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.

論文審査及び最終試験又は学力の確認の結果の要旨

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論文審査の結果の要旨

がんの温熱療法 hyperthermia (HT)は、さまざまな腫瘍に適応されその有用性が認められている。副作用が少なく既存の放射線療法や化学療法との併用によって効果を増強できるが、必然的に熱ショック蛋白(HSP: heat shock protein)が誘導され、細胞保護因子として作用する。特に、HSPファミリーの中でもHSP70はストレス誘導性HSPであり、癌細胞で発現が高く、治療抵抗性に関与していることが知られている。一方、Pifithrin (PFT)-μ は、HSP70と選択的に結合することにより機能を阻害する新規HSP70阻害剤である。本研究では、ヒト前立腺癌細胞におけるHSP70の細胞生存・増殖に及ぼす役割と、HTとPFT-μの併用による抗癌効果を検討し、以下の結果を得た。① small interfering RNA (siRNA)によるHSP70の発現低下は、3種類のヒト前立腺癌細胞株(LNCaP、PC-3、DU-145)の細胞生存率とコロニー形成能を低下させた。② PFT-μ は、全HSPを阻害する Quercetin の 1/10 の濃度で前立腺癌細胞の細胞生存率を低下させた。③ HT（43°Cで2時間）と PFT-μ の併用は、3種類のヒト前立腺癌細胞株の細胞生存率を相乗的または相加的に低下させた。④ HSP70の発現をsiRNAで低下させた前立腺癌細胞では、HTとPFT-μ の併用効果は認めなかった。⑤ 3種類の前立腺癌細胞株の中でLNCaPだけは、HT とPFT-μ の併用による抗癌効果に カスペース依存性のアポトーシスが部分的に関与していた。⑥ Bromodeoxyuridine(BrdU) / 7-amino-actinomycin D(7-AAD)染色による細胞周期解析では、HT と PFT-μ の併用により S期のBrdU陽性細胞が減少し、G2/M期のBrdU陽性細胞が増加していた。⑦ 細胞周期に関連する分子の発現を イムノブロット法で解析したところ、HT と PFT-μ の併用により c-Myc と サイクリンD1 の発現は低下し、$p21^{WAF1/Cip}$ の発現は増加していた。⑧ PC-3細胞を足底部に異種移植したヌードマウスにおいても、HT (43°Cで30分) とPFT-μ の併用効果を認めた。以上より、ヒト前立腺癌に対する HT に PFT-μ を併用する有用性と作用機序が明らかになった。本研究は、前立腺癌に対する HT の治療効果を高めることができる有用な方法を提供する意義のあるものと判断される。

最終試験又は学力の確認の結果の要旨

申請者は、熱ショック蛋白に注目し、前立腺がん細胞を用いた温熱療法に対するHSP70の発現と、その阻害薬の効果について検討した。本研究により、HSP70の阻害が前立腺がん細胞の温熱感受性の増強をもたらすことが証明された。公開審査における質疑応答も的確で背景、関連する分野の知識も充分であり、学位授与に値すると判断した。（主査：礒部　威）

申請者は、3種類のヒト前立腺癌細胞を用いて、前立腺癌細胞が有するアンドロゲン依存性に関わらずHSP70の選択的阻害剤(Pifithrin - μ)により温熱療法の抗腫瘍効果が促進される可能性を示した。発表も適切であり、質疑応答も的確であったので、学位授与に値すると判断した。
（副査：椎名　浩昭）

申請者は、ヒト前立腺癌細胞を用いて、温熱療法とHSP70阻害剤であるPFT-μ の併用による抗腫瘍作用の増強機構の解明を行い、臨床応用にも可能な重要な知見を示した。また関連知識も豊富で質疑応答も的確なため、学位授与に値するものと判断した。（副査：松本　健一）

（備考）要旨は、それぞれ４００字程度とする。