DIPEPTIDYL PEPTIDASES AS SURVIVAL FACTORS IN EWING’S SARCOMA FAMILY OF TUMORS – IMPLICATIONS FOR TUMOR BIOLOGY AND THERAPY

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Running Head: Neuropeptide Y and dipeptidyl peptidases in Ewing’s sarcoma

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Ewing’s sarcoma family of tumors (ESFT) is a group of aggressive pediatric malignancies driven by the EWS-FLI1 fusion protein, an aberrant transcription factor up-regulating specific target genes, such as neuropeptide Y (NPY) and its Y1 and Y5 receptors (Y5Rs). Previously, we have shown that both exogenous and endogenous NPY stimulates ESFT cell death via its Y1 and Y5Rs. Here, we demonstrate that this effect is prevented by dipeptidyl peptidases (DPPs), which cleave NPY to its shorter form, NPY3-36, not active at Y1Rs. We have shown that NPY-induced cell death can be abolished by overexpression of DPPs and enhanced by their down-regulation. Both NPY treatment and DPP blockade activated the same cell death pathway mediated by poly(ADP-ribose) polymerase (PARP-1) and apoptosis-inducing factor (AIF). Moreover, the decrease in cell survival induced by DPP inhibition was blocked by Y1 and Y5R antagonists, confirming its dependence on endogenous NPY. Interestingly, similar levels of NPY-driven cell death were achieved by blocking membrane DPPIV and cytosolic DPP8 and DPP9. Thus, this is the first evidence of these intracellular DPPs cleaving releasable peptides, such as NPY, in live cells. In contrast, another membrane DPP, fibroblast-activation protein (FAP), did not affect NPY actions. In conclusion, DPPs act as survival factors for ESFT cells and protect them from cell death induced by endogenous NPY. This is the first demonstration that intracellular DPPs are involved in regulation of ESFT growth and may become potential therapeutic targets for these tumors.

Ewing’s sarcoma family of tumors (ESFT) is a group of aggressive pediatric malignancies. The characteristic feature of ESFT is a translocation resulting in the fusion of the EWS gene with an ETS transcription factor, most often FLI1. The EWS-FLI1 fusion protein acts as an aberrant transcription factor and is believed to trigger malignant transformation of ESFT cells (1). Recent studies identified multiple molecular targets of the EWS-FLI1 protein, which are up-regulated in ESFT (2,3). One such target, a 36 amino-acid sympathetic neurotransmitter, neuropeptide Y (NPY) and its Y1 and Y5 receptors (Y5Rs) are highly expressed in these tumors and belong to a group of “ESFT signature genes” (3-7). This is particularly intriguing since NPY acting through Y1 and Y5R antagonists, confirming its dependence on endogenous NPY. Interestingly, similar levels of NPY-driven cell death were achieved by blocking membrane DPPIV and cytosolic DPP8 and DPP9. Thus, this is the first evidence of these intracellular DPPs cleaving releasable peptides, such as NPY, in live cells. In contrast, another membrane DPP, fibroblast-activation protein (FAP), did not affect NPY actions. In conclusion, DPPs act as survival factors for ESFT cells and protect them from cell death induced by endogenous NPY. This is the first demonstration that intracellular DPPs are involved in regulation of ESFT growth and may become potential therapeutic targets for these tumors.
dipeptidyl peptidase IV (DPPIV) (4,13). DPPIV is a membrane-bound serine protease, which cleaves proline in the N-terminal penultimate position and modifies the activity of various regulatory peptides and chemokines (14-16). NPY is one of the best substrates of DPPIV. The protease converts full length NPY1-36 to a shorter form, NPY3-36, which is no longer able to bind to the Y1R but retains affinity for all other receptors (14,17). Therefore, in ESFT cells, DPPIV-dependent NPY cleavage may prevent Y1/Y5R-mediated cell death and promote Y2R-dependent angiogenesis, which can be stimulated by NPY3-36.

Recently, new homologues of DPPIV have been discovered, such as membrane fibroblast-activation protein (FAP) and cytoplasmatic dipeptidyl peptidase 8 and 9 (DPP8 and DPP9), which share structural similarities and the same proteolytic activity (15,16,18). All of these enzymes have been implicated in regulation of growth and metastases of many tumors (15,18,19). The increasing interest in these potential therapeutic targets led to the development of numerous DPP inhibitors. For example, a broad range DPP inhibitor, PT-100, has already been tested in clinical trials for the treatment of various cancers (20,21). Surprisingly, however, this compound was introduced to clinics without knowing the exact mechanisms of its actions, which ultimately led to the failure of these trials. Thus, given a role of DPPs in regulation of tumor growth, but also a complex nature of their actions, elucidation of mechanism underlying their effects is essential for further clinical trials. This is particularly important for intracellular DPP8 and DPP9, since their ability to cleave secretory peptides, which are known DPPIV substrates, has been shown only in cell extracts, but never documented in intact cells. Thus, as of now, their natural substrates have not been identified.

Taking into account the known growth-inhibitory effect of NPY in ESFT and its interactions with DPPs, we sought to determine the role of both membrane and intracellular DPPs in regulation of ESFT growth and survival, as well as assess their value as therapeutic targets in these tumors.

**EXPERIMENTAL PROCEDURES**

**Materials-** NPY was purchased from Bachem (San Carlos, CA), Y1R antagonist, BIBP 3226 from Sigma (St. Louis, MO) and Y5R antagonist, CGP71683, from Tocris (Ellisville, MO). The DPP inhibitors – broad range, P32/98, DPPIV-selective, UG92, and DPP8/9 selective, UG93, were received from Probiodrug (22) (Halle, Germany) and FAP-selective inhibitor, 3099, from Dr. Bachovchin (Tufts University, Boston, MA).

**Cell culture-** Human ESFT cell line, SK-N-MC, was obtained from ATCC (Manassas, VA) and cultured in EMEM media with 10% FBS. Other cell lines were obtained and cultured as previously reported (23).

**Cell viability assay-** The cells cultured in 96-well plates were put into 0.25% FBS media and 24h later treated with NPY (10-7M), DPP inhibitors (10-5M) and NPY receptor antagonists (10 -7M). The concentrations were determined based on dose response curves or previous studies (4,13). Cell viability was measured 48h later using MTS-based CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

**siRNA transfection-** Pre-designed siRNAs for NPY, DPPIV, DPP8, DPP9, FAP and negative control were purchased from Applied Biosystems (Foster City, CA). Cells were transfected with 30nM siRNA using Trans IT-TKO reagent (Mirus, Madison, WI). The efficiency of inhibition was tested by real time RT-PCR, Western blot, Neuropeptide Y Enzyme Immunoassay (Bachem, San Carlos, CA) or DPP activity assay. For the survival assay, the transfected cells were cultured for 96h in 1% FBS media with or without NPY or Y1 and Y5R antagonists (10-7M) and then cell viability was assessed as above.

**Real time RT-PCR-** RNA from cultured cells was isolated using High Pure RNA Isolation Kit (Roche Applied Science, Indianapolis, IN) and from tissues using TRI reagent (Sigma, St. Louis, MO). cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) and amplified using iCycler iQ Detection System (Bio-Rad Laboratories, Hercules, CA), TaqMan Universal PCR Master Mix and pre-designed primers and fluorescein-labeled probes (Applied Biosystems, Foster City, CA). The results were calculated by the comparative C_T method using β-actin as a reference gene.

**Mass spectrometry-** Conditioned media collected after 24h culture were subjected to ultrafiltration at
37°C and 2900 rpm using 30Kd cutoff filters. The ultrafiltrate contained around 7mg/dL protein plus peptides which include NPY_1-36 and NPY_3-36. These were then quantified using multiple reaction mode monitoring. The MRM for NPY_1-36 was 1068.8/70.1 and for NPY_3-36 was 803.4/70.1 on the API-4000 Tandem Mass Spectrometer (AB Sciex, Foster City, CA). Deuterated NPY1-36 was used as internal standard (MRM 857.1/70.1).

**DPP activity**- ESFT cells or xenograft tissues were lysed in 0.1% Triton X-100. DPP activity was measured calorimetrically at 405nm, using 1mM p-nitroanilide (pNA)-conjugated Gly-Pro dipeptide substrate (Sigma, St. Louis, MO) in 200mM Tris-HCl, pH 8.5, according to (24). The activity of particular DPPs was determined based on the differences between DPP activities with or without selective DPP inhibitors (10^-5M).

**DPPIV mRNA transfection**- pGEM4Z plasmid encoding rat DPPIV cDNA (25) was linearized with HindIII restriction enzyme and used as a template for the in vitro transcription reaction performed using mMESSAGE mMACHINE® SP6 Kit (Applied Biosystems, Foster City, CA). The Xenopus elongation factor 1α mRNA served as a control mRNA. SK-N-MC cells plated into 96-well plates were transfected with 2ng/μl of DPPIV or control mRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 18h after transfection, the cells were assayed for the DPP activity and treated in 2.5% FBS media with NPY or Y1 and Y5R antagonists (10^-7M). 48h later, cell viability was assessed as above. For the co-transfection experiments, DPPIV mRNA was combined with 30nM negative control siRNA or DPPIV siRNA (Applied Biosystems, Foster City, CA) and transfected as above.

**Nuclear extract isolation and Western blot**- ESFT cells were treated with NPY (10^-7M) with or without Y1 and Y5R antagonists (10^-7M) in 0.25% FBS media. 1h or 8h after treatment, the nuclear extracts were isolated using NE-PER nuclear and Cytoplasmic Extraction Kit (Thermo Scientific Rockford, IL). SK-ES cells were transfected with desired siRNA and 24h after transfection treated in 1% FBS media with Y1 and Y5R antagonist (10^-7M and 10^-8M, respectively). Approximately 54h after transfection, the nuclear extracts were isolated, as above. The Western blot on nuclear extracts was performed using rabbit polyclonal anti-apoptosis inducing factor (AIF) antibody (Cell Signaling Technology, Inc., Beverly, MA), while cytosolic fraction was used for detection of poly(ADP-ribose) (PAR) with rabbit polyclonal antibody (BD Pharmingen, San Diego, CA). Immunoblotting with rabbit polyclonal antibodies against DPPIV, DPP8, DPP9 (Abcam, Cambridge, MA), cleaved PAR polymerase-1 (PARP-1; Cell Signaling Technology, Beverly, MA) and mouse monoclonal anti-FAP antibody (Abcam, Cambridge, MA) were performed on whole cell extracts. Mouse monoclonal anti-β-actin antibody (Sigma, St. Louis, MO) was used as a control. Densitometry was performed using NIH Scion Image software (Scion Corp., Frederick, MD).

**Colony formation on soft agar**- SK-ES cells were resuspended in 0.3% agar (2 x 10^4 cells/ml), and overlaid onto 0.5% agar in 6-well plates in triplicates. Once the agar solidified, the medium with the desired treatments was added and changed daily for 5 days. The colonies were stained 2 weeks later using 0.005% crystal violet for 1 hour at 37°C and number of colonies were counted using Image J.

**Nude mice xenograft model**- Seven-ten week old nude mice (Taconic, Hudson, NY) were subcutaneously injected into their right flank with 2 x 10^6 of SK-ES cells suspended in 0.1 ml of Matrigel (BD Biosciences, San Diego, CA). 5 days after tumor cell inoculation, the daily treatment with NPY (10^-7M) with or without P32/98 (10^-5M), administered as local injection (approximately 1 cm from the tumor) of 100μl solution in saline, or with saline alone was started. Tumor size was measured periodically and volume calculated by the formula: 0.44 x length x width^2 (26). The SK-N-MC xenograft experiment and TUNEL staining were described previously (4).

**Statistical analysis**- Statistical analysis was performed using SigmaStat® software. One-way repeated measure ANOVA with post-hoc t-test (P<0.05) using Dunnett’s method was used for data comparison and analysis. Data is presented as mean ± standard errors for indicated numbers of repetitions. For the analysis of the SK-ES xenografts, the log-transformed tumor volumes were compared using linear mixed models to account for potential experiment effect and repeated measures of each animal.

**RESULTS**
ESFT cell lines vary in response to NPY. Previously, we have shown that NPY stimulates death of SK-N-MC ESFT cells (4,13). However, not all ESFT cell lines are equally responsive to NPY. While NPY treatment reduced the number of viable SK-N-MC cells with maximum effect at concentration 10^{-7}M, no statistically significant effect was observed in SK-ES cells (Fig. 1A). In line with this, in the panel of nine ESFT cell lines, only three responded to NPY (10^{-7}M) (Fig. 1B).

Similar differences were observed in response to endogenous NPY. In both SK-N-MC and SK-ES cell lines, two different NPY siRNAs exerted comparable inhibitory effects on expression and release of the tumor cell-derived NPY (supplementary Fig. 1). In SK-N-MC cells, this reduction in NPY levels led to a significant increase in the number of viable cells, suggesting that under basal conditions the endogenous peptide decreases cell survival (Fig. 1C). The effect of NPY siRNA was reversed by treatment with exogenous NPY, which confirmed its specificity. In contrast, NPY siRNA in SK-ES cells had no effect on cell survival (Fig. 1C). Thus, in the cells non-responsive to exogenous NPY, the endogenous peptide also does not affect cell viability.

**ESFT cell lines not responsive to NPY release high levels of NPY_{3-36}.** Since ESFT cell lines did not vary significantly in Y1 and Y5R expression (data not shown), we compared by mass spectrometry the forms of NPY released by ESFT cells in responsive and non-responsive cells. In SK-N-MC conditioned media, NPY was detected mostly in its intact form, NPY_{1-36}. In contrast, approximately 50% of NPY released by SK-ES cells was in its cleaved form, NPY_{3-36}, inactive at Y1Rs (Fig. 2A). The relevance of these findings was confirmed by the fact that in SK-N-MC cells, the decrease in cell viability was achieved with NPY_{1-36}, but not with NPY_{3-36} (Fig. 2B). Since NPY_{3-36} is a product of DPPIV cleavage, the above results suggested that the lack of the responsiveness to NPY in some ESFT cells might result from elevated DPPIV-like activity.

**ESFT cell lines vary in expression and activity of DPPs.** As suggested by previous experiments, we evaluated the expression and activity of DPPIV and its analogs in nine investigated ESFT cell lines. Average DPPIV mRNA and activity levels were significantly higher in the cells non-responsive to NPY (5838, A4573, ES-925, SK-ES, TC-32, TC-71), as compared to those responsive to the peptide (MMHES1, SK-N-MC and RDES) (Fig. 2C). Moreover, the non-responsive cells had elevated DPP8/9 activity, despite a lack of differences in mRNA levels of these cytosolic DPPs (Fig. 2C). FAP mRNA levels were highly variable and no significant difference between responsive and non-responsive cells was observed. In addition, under our culture conditions, no FAP activity was detected in ESFT cells.

**DPPIV overexpression abolishes the effect of NPY in responsive cells.** To prove that the observed differences in DPP levels regulate the response of ESFT cells to NPY, SK-N-MC cells were transfected with DPPIV mRNA, which led to over a six-fold increase in the protease activity (Fig. 3A). As a result, the cells overexpressing DPPIV lost their original responsiveness to exogenous NPY, while both non-transfected and transfected with control mRNA cells maintained their Y1/Y5R-dependent responses. Co-transfection of DPPIV mRNA with DPPIV siRNA restored the response to NPY, confirming the specificity of the observed effects (Fig. 3A).

**Knockdown of DPPs restores effect of NPY in non-responsive cells.** To determine if down-regulation of DPP expression in cells not responding to NPY will render them responsive, SK-ES cells were transfected with siRNAs targeting particular DPPs. Real time RT-PCR analysis confirmed significant and specific knock-down of selected DPPs (Supplementary Fig. 2), which resulted in a decrease in DPP protein levels and overall reduction of DPP activity with DPPIV, DPP8 and DPP9 siRNA transfections (Fig. 3B). Consistent with the lack of FAP activity in ESFT cells, FAP siRNA had no effect on overall DPP activity despite the effective inhibition of enzyme expression at the mRNA and protein levels (Supplementary Fig. 2, Fig 3B). In agreement with changes observed in DPP activity, transfections with DPPIV, DPP8 and DPP9 siRNAs, but not FAP siRNA, significantly decreased the number of viable SK-ES cells (Fig. 3C). Furthermore, all of these effects were blocked by NPY receptor antagonists – Y1R antagonist alone or in combination with Y5R antagonist. Thus, the effect of DPP siRNAs on SK-ES cell survival appears to be dependent on endogenous NPY. The level of cell death induced by DPP siRNAs was not...
increased by exogenous NPY, suggesting that the autocrine peptide is sufficient to saturate the receptors. The fact that the Y1R antagonist was equally effective alone, as it was in combination with the Y5R antagonist, indicated that blocking Y1Rs is sufficient to prevent the NPY effect in ESFT cells. Thus, even though both Y1R and Y5R seem to contribute to NPY actions (4), activation of Y1Rs is indispensable for NPY-induced cell death in ESFT cells. This observation further confirmed the crucial role of DPPs, which convert NPY to its Y1R-inactive form, in preventing this effect.

Both NPY and DPP siRNAs stimulate cell death via AIF-dependent pathway. Previously, we have shown that the growth-inhibitory effect of NPY in SK-N-MC depends on cell death, as shown by increased TUNEL staining both in vitro and in vivo (4). However, activation of the classical apoptotic pathway (caspase 3/7) was modest and observed late after NPY treatment. Instead, we observed molecular events consistent with AIF-mediated caspase-independent cell death (Fig. 4A) (27-30). In NPY-treated SK-N-MC cells, the elevated activity of PARP-1, measured by formation of PAR polymers, was detected 1h after treatment and followed by an increase in nuclear levels of AIF 57 (Fig. 4B). This effect was blocked by Y1/Y5R antagonists (Fig. 4B).

In SK-ES cells, similar increases in PAR and AIF 57 levels were observed upon transfection with DPPIV, DPP8 and DPP9 siRNA, but not FAP siRNA (Fig. 4C). However, treatments with the same siRNAs in the presence of the Y1/Y5R antagonists did not cause a significant increase in AIF levels (Fig. 4D). Thus, changes in PARP-1 activity and nuclear levels of AIF 57 mimic the effects of NPY and DPP siRNAs on ESFT cell survival, and suggest caspase-independent programmed cell death as their main mechanism.

Selective DPP inhibitors mimic the effects of DPP siRNAs. Although the majority of DPPIV actions are due to its enzymatic activity, some of these result from its binding to other regulatory proteins and extracellular matrix (31). Thus, we used selective DPP inhibitors to confirm that the effects observed with DPP siRNAs depend on changes in DPP activity. To this end, we chose three representative cell lines with various levels of NPY release and DPP activity (Fig. 5A).

In SK-ES cells with high DPP activity and high NPY release (Fig. 5A), broad range DPP inhibitor, P32/98, as well as DPPIV-selective (UG92) and DPP8/9 selective (UG93) inhibitors significantly decreased SK-ES cell viability to levels consistent with those achieved by NPY treatment (approximately 30% inhibition) (Supplementary Fig. 3, Fig. 5B). As observed with DPP siRNAs, these effects were blocked by Y1R antagonist, but not enhanced with exogenous NPY. On the other hand, NPY did not exert a significant inhibitory effect. A similar pattern was observed with PARP-1 activity. Although some increase in PAR polymer formation was observed 1h after treatment with NPY, its levels were higher upon P32/98 administration (Fig. 5B). These results are consistent with the high levels of endogenous NPY and high DPP activity present in SK-ES cells.

In SK-N-MC cells with low NPY release and low DPP activity (Fig. 5A), exogenous NPY significantly decreased the number of viable cells (Fig. 5C). In contrast, broad range DPP inhibitor, P32/98, as well as selective DPPIV inhibitor, UG92, had no effect on cell survival, independent of the presence of exogenous NPY. These results were consistent with PARP-1 activation observed after treatment with NPY, but not with P32/98 alone (Fig. 5C). Selective DPP8/9 inhibitor, UG93, exerted a modest (approx. 15%) inhibitory effect even without exogenous NPY that was blocked by Y1R antagonist. Moreover, inhibition of DPP8/9 augmented the effect of exogenous NPY up to 40%, when UG93 and NPY were applied in combination.

To confirm that the observed effects of DPP inhibitors are NPY-dependent, we used ES925 cells, which have high DPP activity, but no detectable NPY release (Fig. 5A). Indeed, in these cells, neither NPY nor DPP inhibitors alone exerted an effect on cell viability (Fig. 5D). However, the Y1R-dependent decrease in cell survival was observed when DPP inhibitors were given in combination with exogenous NPY. Consistently, PARP-1 activation was detected only upon treatment with both NPY and P32/98. Thus, the presence of NPY is necessary for DPP inhibitor-induced cell death.

DPP inhibitor, P32/98, inhibits colony formation of SK-ES cells. The effect of DPP inhibition on SK-ES cells was also tested using a
colony formation assay in soft agar. As observed with MTS assay, a broad range DPP inhibitor, P32/98, significantly decreased the number of SK-ES colonies. The effect of DPP inhibitor on colony formation was more pronounced than that observed under monolayer culture conditions (60% versus 25% inhibition, respectively). On the other hand, exogenous NPY had only a modest effect, which did not reach statistical significance (Fig. 6A). The growth-inhibitory effect of P32/98 was blocked by Y1R antagonist alone, or in combination with Y5R antagonists (Fig. 6B). Therefore, the colony formation assay confirmed the results obtained in monolayer culture.

Effect of NPY on ESFT cell survival is enhanced in vivo. SK-N-MC and SK-ES xenografts seemed to recapitulate features of these cells cultured in vitro. Both NPY level and DPP activity in plasma of mice bearing SK-ES tumors were elevated, as compared to plasma of animals with SK-N-MC xenografts (Fig. 7A). These systemic changes reflect a high release of NPY and shedding of DPPIV from SK-ES tumors.

Surprisingly, treatment with NPY alone resulted in significant inhibition of SK-ES xenograft growth (P=0.0016), which was associated with increased levels of cell death, as measured by DNA fragmentation (TUNEL) and PARP-1 cleavage, both of which are late events in AIF-mediated cell death (29,30) (Fig. 4A, 7B-D). However, we were not able to achieve successful DPP inhibition in vivo with P32/98, despite testing different doses and routes of administration. Paradoxically, at the end of the experiment, the DPP activity in P32/98-treated tumor tissues was significantly higher than in control, which was associated with elevated mRNA levels for all investigated DPPs (Supplementary Fig. 4).

DISCUSSION

Recent microarray data identified NPY and its Y1 and Y5Rs as “Ewing’s sarcoma signature genes” up-regulated by EWS-FLI1 fusion protein. However, a clinically relevant role of NPY in ESFT has not been elucidated. The cell death induced by endogenous NPY previously reported by us (4) seems paradoxical and raises the question of how ESFT cells are protected from its effect. The data presented here suggest that high levels of DPP expression in ESFT cells provide a survival mechanism for these cells. We have shown that not only membrane DPPIV, but also its intracellular homologs, DPP8 and DPP9, are actively involved in preventing NPY-induced cell death. This is the first demonstration that these enzymes are involved in regulation of ESFT growth and the first evidence that the intracellular DPPs are able to cleave releasable peptides in intact cells.

We have shown that NPY directly stimulates cell death only in ESFT cells with low DPP activity. In these cells, NPY is released mainly in its intact NPY1-36 form, which can activate Y1R/Y5Rs. We have identified PARP-1 and AIF-dependent programmed cell death as the primary mechanism of NPY actions in ESFT cells. This observation is consistent with the fact that the majority of known ESFT cell lines, including those used in our study, lack a functional p53 pathway (32), which is necessary for intrinsic activation of caspase-dependent apoptosis. In such cells with impaired apoptotic pathways, AIF serves as a substitute mechanism of executing cell death (27,28). Moreover, PARP-1 and AIF activation is triggered by an increase in intracellular calcium, a classical signaling event induced by NPY in various cells, including SK-N-MC (27,33,34).

In the cells with high DPP activity, a significant amount of NPY is released in its NPY1-36 form, which does not activate Y1Rs and, consequently, does not stimulate cell death. However, the effect of NPY can be restored by blocking DPPs with selective inhibitors or siRNAs. Interestingly, similar effects were observed for the membrane-bound DPPIV as for cytosolic DPP8 and DPP9. While NPY is a well established substrate for DPPIV (17), the natural substrates of the cytosolic DPPs have not been identified. Both DPP8 and DPP9 have been shown to cleave NPY in cell extracts (22,35), however, the ability of cytosolic enzymes to process releasable peptides under physiological conditions is highly controversial. Recently, DPP8 has been shown to cleave certain chemokines in vitro and implicated in the inactivation of internalized chemokine/receptor complexes, however no direct proof for the latter was provided (36). Here, we have shown that blocking DPP8 and DPP9 activates the same cell death pathway as NPY and that all of these effects can be blocked by NPY receptor antagonists. Thus, we provide the first
evidence that NPY is a natural substrate of these intracellular DPPs. This phenomenon can be explained by the fact that NPY is present in various intracellular compartments, such as the cytosol, nucleoplasm and mitochondria (37-39). Moreover, Y1Rs have been identified on the nuclear membrane, suggesting an intracellular mode for NPY actions (37). Thus, cytosolic DPPs may be potentially involved in regulating such actions of the peptide. Although the efficiency of NPY cleavage by DPP8 and DPP9 is approximately two-fold lower than that of DPPIV (35), DPP8/9 accounts for approximately 80% of overall DPP activity in ESFT cell extracts (13). Due to this high representation of DPP8/9, these proteases may still significantly contribute to NPY cleavage, despite their lower activity limited to the intracellular pool of the peptide. This was observed in SK-N-MC cells, in which DPP8/9 selective inhibitor significantly inhibited cell survival in an NPY-dependent manner, while the DPPIV selective inhibitor had no effect.

FAP, like DPPIV, is a membrane protease. However, its substrate specificity varies significantly from DPPIV. Unlike other DPPs, FAP has endopeptidase activity, while its DPP activity is lower than that of DPPIV for most substrates (40,41). In the panel of ESFT cells, we did not detect significant activity of FAP. However, its catalytic efficiency for the hydrolysis of Gly-Pro dipeptide used in our assays is approximately 100-fold lower than observed for DPPIV (40,41). Moreover, the efficiency of FAP cleavage for dipeptides with tyrosine in the first position, as in NPY, is among the lowest observed for various P2-Pro1 combinations (41). Therefore, it is very unlikely that FAP plays a significant role in NPY processing.

Despite the fact that FAP did not seem to be involved in the regulation of ESFT growth and survival, its role in these tumors cannot be underestimated. Although we did not detect a significant FAP-dependent DPP activity in non-confluent ESFT cells, it appeared to increase in confluent cells, while DPPIV activity decreased (data not shown). Thus, DPPIV may be important under conditions promoting cell proliferation, while FAP may be involved in other functions of ESFT cells, such as invasiveness and motility. Importantly, these actions of both FAP and DPPIV are often not dependent on their DPP activity, but rather their binding properties (31,42,43).

Actions of DPPs may be further altered in vivo due to their high expression in stromal cells, such as tumor-associated fibroblasts, endothelial and immune cells (43-46). For example, we have shown that DPPIV activity in cell extracts from TC32 ESFT cells accounts for only 20% of total DPP activity, while in extracts from TC32 xenograft tissue DPPIV-dependent activity raises to 40% (13). Thus, the relative contribution of particular DPPs may change significantly in the tumor tissue, as compared to the isolated tumor cells. Moreover, in the tumor microenvironment DPPs may acquire new functions dependent on interactions with host cells, extracellular matrix and stroma-derived proteolytic substrates, such as regulation of cell invasiveness and anti-tumor immune response (19,21,45).

The importance of tumor environment and clinical relevance of our data is further supported by the fact that the effect of NPY and DPP inhibitor was significantly enhanced in 3-D culture and in vivo. As shown previously, relatively modest effect of NPY on SK-N-MC cell survival in vitro translated to a substantial inhibition of tumor growth in vivo (4). Similarly, in SK-ES xenografts, NPY inhibited tumor growth, despite no significant effect on survival of these cells in culture. Thus, prolonged treatment with high doses of NPY in the context of tumor microenvironment may partially overcome the protective effect of DPPs and lead to effective activation of Y1R/Y5R-mediated apoptosis. These results are consistent with our observations in neuroblastoma, where the effect of NPY on tumor growth was also much more dramatic in vivo, than in vitro (47).

Taking into account significant growth-inhibitory effects of NPY in vivo, the successful inhibition of DPP activity in combination with NPY treatment should lead to pronounced reduction of the tumor growth rate. Moreover, in vivo, DPP inhibitors should not only enhance the Y1R/Y5R-mediated cell death of tumor cells, but also impair Y2R-mediated angiogenesis, normally favored by DPPs (4). Unfortunately, our attempts to use a broad range DPP inhibitor, P32/98, in vivo were not successful, since prolonged treatment with P32/98 triggered up-regulation of the DPP expression and activity to the levels higher than in control. Therefore, careful
pharmacodynamic assays will be important with clinical use of these agents against tumors.

Even though we were not able to attain an effective DPP inhibition with P32/98, this may be achieved with more potent inhibitors. Recent years have brought a strong interest in DPPIV and its homologs, which led to the development of numerous highly selective and potent DPP inhibitors. For example, DPPIV-selective inhibitors, Sitagliptin and Vildagliptin, are already used for the treatment of diabetes (48), while broad range DPP inhibitor, PT-100, has been tested in clinical trials for various types of cancer (20,21,49). Using such potent inhibitors in an appropriate treatment regime may allow overcoming or avoiding induction of DPPs observed in P32/98-treated tumors.

In summary, we have shown that both DPPIV and its cytosolic homologs, DPP8 and DPP9, serve as survival factors for ESFT cells, protecting them from NPY-induced cell death mediated by Y1R/Y5Rs. This is the first demonstration that intracellular DPPs are involved in the regulation of ESFT growth, and the first evidence that NPY is their natural substrate. These findings indicate that targeting DPPs in vivo may enhance the growth-inhibitory effect of NPY and become a new therapeutic strategy for ESFT. Moreover, given pleiotropic actions of NPY and its processing enzymes, DPPs, continued explorations of this EWS-FLI1-driven pathway may also open other therapeutic opportunities for ESFT patients.

REFERENCES


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FOOTNOTES

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The abbreviations used are: ESFT, Ewing’s sarcoma family of tumors; NPY, neuropeptide Y; DPPs, dipeptidyl peptidases; AIF, apoptosis inducing factor; DPPIV, dipeptidyl peptidase IV; FAP, fibroblast-activation protein; DPP8, dipeptidyl peptidase 8; DPP9, dipeptidyl peptidase 9; PAR, poly(ADP-ribose); PARP-1, poly(ADP-ribose) polymerase 1.

FIGURE LEGENDS

Fig. 1. ESFT cell lines vary in response to NPY. A. SK-N-MC and SK-ES cells were treated for 48h with NPY at concentrations ranging from $10^{-10}$M to $10^{-7}$M. The number of viable cells was measured by MTS assay. The decrease in cell viability was observed in SK-N-MC, but not SK-ES cells. B. Nine ESFT cell lines were treated with NPY at concentration $10^{-7}$M for 48h and the cell viability was measured as above. Only three tested cell lines responded to NPY. C. SK-N-MC cells were transfected with two different NPY siRNAs or non-blocking siRNA (negative control-NC) at concentration 30nM. The cells were cultured for 96h, with or without NPY ($10^{-7}$M), and then the number of viable cells was assessed by MTS assay. NPY knock-down increased viability of SK-N-MC cells, which was abolished by exogenous NPY. No effect of NPY siRNA on SK-ES cell survival was observed. For all these experiments, the data represent an average from at least three independent experiments, six wells per treatment each.

Fig. 2. Endogenous NPY and DPPs in ESFT cell lines. A. Conditioned media from SK-N-MC and SK-ES cells were collected after 24h culture and the forms of released NPY analyzed by mass spectrometry. NPY detected in culture media of SK-N-MC cells was mostly in its intact form (NPY$_{1-36}$), while approximately 50% of NPY present in SK-ES cell culture media was cleaved to NPY$_{3-36}$, inactive at
Y1Rs. B. SK-N-MC cells were treated with NPY1-36 or NPY3-36 at concentrations 10^{-7}M for 48h. Then, the cell viability was assessed by MTS assay. A significant decrease in the number of viable cells was induced by NPY1-36, while NPY3-36 had no effect. C. mRNA levels of DPPs were measured in nine ESFT cell lines by real-time RT-PCR, while their selective activities were assessed based on pNA-Gly-Pro cleavage in the absence or presence of specific DPP inhibitors. The average mRNA and activity levels were compared between NPY-non-responsive (5838, A4573, ES-925, SK-ES, TC-32 and TC-71) and NPY-responsive (MMH-ES1, SK-N-MC, RDES) ESFT cell lines. DPPIV mRNA and activity, as well as combined DPP8/9 activity were significantly elevated in non-responsive cells.

**Fig. 3.** The effect of NPY on ESFT cell survival depends on DPP expression levels. A. SK-N-MC cells were transfected with 2ng/μl of DPPIV or control mRNA and cultured for 48h with or without Y1 and Y5R antagonists at concentrations 10^{-7}M. As a control, DPPIV mRNA was co-transfected with 30nM DPPIV or negative control (NC) siRNA. 48h after transfection, DPPIV activity was measured by pNA-Gly-Pro cleavage with or without DPPIV-specific inhibitor and cell viability assessed by MTS assay. DPPIV overexpression significantly increased its activity and abolished the Y1R/Y5R-dependent decrease in cell viability induced by NPY in control cells. Co-transfection with DPPIV siRNA restored the growth inhibitory effect of NPY. B. SK-ES cells were transfected with 30nM NC or DPP-specific siRNAs. 48h after transfection the efficiency of the DPP knock-down was assessed by Western blot with DPP-selective antibodies and total DPP activity measured by pNA-Gly-Pro cleavage. While all siRNAs were effective on the protein level, DPPIV, DPP8 and DPP9 siRNAs, but not FAP siRNA, decreased DPP activity. C. SK-ES cell viability was assessed by MTS assay 96 after transfection with DPP siRNAs and culture with or without NPY or Y1R and Y5R antagonists (10^{-7}M). DPPIV, DPP8 and DPP9 siRNAs reduced number of viable SK-ES cells, which was blocked by Y1 and Y5R antagonists. No effect of FAP siRNA on cell SK-ES cell survival was observed.

**Fig. 4.** NPY and DPP siRNAs induce caspase-independent cell death. A. Caspase-independent programmed cell death is triggered by factors increasing intracellular calcium levels, which leads to formation of reactive oxygen species (ROS), DNA damage and PARP-1 activation resulting in formation of PAR polymers. This, in turn leads to further deregulation of mitochondrial calcium, cleavage of AIF to AIF 57, which translocates to the nucleus and induces DNA fragmentation. In the end stages of this process, PARP-1 cleavage is observed. B. SK-N-MC cells were treated with NPY with or without Y1R and Y5R antagonists (10^{-7}M). 1h and 8h after treatment nuclear and cytosolic extracts were collected and levels of PAR and AIF assessed by Western blot. 1h after treatment, presence of PAR polymers in cytosolic extracts was observed, while at 8h increase in the nuclear levels of AIF 57 was detected. This effect was blocked by Y1R and Y5R antagonists (10^{-7}M). C-D. SK-ES cells were transfected with 30nM negative control (NC) or DPP siRNAs with or without Y1R and Y5R antagonists (10^{-7}M). 48h later, cytosolic and nuclear extracts were collected and levels of PAR polymers and AIF 57 assessed, respectively. DPPIV, DPP8 and DPP9 siRNA transfections increased the levels of PAR and AIF 57 (C). No significant increases in nuclear levels of AIF 57, as measured by densitometry, were observed when SK-ES cells were transfected with DPP siRNAs in the presence of Y1R and Y5R antagonists (D). For B and D, the graphs represent average densities of AIF 57 normalized to β-actin from three independent experiments. Representative Western blots are shown.
Fig. 5. Effects of DPP inhibitors on ESFT cells. A. DPP activity in extracts from three ESFT cell lines was measured by pNA-Gly-Pro cleavage with or without DPPIV- and DPP8/9-specific inhibitors. NPY levels were measured by ELISA in ESFT cell conditioned media after 24h of culture. B-D. ESFT cell were treated for 48h with DPP inhibitors (10^{-5}M), with and without NPY or Y1R antagonist (10^{-7}M), and then the number of viable cells was measured by MTS assay. PAR polymer accumulation was assessed by Western blot in cell extracts collected 1h after treatment with NPY (10^{-7}M) with or without broad range DPP inhibitor, P32/98 (10^{-5}M). In SK-ES cells (B), which contain high levels of endogenous NPY and high DPP activity, exogenous NPY did not exert a growth-inhibitory effect, while DPP inhibitors alone significantly decreased cell viability. This effect was blocked by Y1R antagonist. In SK-N-MC cells (C), which present with low levels of endogenous NPY and low DPP activity, exogenous NPY decreased cell survival, while broad range and DPPIV-specific inhibitors had no effect. DPP8/9-specific inhibitor, UG93, significantly decreased the number of viable cells and enhanced the effect of NPY alone, which was blocked by Y1R antagonist. In ES925 cells (D), which do not release NPY and possess high levels of DPPs, the decrease in cell survival was achieved only when exogenous NPY was applied with DPP inhibitors. Similar patterns of responses were observed with PAR polymer formation.

Fig. 6. NPY and DPP inhibitors reduce the colony formation of SK-ES cells. A. SK-ES cells were plated in soft agar in the presence of NPY (10^{-7}M), with or without broad range DPP inhibitor, P32/98 (10^{-5}M). Two weeks after plating, a significant decrease in the number of colonies was observed with P32/98, but not NPY treatment. B. The colony formation assay was performed in the presence of P32/98 (10^{-5}M) with or without Y1 and Y5R antagonists (10^{-7}M), which blocked its effect. The data represent an average of three experiments, three wells per treatment each.

Fig. 7. NPY inhibits growth of ESFT xenografts in vivo. A. SK-N-MC and SK-ES cells were subcutaneously injected into nude mice and tumors grown for approximately 3-4 weeks. At the end of the experiments, NPY levels and DPP activity were measured in plasma of the animals by ELISA and pNA-Gly-Pro cleavage, respectively. Mice bearing SK-ES xenografts had elevated plasma NPY and DPP activity levels, as compared to animals with SK-N-MC tumors. B-D. SK-ES xenografts were treated with daily injections of NPY (10^{-7}M) or saline (n=12 per group) for two weeks. Tumor growth rates were compared based on periodical measurements of their volumes (B). DNA fragmentation was assessed in tumor tissues by TUNEL (C), while cleaved PARP-1 was detected in extracts from SK-ES xenografts by Western blot (D). Significant inhibition of SK-ES xenografts growth was associated with increased levels of cell death, as measured by TUNEL and PARP-1 cleavage (Western blot of representative samples shown).
Figure 1

A. 

B. 

C. 

* p<0.05, as indicated
**Figure 2**

**A.**

![Graph showing NPY (ng/ml) in SK-N-MC and SK-ES cells.](image)

NPY (ng/ml)

- SK-N-MC: 0.2
- SK-ES: 0.1

Ratio: NPY1-36 / NPY3-36

**B.**

![Bar graph showing number of viable cells (% of control) for Control, NPY 1-36, and NPY 3-36.](image)

- Control: 100
- NPY 1-36: 80
- NPY 3-36: 100

* p<0.05, as compared to control

**C.**

![Graph showing mRNA levels relative to β-actin for DPPIV, DPP8, DPP9, and FAP.](image)

- DPPIV: 1.01E-03
- DPP8: 1.01E-03
- DPP9: 1.01E-03
- FAP: 1.01E-03

* p<0.05, as indicated
Figure 3

A. SK-N-MC

<table>
<thead>
<tr>
<th>mRNA:</th>
<th>none</th>
<th>control</th>
<th>DPPIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPP IV activity (pmol/min/mg of protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of viable cells (% of control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of viable cells (percent of control)</td>
<td></td>
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</tr>
</tbody>
</table>

B. SK-ES

DPP-specific siRNA:  -  +  -  +  -  +  -  +
DPP-specific Ab:     
β-actin:             

<table>
<thead>
<tr>
<th>siRNA:</th>
<th>NC</th>
<th>DPP IV</th>
<th>DPP8</th>
<th>DPP9</th>
<th>FAP</th>
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</thead>
<tbody>
<tr>
<td>Total DPP activity (pmol/min/mg of protein)</td>
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<td></td>
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<td></td>
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</tbody>
</table>

C.

<table>
<thead>
<tr>
<th>siRNA:</th>
<th>NC alone</th>
<th>DPP IV alone</th>
<th>DPP8 alone</th>
<th>DPP9 alone</th>
<th>FAP alone</th>
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</thead>
<tbody>
<tr>
<td>Number of viable cells (% of control)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* p<0.05, as compared to negative control (NC) siRNA alone
# p<0.05, as compared to DPP siRNA alone
Figure 4

A. Increased intracellular Ca$^{2+}$
   ↓
   ROS and DNA damage
   ↓
   PARP-1 activation in the nucleus
   ↓
   PAR polymer accumulation
   ↓
   Cleavage of mitochondrial AIF to AIF 57 and transport to the nucleus
   ↓
   Caspase activation and PARP-1 cleavage
   ↓
   DNA fragmentation
   → Cell death

B. SK-N-MC

<table>
<thead>
<tr>
<th>siRNA:</th>
<th>NC</th>
<th>DPPIV</th>
<th>NPY1h</th>
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<tbody>
<tr>
<td>AIF 57</td>
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<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
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</tbody>
</table>

C. SK-ES

<table>
<thead>
<tr>
<th>siRNA:</th>
<th>NC</th>
<th>DPPIV</th>
<th>DPP8</th>
<th>DPP9</th>
<th>FAP</th>
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</thead>
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<tr>
<td>AIF 57</td>
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<td>β-Actin</td>
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D. siRNA:  

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<tr>
<th>Y1R/Y5R Ant:</th>
<th>NC</th>
<th>+</th>
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<th>+</th>
<th>NC</th>
<th>+</th>
<th>NC</th>
<th>+</th>
<th>NC</th>
<th>+</th>
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<tbody>
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<td></td>
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<tr>
<td>β-Actin</td>
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</table>

* p<0.05, as indicated

PARP-1 activation in the nucleus
PAR polymer accumulation
Cleavage of mitochondrial AIF to AIF 57 and transport to the nucleus
Caspase activation and PARP-1 cleavage
DNA fragmentation
Cell death

AIF 57 protein levels
Ratio to Control

AIF 57
β-Actin

AIF 57
β-Actin

AIF 57
β-Actin

AIF 57
β-Actin

AIF 57
β-Actin

DPP siRNA
DPP siRNA + Y1R/Y5R Ant

AIF 57 protein levels
Ratio to Control

siRNA:
NC
DPPIV
DPP8
DPP9
FAP

* p<0.05, as compared to negative control (NC) siRNA
Figure 5

A. Number of viable cells (% of control) 
- SK-ES: Control, NPY, P32/98 (broad range), Y1R Ant, NPY + Y1R Ant
- SK-N-MC: Control, NPY, P32/98, UG92, UG93
- ES925: Control, NPY, P32/98, Y1R Ant, NPY + Y1R Ant

B. SK-ES
- DPP inhibitor: None, NPY, P32/98, Y1R Ant, NPY + Y1R Ant

C. SK-N-MC
- DPP inhibitor: None, NPY, P32/98, UG92, UG93

D. ES925
- DPP inhibitor: None, NPY, P32/98, UG92, UG93

* p<0.05, as compared non-treated control
# p<0.05, as indicated
Figure 6

A.

Control

NPY

P32/98

NPY+P32/98

B.

Control

P32/98

P32/98 + Y1R Ant

P32/98 + Y1R/Y5R Ant

* p<0.05, as compared to control

* p<0.05, as indicated

Number of colonies (fold-increase as compared to control)
Figure 7

A. 

**Bar graph showing NPY-ir content and Total DPP activity.**

<table>
<thead>
<tr>
<th></th>
<th>SK-N-MC</th>
<th>SK-ES</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY-ir (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total DPP activity (pmol/min/mg of protein)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05, as indicated

B. 

**Graph showing tumor volume (fold-increase) over days after tumor cell injection.**

- Control
- NPY

p=0.0016, for comparison between control and NPY-treated tumors

C. 

**Images and bar graph showing % of area stained.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NPY</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of area stained</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05, as indicated

D. 

**Western blot images showing Cleaved PARP-1 and β-actin.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NPY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved PARP-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
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<td></td>
</tr>
</tbody>
</table>

*p<0.05, as indicated*
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Congyi Lu, Jason U. Tilan, Lindsay Everhart, Magdalena Czarnecka, Steven J. Soldin, Damodara R. Mendu, Dima Jeha, Jailan Hanafy, Christina K. Lee, Junfeng Sun, Ewa Izycka-Swieszczewska, Jeffrey A. Toretsky and Joanna Kitlinska

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