-μ.

Finally, we evaluated whether combination therapy with HT and PFT-μ exerted an antitumor effect on established human prostate cancer in a xenograft mouse model. Although the local administration of PFT-μ had no antitumor effect but, rather, promoted the tumor growth, and HT decreased the tumor growth slightly but not significantly, the combination therapy with HT and PFT-μ significantly suppressed the tumor growth of PC-3 compared with the control group.

Among a variety of cancer types, HT is applicable especially to prostate cancer. However, HT inevitably evokes stress responses in cancer cells. In this study, we showed that combination therapy with HT and PFT-μ significantly decreased the cell viability of three prostate cancer cell lines compared to treatment with either alone. Regarding the underlying mechanism, we tested two possibilities; *i.e.*, cell death and cell growth arrest, and found that the combination therapy can induce cell death, partially caspase-dependent, and growth arrest in cancer cells.

CONCLUSION

In conclusion, we investigated the sensitizing effect of PFT-μ, a small molecule inhibitor of HSP70, when human prostate cancer cells were treated with HT. Our findings suggest that PFT-μ effectively enhances HT-induced antitumor effects both *in vitro* and *in vivo*, and that PFT-μ is a promising agent for use in combination with HT to treat prostate cancer.