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Research paper

VIP and PACAP analogs regulate therapeutic targets in high-risk neuroblastoma cells

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ABSTRACT

Neuroblastoma (NB) is a pediatric cancer. New therapies for high-risk NB aim to induce cell differentiation and to inhibit MYCN and ALK signaling in NB. The vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptide (PACAP) are 2 related neuropeptides sharing common receptors. The level of VIP increases with NB differentiation. Here, the effects of VIP and PACAP analogs developed for therapeutic use were studied in *MYCN*-amplified NB SK-N-DZ and IMR-32 cells and in Kelly cells that in addition present the F1174L *ALK* mutation. As previously reported by our group in IMR-32 cells, VIP induced neuritogenesis in SK-N-DZ and Kelly cells and reduced MYCN expression in Kelly but not in SK-N-DZ cells. VIP decreased AKT activity in the *ALK*-mutated Kelly cells. These effects were PKA-dependent. IMR-32, SK-NDZ and Kelly cells expressed the genes encoding the 3 subtypes of VIP and PACAP receptors, VPAC1, VPAC2 and PAC1. In parallel to its effect on MYCN expression, VIP inhibited invasion in IMR-32 and Kelly cells. Among the 3 PACAP analogs tested, [Hyp^2]PACAP-27 showed higher efficiency than VIP in Kelly cells. These results indicate that VIP and PACAP analogs act on molecular and cellular processes that could reduce aggressiveness of high-risk NB.

1. Introduction

Neuroblastoma (NB) is an embryonic tumor derived from neural crest cells. It is the most common extracranial solid tumor in infants and children, and it accounts for approximately 15% of deaths due to pediatric tumors [1,2]. NB is characterized by a large clinical heterogeneity. Indeed, these tumors may regress completely or differentiate into a benign ganglioneuroblastoma without treatment while metastatic NB in children older than 18 months at diagnosis are lethal for most patients despite aggressive multimodal therapy [3].

The amplification of the *MYCN* locus is found in about 25% of NB and is strongly related to poor clinical outcome [1,2]. The involvement of *MYCN* in NB tumorigenesis was demonstrated in transgenic mice overexpressing *MYCN* in neural crest cells [4]. High expression of the transcription factor MYCN disrupts the cell-cycle exit and terminal differentiation that occur during normal neuroblast development [5,6]. MYCN also plays an important role in cell invasion by directly or indirectly modulating specific target genes involved in cell adhesion, motility and matrix degradation [7].

The tyrosine kinase receptor ALK (anaplastic lymphoma kinase) collaborates with MYCN for the development of most aggressive NB [8]. The F1174L *ALK* mutation is associated preferentially with *MYCN* amplification in NB and induces activation of the PI3K/AKT pathway [9,10], leading to oncogenic stabilization of MYCN protein [11]. Poorly differentiated NB with high expression of MYCN and the F1174L *ALK* mutation define a subset of patients with high-risk NB [9,12].

Treating patients with high-risk NB is a challenge because about one-third of them do not respond completely to therapy [13,14]. New therapies inducing differentiation of NB cells and inhibit-

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ing specific signaling pathways, angiogenesis and ALK and MYCN expression are in development [7,15,16].

The vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptide (PACAP) are 2 related neuropeptides that share common receptors, named VPAC1 and VPAC2. These two receptors bind VIP and PACAP with similar high-affinity. Some isoforms of the PACAP-specific PAC1 receptor, generated by alternative splicing of the PAC1 primary transcript, have been demonstrated to also bind VIP with high-affinity [17]. VPAC1, VPAC2 and PAC1 mainly activate the protein kinase A (PKA) and protein kinase C (PKC) signaling pathways, which induces regulation of gene expression [18]. VIP and PACAP are involved in the development of the nervous system. Indeed, VIP regulates the proliferation and the differentiation of neuronal precursors *in vitro* and *in vivo*, and is involved in the growth, differentiation and maintenance of neurons [19–21]. VIP can also regulate proliferation, differentiation and migration of various tumor cells [21,22]. In NB cell lines, this neuropeptide induces both inhibition of growth and morphological differentiation [23]. The rate of VIP increases with the degree of differentiation of NB and is associated with a good prognosis [24–26]. Our group demonstrated that VIP induces morphologic differentiation (neuritogenesis) and reduces *MYCN* expression in the high-risk NB IMR-32 cells with *MYCN* amplification [27]. Then VIP can regulate several processes that are the targets of new therapeutic strategies for high-risk NB. VIP can also up- or down-regulate invasion of different cancer cells depending of their tissue of origin [28–32]. However, its effect on NB cell invasion is unknown.

In the present study, we analyzed the effect of VIP on different molecular and cellular processes, *i.e.*, neuritogenesis, *MYCN* expression, AKT activity and cell invasion, in high-risk human NB cell lines with *MYCN* amplification, one of them also presenting the F1174L *ALK* mutation. Furthermore, the activity of peptide analogs of PACAP developed for therapeutic use was tested. These PACAP analogs were initially selected from a quite important library of modified peptides and their effects on neurite outgrowth and neuroprotection were demonstrated *in vitro* and *in vivo* [33–35].

2. Materials and methods

2.1. Cell culture

Kelly and IMR-32 NB cells (obtained from ECACC and ATCC, respectively) were routinely seeded at a density of 10^6 cells/25 cm^2 flask in RPMI 1640 medium or DMEM medium 4.5 g/L glucose, respectively, containing GlutaMAX™ (Gibco) and supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). These cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. SK-N-DZ cells (obtained from ATCC) were routinely seeded at a density of 10^6 cells/25 cm^2 flask in DMEM medium with GlutaMAX supplemented with 10% fetal calf serum (FCS) and 1% non essential amino acids (Sigma). SK-N-DZ cells were incubated at 37 °C in a humidified atmosphere of 10% CO_2 in air. For all cell lines, medium was changed every 3 days. Passages were performed once per week using Trypsin/EDTA (Invitrogen).

2.2. Peptides

The VIP, PACAP-38, PACAP-27 and secretin were from Neosystem while analogs were synthesis as previously described [36]. Neuropeptides and analogs were diluted to a concentration of 10^{-4} M in pure water or DMEM.

2.3. Treatments

Kelly, IMR-32 cells were seeded at a density of 2×10^6 and SK-N-DZ cells at 0.5×10^6 cells/25 cm^2 flask in the media mentioned above except that they were supplemented with 5% FCS. After 2–3 days in culture, cells were treated with the neuropeptide VIP at a concentration of 10^{-6} M or less. An equal volume of phosphate buffered saline (PBS) was added to control cells. Treatments with secretin were performed at the concentration of 10^{-6} M, and those with PACAP-38, PACAP-27 and PACAP analogs at 10^{-7} M or less. For experiments in the presence of signaling pathway inhibitors, H-89 dihydrochloride, Akt Inhibitor VIII Isozyme-Selective and bisindolylmaleimide I (Calbiochem) were added 20 min before the addition of VIP, at a concentration of 1 and 10 μM.

2.4. Micrographs of cells

Video images of cells were captured using a digital camera connected to a phase-contrast microscope (Olympus). The quantification of the number of cells with neurites and the measure of neurite length were performed using ImageJ software.

2.5. Western immunoblot analysis

After treatment, protein extracts and western immunoblotting were performed as previously described [37]. Antibodies anti-MYCN NCM II 100 (1:100; Calbiochem), anti-GAPDH (1:160,000; ABCAM), anti-phospho AKT (1:1000; Cell Signaling Technology), anti-PAN AKT (1:1000; Cell Signaling Technology) and goat anti-rabbit or goat anti-mouse secondary antibodies (1:20,000; Calbiochem) conjugated to horseradish peroxydase were diluted in blocking solution. After ECL Prime (Amersham) detection, the blots were exposed to Hyperfilms (Amersham) and signals were quantified using the software ImageJ.

2.6. cDNA synthesis

Total RNA was isolated by using the GenElute™ Mammalian Total RNA kit (Sigma–Aldrich) and cDNA were synthetized as previously described [37].

2.7. RT-PCR, real-time RT-PCR and quantification

RT-PCR and real-time RT-PCR were performed as previously described [38]. RT-PCR were carried out using GoTaq® DNA polymerase (Promega) and RT-qPCRs were performed with the LightCycler System (Roche Molecular Biochemicals) by using the «SYBR Premix Ex Taq kit» (Takara). GAPDH mRNA levels were used as an internal control and for normalization of the mRNA levels between samples for real-time RT-PCR. MYCN, VPAC1, VPAC2, PAC1 and GAPDH primers used were as followed (5′–3′): MYCN forward: CGACCACAAGGCCCTCAGT, MYCN reverse: TGACCACGTCGATTTCTTCCT, VPAC1 forward: ACAAGGCAGCGAGTTTGGAT, VPAC1 reverse: GTGCAGTGGAGCTTCCTGAAC, VPAC2 forward: CGTGAACAGCATTCACCCAGAAT, VPAC2 reverse: CGTGACGGTCTCTCCCACAT, PAC1 forward: TTCTGGCTGTTCATCGAGGG, PAC1 reverse: CCAGCAGCCTGTGTCATCAAA, GAPDH forward: GAAGGTGAA-GGTCGGAGTCA, GAPDH reverse: GACAAGCTTCCCGTTCTCAG. The cycle program for RT-PCR was: one cycle at 95 °C for 2 min, 40 cycles (or 25 cycles for GAPDH) at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and one cycle at 72 °C for 2 min. PCR products were resolved and visualized on a 3% agarose gel containing SYBR Green (Invitrogen). The cycle program for real-time RT-PCR was: 5 s at 95°C, 5 s at 60°C and 10 s at 72°C.