Valproic Acid Enhances the Anti-tumor Effect of Pegylated Interferon-α Towards Pancreatic Cancer Cell Lines

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Abstract. *Background: Valproic acid (VPA) acts as a specific inhibitor of class I HDACs and it use has been proven to be safe since a long time. Materials and Methods: In the present study, we investigated the effect of VPA in the combination with pegylated interferon-α (PEG-IFNα) in inhibition of cell proliferation of human pancreatic cancer cell lines. Results: VPA enhanced the effect of PEG-IFNα, and the effect was decreased by the caspase inhibitor. VPA alone and VPA in combination with PEG-IFNα increased the expression of interferon-α receptor and interferon regulatory factor 8. Conclusion: The combination of VPA and PEG-IFNα can be useful for the treatment of pancreatic cancer.*

Pancreatic cancer is the fifth leading cause of death related to cancer in Japan. Surgery is the sole curative therapy, and for advanced pancreatic cancer, surgical resection is followed by adjuvant chemotherapy. However, the response rates to chemotherapy are below 20%, with a median life expectancy of 19 months (1, 2). The statistics for second-line therapy are even more dismal, with response rates <10% and median survival of 4 months (3). So, it is imperative to improve the chemotherapy in the management of pancreatic cancer. Recently, it has been reported (4) that interferon-α (IFNα) in combination with adjuvant chemoradiotherapy improved 5-year survival to 55%, and IFNα seems to play a crucial role in these treatment regimens (5).

There is growing interest in histone deacetylase (HDAC) inhibitors as anticancer drugs due to their ability to induce cell differentiation, growth arrest and apoptosis (6). Acetylation and de-acetylation of histones play an important role in the regulation of gene transcription and in the modulation of chromatin structure (7, 8). HDAC activity has been identified in several types of cancers (9, 10, 11) and a number of HDAC inhibitors have been characterized to inhibit tumor growth *in vitro* and *in vivo* (12, 13). HDAC inhibitors comprise of a diverse range of unrelated compounds that all induce an accumulation of hyperacetylated histones, resulting in various biological effects (*e.g.*, cell-cycle arrest, cell death, or differentiation) (14). These outcomes are to a large extent cell-type specific and have raised the potential that HDAC inhibitors may represent a promising new target of anticancer drugs.

Valproic acid (VPA) is a well-established drug in the long-term therapy of epilepsy, and has been safely used since a long time. In addition, VPA acts as a specific inhibitor of class I HDACs and induces proteasomal degradation of HDAC2, leading to cellular differentiation, growth arrest, and death *in vivo* and *in vitro* (15, 16). In the present study, we describe the anticancer effects of VPA in combination with pegylated interferon-α (PEG-IFNα) on pancreatic cancer cell lines.

Materials and Methods

Cell culture. Human pancreatic cancer cell lines BxPC3 and SUIT-2 were purchased from ATCC (American Type Culture Collection, Manassas, VA, United States). Tumor cells were cultured in RPMI-1640 medium (Sigma-Aldrich Corporation, St. Louis, MO, United States) supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified, 5% CO_2 incubator.

Cell proliferation assay. The effect of VPA and PEG-IFNα on cell proliferation was determined by WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) assay, as described previously (17). Cell proliferation was measured using a Cell Counting Kit-8 (Dojindo, Machidahara, Kumamoto, Japan). Experiments were carried-out in triplicate. Briefly, tumor cells (10^4 cells) were treated with VPA (Sigma-Aldrich Corporation, St. Louis, MO, United

Abbreviations: VPA, Valproic acid; PEG-IFNα, pegylated interferon α; IFNAR, interferon alpha receptor; IRF8, interferon regulatory factor 8.

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States) at a final concentration of 0.5, 1.0, 1.5 or 3.0 mM for 3 days. Tumor cells were also treated with PEG-IFNα (Schering-Plough, Kenilworth, NJ, United States) at final concentrations of 10^3, 10^4 and 10^5 U/ml). Controls were untreated. In the combination group, tumor cells were treated with VPA and PEG-IFNα and compared to cells treated with VPA or PEG-IFNα alone, or controls. In addition, to investigate the effect of caspase inhibition, the caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]–fluoromethylketone (Z-VAD-FMK), (Promega Corporation, Madison, WI, United States) was added to the cells (10 μM), and cell proliferation was measured using the Cell Counting Kit-8. All experiments were done in triplicate and the representative data is shown.

Caspase 3 and 7 activity. To investigate the effect of VPA and PEG-IFNα on caspase 3 and 7 activity, the Caspase-Glo3/7 Assay kit (Promega Corporation, Madison, WI, United States) was used. After treating tumor cells with VPA and PEG-IFNα for 72 h, Caspase-Glo3/7 Assay kit was used as described in the manufacturer's protocol.

Real-Time PCR. Quantitative real-time RT-PCR was performed on the ABI Prism 7,500 using the commercially available gene expression assay for interferon-α receptor (*IFNAR1, IFNAR2*), interferon regulatory factor-8 (*IRF8*) and glyceraldehyde phosphate dehydrogenase (GAPDH) (Hs00265057_m1, Hs00175238_m1, Hs00174198_m1 and Hs99999905_m1). A 25 ml final reaction volume containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, Unites States), 1x Multiscribe with RNase inhibitors, and 1x gene expression assay was used to amplify 25 ng of total RNA with the following cycling conditions: 30 min at 48˚C, 10 min at 95˚C, then 50 cycles of 95˚C for 15 sec, and 60˚C for 1 min. The 7,500 Sequence Detection System 1.3.1 software automatically determined fold change for each gene in each sample using the $\Delta\Delta_{CT}$ method (18). Samples were quantified by dividing each mRNA level with the signal obtained for the GAPDH standard.

Statistical analysis. Statistical comparisons of mean values was performed by one-way ANOVA. Statistical analysis was performed using the StatView 5.0J software (SAS Institute, Inc., Cary, NC, United States). A *p*-value of less than 0.05 was considered statistically significant.

Results

Combination of VPA and PEG-IFNα inhibits cell proliferation in pancreatic cancer cell lines. To examine whether VPA and PEG-IFNα can inhibit cell proliferation, and to test whether VPA can enhance the effect of PEG-IFNα, a cell proliferation assay was employed. BxPC3 or SUIT-2 cells (10^4 cells) were treated with VPA and/or PEG-IFNα at various concentrations for 72 h. As shown in Figure 1, VPA at the 0.5 mM concentration alone did not significantly decrease cell proliferation of BxPC3 and SUIT-2. On the other hand, PEG-IFNα of 10^3 U/ml significantly decreased cell proliferation of BxPC3 and SUIT-2. VPA enhanced the effect of PEG-IFNα in BxPC3 cell after 72 h treatment. These data indicate that VPA in conjunction with PEG-IFNα could synergistically inhibit pancreatic cancer cell proliferation.

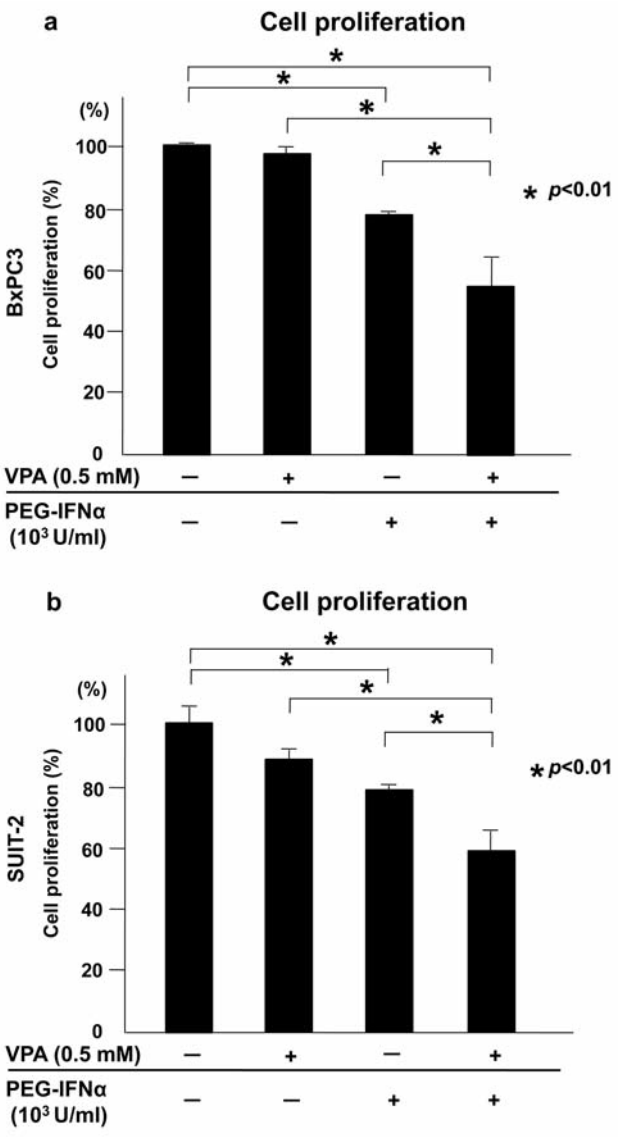

Figure 1. *The effect of VPA and/or PEG-IFNα in inhibiting cell proliferation of the BxPC3 (a) and SUIT-2 (b) human pancreatic cancer cell lines. VPA enhanced the anti-tumor effect of PEG-IFNα in BxPC3 cell after 72 h treatment. Results are expressed as the percentages of live cells compared to those without VPA and/or PEG-IFNα treatment. The error bars indicate the standard deviation. *p<0.05 vs. control and single-agent therapy.*

The anti-proliferating effect of VPA and PEG-IFNα depends on the caspase pathway. The role of the caspase pathway was examined in the anti-tumor effect of VPA and PEG-IFNα. We used the caspase inhibitor, Z-VAD-FMK, with VPA and/or PEG-IFN α. As shown in Figure 2, after a 72 h treatment, with or without Z-VAD-FMK, caspase inhibition decreased the anti-tumor effect of the combination of VPA and PEG-IFNα. This suggests that the anti-tumor effect of

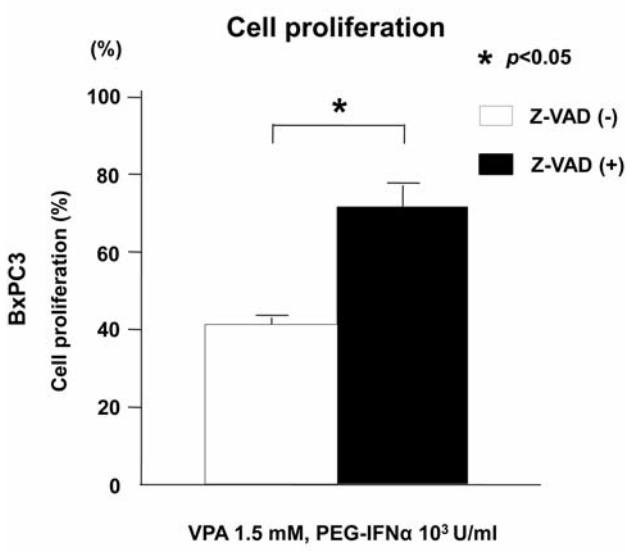

Figure 2. *Effect of the caspase inhibitor in anti-tumor effect of VPA and/or PEG-IFNα. By using caspase inhibitor Z-VAD-FMK, the effect of VPA and PEG-IFNα in inhibiting cell proliferation was decreased. Results are expressed as the percentages of live cells compared to the control group. The error bars indicate the standard deviation.*

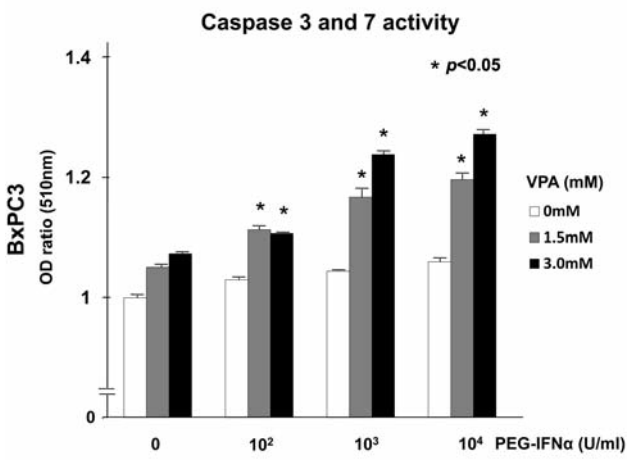

Figure 3. *Caspase 3 and 7 activity induced by VPA and/or PEG-IFNα. The combination of VPA and PEG-IFNα increased the caspase 3 and 7 activity. The error bars indicate the standard deviation. *p<0.05 vs. control.*

the combination effect of VPA and PEG- IFN α could depend on the caspase pathway.

Combination of VPA and PEG-IFNα enhances caspase 3/7 activity. Based on the result shown in Figure 2 we measured the caspase 3 and 7 activity to confirm the role of the apoptosis pathway in pancreatic cancer cells treated with VPA and/or PEG-IFNα. BxPC3 cells (10^4 cells) were treated with VPA and/or PEG-IFNα at a various concentrations. After 72 h in culture, caspase 3 and 7 activities were measured. VPA-alone and PEG-IFNα-alone did not significantly increase caspase 3 and 7 activities. However, the combination of VPA and PEG-IFNα caused significant increases in caspase 3/7 activities (Figure 3).

Combination of VPA and PEG-IFNα induced the expression of IFNAR and IRF8. In addition to apoptosis *via* the caspase pathway, we investigated the effect of VPA and PEG-IFNα towards sensitivity of interferon-α, and the restoration of IRF8 function in pancreatic cancer cells. BxPC3 and SUIT-2 cells were treated with VPA and/or PEG-IFNα for 72 h. After 72 h, total RNA was obatined from each group, and mRNA expression of *IFNAR1*, *IFNAR2* and *IRF8* were measured by real-time PCR. As shown in Figure 4, VPA significantly increased the expression of *IFNAR1, IFNAR2* and significantly increased the expression *IRF8* (over 300-fold change). Moreover, VPA with PEG-IFNα further increased mRNA expression of *IFNAR1, IFNAR2* and *IRF8* compared to VPA-alone.

Discussion

In the present study we showed that VPA enhances the anti-tumor effect of PEG-IFNα *via* the caspase pathway, and also induces the expression of the IFNAR and IRF8 against the pancreatic cancer cell lines BxPC3 and SUIT-2. A previous report described the potent anti-tumor effects of VPA against various cancers such as leukemia, prostate cancer, colon cancer, and breast cancer. There were encouraging results in early clinical trials of VPA, either alone or in combination with de-methylating and/or cytotoxic agents. In addition, whole-genome expression by microarray analysis from the primary tumors of patients treated with VPA showed significant up-regulation of hundreds of genes involved in ribosomal proteins, oxidative phosphorylation, mitogen-activated protein kinase signaling, focal adhesion, cell cycle, antigen processing and presentation, proteasome, apoptosis, phosphoinositide 3-kinase (PI3K) signaling, wnt signaling, calcium signaling, transforming growth factor-β (TGF-β) signaling, and ubiquitin-mediated proteolysis, among others (19). In this study VPA-alone (0.5 mM) did not significantly inhibit cell proliferation of pancreatic cancer cell lines. However, in the presence of 0.5 mM VPA, the anti-tumor effect of PEG-IFNα was significantly increased. Because VPA at a dose of 0.5-1.0 mM corresponds to plasma levels in patients treated for epilepsy, and does not result in any serious side-effects, we suggest that VPA may be a promising drug for clinical trials for pancreatic cancers

Since there is evidence of cancer immunosurveillance, new chemotherapeutic protocols include immunomodulatory agents such as interferon-α in their treatment regime. *In vitro*

and *in vivo* studies have demonstrated the efficacy of type I IFNs (*e.g.*, IFN-α, IFN-β, IFN-γ, and IFN-κ), in the treatment of several tumors (20-24). In addition, in previous studies, it has been reported that PEG-IFNα i) induces apoptosis through the TRAIL pathway (25), ii) inhibits NF-κB (26), and iii) inhibits angiogenesis (inhibition of bFGF, IL-8, MMP-2, MMP-9) (27). Because some of these pathways are common between IFN-α and HDAC inhibitors, in the current study we focused on the anticancer effect of VPA and PEG-IFNα.

PEG-IFNα (10^3 U/ml) alone significantly inhibited the cell proliferation of BxPC3 and SUIT-2. Moreover, in combination with VPA (0.5 mM) and PEG-IFNα (10^3 U/ml), VPA enhanced the effect of PEG-IFNα in BxPC3 and SUIT-2 cells after 72 h in culture. Next, we focused on the caspases, and we used the caspase inhibitor, Z-VAD-FMK, to inhibit the caspase pathway. It was shown that the caspase inhibitor decreased the effect of VPA and PEG-IFNα on the inhibition of cell proliferation. We also examined caspase 3 and 7 activities to confirm that VPA and PEG-IFNα stimulated the caspase pathway. The combination of VPA and PEG-IFNα increased caspase 3 and 7 activities although VPA- or PEG-IFNα-alone did not significantly induce the caspase 3 and 7 activation. From these results, we conclude that the effect of VPA and PEG-IFNα depends on the caspase pathway. It is well-known that the representative apoptosis pathway consists of death ligands (TNF-α, FasL, TRAIL), death receptors (TNFR, Fas, DR3, DR4, and DR5) and the caspase pathway. In this cascade, it has been reported that a HDAC inhibitor induced the death ligands and receptors (28, 29), and IFNα induced the death ligands (30). The present study revealed an activation of caspase 3 and 7 activities, therefore we need to confirm the effect of VPA and PEG-IFNα against death ligands and receptors in further studies.

In a previous report regarding the combination of VPA and IFNα, it was shown that VPA and IFNα synergistically decreased the growth of neuroblastoma (31) and renal cancer (32). In these reports, they suggested that VPA and IFNα could affect cell-cycle regulation. They showed that the decrease of CDK2 and cyclin D3, and the increase of p21 and Rb occurred in a combination of VPA and IFNα. We also need to consider this point in future studies.

In our study, we investigated the expression of IFNAR and IRF8 as a new effect of VPA. VPA alone did not significantly decrease the cell proliferation of BxPC3 and SUIT-2, but enhanced the effect of PEG-IFNα. So, we hypothesized that VPA could improve the sensitivity of IFNα in pancreatic cancer cell lines. Human pancreatic cancer cell lines express IFNAR (33), and recent *in vitro* studies showed that IFNs have anti-proliferative effects on pancreatic cancer cell lines expressing IFNAR (34-36). It is well-known that IFNAR families form a high affinity-binding site and initiate signal transduction, leading to the induction of IFN-responsive genes, perhaps including the genes related to apoptosis pathway such as death ligands. The soluble form may act as a regulator of free IFNs and, depending on the concentration, leads to the neutralization or even enhancement of IFN bioactivity (37, 38). In our data, the use of PEG-IFNα did not significantly increase the IFNAR expression in BxPC3 and SUIT-2. However, VPA significantly increased IFNAR expression and the combination of VPA and PEG-IFNα up-regulated the expression of IFNAR mRNA level. One possible reason is that VPA enhanced the anti-tumor effect of PEG-IFNα.

IRF8, also known as IFN consensus sequence-binding protein, is a transcription factor of the IRF family, which also includes IRF1, IRF2, IRF3, IRF4/lymphoid-specific IRF/pip/ICSAT, IRF5, IRF6, IRF7, and ISGF3g (39). IRF8 is regulated by type-I IFNs, and it was reported that IRF8 is one of the cascade factors whose expression level is regulated by IFN-γ (40), and IFN-α and IL-12 activate IRF-8 gene expression in human NK and T cells (41). Recently, IRF8 expression has been shown in human cancer cell lines originating from solid tissues (42, 43), and disruption of IRF8 function in solid tumor cells inhibits tumor cell sensitivity to apoptosis induction. Furthermore, IRF8 is thought to be one of the cancer suppressing genes because IRF8-knockout mice present with myelomas (44). In cancer cells the IRF8 gene is often methylated, which inhibits its function (45), so we hypothesized that a HDAC inhibitor could recover the function of IRF8 and up-regulate the anti-tumor action of IFNα through apoptosis. In our data, IRF8 expression was significantly up-regulated by VPA alone or with the combination of VPA and PEG-IFNα. This suggests that VPA restored the IRF8 function in cancer cells through HDAC inhibition, and IRF8 expression was dramatically induced. However, further studies are needed to determine why the combination of VPA and PEG-IFNα increased expression more than VPA alone. However, it does suggest that the combination of VPA and PEG-IFNα induced the apoptosis through IRF8.

In conclusion, our data suggest that VPA enhances the anti-tumor effect of PEG-IFNα against pancreatic cancer cell lines, through inducing apoptosis *via* the caspase pathway, by recovering IRF8 function, and by improving the sensitivity for interferon-α. This suggests that the combination therapy of VPA and PEG-IFNα may be useful for the treatment of pancreatic cancer.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this manuscript.

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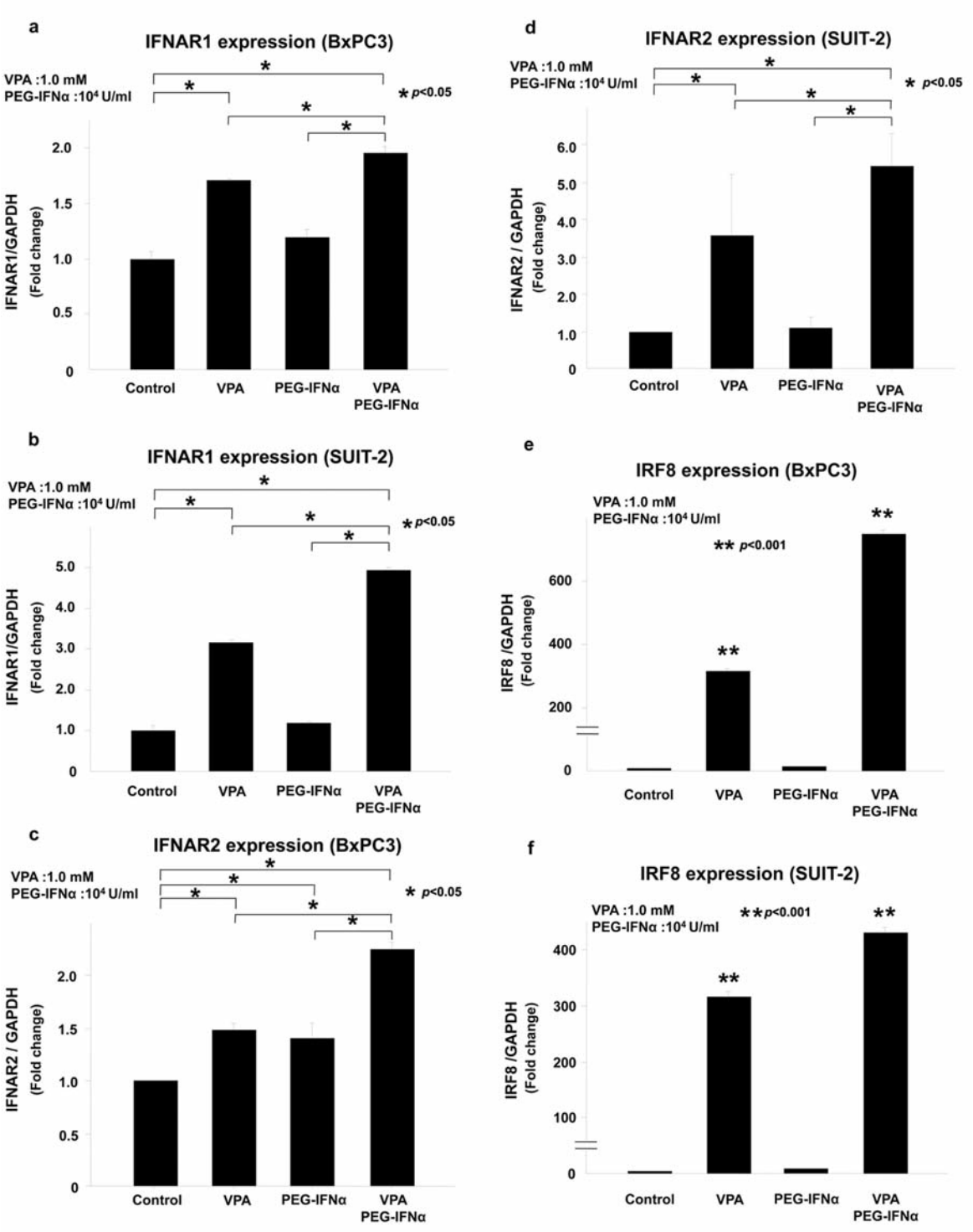

Figure 4. *a. The expression of IFNAR1 was induced by VPA in BxPC3 cells. Moreover, VPA with PEG-IFNα induced more IFNAR1 expression than VPA or PEG-IFNα alone. b. The expression of IFNAR1 was induced by VPA in SUIT-2 cells. Moreover, VPA with PEG-IFNα induced more IFNAR1 expression than VPA or PEG-IFNα alone. c. The expression of IFNAR2 was induced by VPA or PEG-IFNα in BxPC3 cells. Moreover, VPA with PEG-IFNα induced more IFNAR2 expression than VPA or PEG-IFNα alone. d. The expression of IFNAR2 was induced by VPA in SUIT-2 cells. Moreover, VPA with PEG-IFNα induced more IFNAR2 expression than VPA or PEG-IFNα alone. e. The expression of IRF8 was induced by VPA in BxPC3 cells. Moreover, VPA with PEG-IFNα induced more IRF8 expression than VPA alone. f. The expression of IRF8 was induced by VPA in SUIT-2 cells. Moreover, VPA with PEG-IFNα induced more IRF8 expression than VPA alone. The error bars indicate the standard deviation. *p<0.05 vs. control and single-agent therapy, **p<0.01 vs. control.*

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