Pituitary Adenylate Cyclase-Activating Polypeptide Is a Potent Inhibitor of the Growth of Light Chain-Secreting Human Multiple Myeloma Cells

Min Li, Shirley Cortez, Tomoya Nakamachi, Vecihi Batuman, and Akira Arimura
Department of Medicine, School of Medicine, Tulane University Health Sciences Center, New Orleans, Louisiana

Abstract
Multiple myeloma represents a malignant proliferation of plasma cells in the bone marrow, which often overproduces immunoglobulin light chains. We have shown previously that pituitary adenylate cyclase-activating polypeptide (PACAP) markedly suppresses the release of proinflammatory cytokines from light chain-stimulated human renal proximal tubule epithelial cells and prevents the resulting tubule cell injury. In this study, we have shown that PACAP suppresses the proliferation of human κ and λ light chain-secreting multiple myeloma–derived cells. The addition of PACAP suppressed light chain-producing myeloma cell–stimulated interleukin 6 (IL-6) secretion by the bone marrow stromal cells (BMSCs). A specific antagonist to either the human PACAP-specific receptor or the vasoactive intestinal peptide receptor attenuated the suppressive effect of PACAP on IL-6 production in the adhesion of human multiple myeloma cells to BMSCs. The secretion of IL-6 by BMSCs was completely inhibited by 10−9 mol/L PACAP, which also attenuated the phosphorylation of both p42/44 and p38 mitogen-activated protein kinases (MAPK) as well as nuclear factor-κB (NF-κB) activation in response to the adhesion of multiple myeloma cells to BMSCs, whereas the inhibition of p42/44 MAPK signaling attenuated PACAP action. The signaling cascades involved in the inhibitory effect of PACAP on IL-6-mediated paracrine stimulation of light chain-secreting myeloma cell growth was mediated through the suppression of p38 MAPK as well as modulation of activation of transcription factor NF-κB. These findings suggest that PACAP may be a new antitumor agent that directly suppresses light chain-secreting myeloma cell growth and indirectly affects tumor cell growth by modulating the bone marrow milieu of the multiple myeloma. (Cancer Res 2006; 66(17): 8796-803)

Introduction
A spectrum of plasma cell disorders from monoclonal gammapathy of undetermined significance evolves to rapidly progressive myeloma. Multiple myeloma is a B-cell malignancy characterized by excess expansion of malignant plasma cells in close association with stromal cells in the bone marrow and often overproduces immunoglobulin light chains. The increased amounts of malignant plasma cells in bone marrow cause serious medical complications, including skeletal destruction, renal failure, anemia, and hypercalcemia. A complex network of cytokines and cell adhesion molecules is maintained by bone marrow stromal cells (BMSCs), which regulate the proliferation, survival, and the function of myeloma cells (1, 2). The disease course is variable but remains incurable despite the use of conventional and high-dose chemotherapy with hematopoietic stem cell transplantation; therefore, novel therapeutic approaches are urgently needed in clinical settings. The establishment of new strategies that increase the specificity of the treatment and minimize undesirable toxicity to normal cells is needed to improve the prognosis of multiple myeloma.

Human cytokines have been known to play a major role in the growth and prevention of apoptosis of tumor cells in myeloma patients. The adhesion of human myeloma cells to the BMSCs triggers both adhesion and expression of cytokines from the stromal cells that in turn stimulate cytokine-mediated local myeloma cell growth, survival, drug resistance, and migration. Specifically, interleukin 6 (IL-6) promotes multiple myeloma cell growth, survival, and drug resistance (3–6), whereas vascular endothelial growth factor induces multiple myeloma cell migration (7). More precisely, the adhesion of multiple myeloma cells to BMSCs triggers nuclear factor-κB (NF-κB)–dependent transcription and secretion of IL-6 (8), whereas inhibition of NF-κB activity eliminated this response (9, 10). On the other hand, tumor necrosis factor-α (TNF-α) secreted by the multiple myeloma cells in the bone marrow milieu activates NF-κB, thereby modulating the expression of adhesion molecules on both multiple myeloma cells and BMSCs as well as inducing IL-6 transcription and secretion in BMSCs (8, 9, 11). Many of these same cytokines also contribute to osteolytic bone destruction. The cells involved in myeloma bone loss make large amounts of cytokines that are capable of stimulating myeloma growth and preventing apoptosis (12–15). Therefore, the stroma, osteoblasts, osteoclasts, and tumor cells all contribute to myeloma growth. Moreover, the bone marrow microenvironment also confers drug resistance in multiple myeloma cells via at least two different mechanisms: adhesion of multiple myeloma cells to fibronectin confers cell adhesion-mediated drug resistance, associated with induction of p27Kip1 and G1 growth arrest (16, 17), and cytokines (IL-6 and insulin-like growth factor 1) in the bone marrow milieu induce Janus-activated kinase 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3), phosphatidylinositol 3-kinase (PI3K)/PI3K target (Akt) signaling, or both, which mediates resistance to conventional therapies (4, 18, 19). The understanding that has been gained recently into the biology of myeloma has led to the development of biological therapeutics, such as thalidomide and bortezomib, which target not only the multiple myeloma cell but also multiple myeloma cell-host interactions and cytokines in the bone marrow microenvironment. These agents have shown remarkable activity...
against refractory multiple myeloma in early clinical studies, but prolonged drug exposure may result in the development of de novo drug resistance and side effects (2, 20–22). Therefore, the identification and validation of additional novel-targeted therapies to overcome drug resistance and improve patient outcome are needed.

The apparent pivotal role of cytokine-mediated multiple myeloma cell growth in the bone marrow milieu prompted us to look into the inhibitory effect of pituitary adenylate cyclase-activating polypeptide (PACAP). PACAP is a 38-amino acid pleiotropic neuroendocrine peptide originally isolated from ovine hypothalami based on its ability to stimulate adenylate cyclase in the pituitary cell cultures (23). PACAP is a new member of the secretin, glucagon, and vasoactive intestinal peptide (VIP) family of peptides, with the highest sequential similarity to VIP. PACAP can function as a neurotransmitter, neuromodulator, neurotrophic factor, vasodilator, noncholinergic catecholamine secretagogue, and also a modulator of immune cells by controlling the homeostasis. Recently, PACAP has been shown to have potent immunomodulatory effects on various immune responses by suppressing or stimulating the production of proinflammatory cytokines (24, 25). The immune modulatory effects of PACAP have attracted interest in this natural peptide as potential treatment for a variety of autoimmune and inflammatory disorders.

Chronic renal failure is often associated with human multiple myeloma and myeloma kidney, caused by the pathogenic effects of the overproduction of κ or λ light chain immunoglobulin proteins by cancerous plasma cells (26–28). To date, no therapy adhering to the BMSC in production of cytokines to determine the myeloma cell growth, its use as an effective renoprotectant in myeloma kidney would be seriously compromised. The present study addressed this issue of the direct and indirect effects of PACAP Inhibits Myeloma Cell Growth.

Materials and Methods

**Kinase inhibitors and receptor antagonists.** The MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) 1 inhibitor PD98059 and the p38 MAPK inhibitor SB202190 were purchased from Sigma (St. Louis, MO). The NF-κB inhibitor PDTC, the protein kinase A inhibitor H89, and the phospholipase C (PLC) inhibitor U73122 were also purchased from Sigma. These inhibitors were dissolved in DMSO, and the final concentration of DMSO was adjusted to be <0.01%. In each experiment using these inhibitors, the vehicle contained the same concentration of DMSO as was used for the treatment groups. The PAC1 and VPAC2 receptor antagonist PACAP(6-38) was obtained from American Peptide Co. (Sunnyvale, CA). The potent VPAC1-R-specific antagonist Ac-His-D-Phe2,K15,R16,L27 VIP(1-7)/GRF(8-27) (PG97-269) and the VPAC2-R-specific antagonist PG99-485 were provided by Drs. P. Gourlet and P. Robberecht (Université Libre, Brussels, Belgium), and Dr. A. Miyata (Kagoshima University, Kagoshima, Japan) provided us with the PAC1-R-specific antagonist M65.

**Cell cultures.** The NCI-H929 (H929) and IM-9 human multiple myeloma–derived cell line that produces the κ light chains and RPMI 8226 and U266/B1 (U266) human multiple myeloma cells producing the λ light chains were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 containing 10% noninactivated fetal bovine serum (FBS) and 0.05 mmol/L 2-mercaptoethanol in a humidified atmosphere of 95% air-5% CO2. Human BMSCs were provided by the Center for Gene Therapy, Tulane University Health Sciences Center (New Orleans, LA) and cultured in MEM containing 16.5% FBS. To evaluate cytokine secretion in BMSCs adherent to multiple myeloma cells, the passages 3 to 5 normal human BMSCs were cultured (1 × 10^5 cells/mL) for 24 hours to obtain a confluent monolayer. After BMSCs became confluent, the multiple myeloma cell (H929) suspension (4 × 10^5–5 × 10^6 cells/mL) was then added directly onto BMSCs. After incubation at 37°C for 24 hours, the supernatants were collected, and the remaining cells were used for the analyses of kinases. Human cytokine secretion in the supernatants of BMSCs cultured in medium or with multiple myeloma cells were determined using ELISAs.

**Growth inhibition assay in multiple myeloma cells.** The effects of PACAP or dexamethasone on H929, IM-9, RPMI 8226, and U266 multiple myeloma cell growth were determined using a colorimetric assay for the quantification of cell proliferation, based on the measurement of bromodeoxyuridine (BrdUrd) incorporation during DNA synthesis (Roche Applied Science, Penzberg, Germany). All experiments were repeated at least thrice, and the experimental condition was repeated in quadruplicate wells for each experiment.

**Treatments of human multiple myeloma cell-BMSC cocultures.** To evaluate the production of cytokines and signaling in multiple myeloma cells that are adherent to BMSCs, we used human H929 multiple myeloma cells cultured in BMSC-coated 12-well plates with serum containing fresh medium in the presence or absence of various concentrations of PACAP or dexamethasone as well as kinase inhibitors, transcription factor inhibitors, or receptor antagonists. After exposure to the corresponding test substances for 24 hours, culture supernatants were harvested and stored at −70°C for cytokine assays. After the media were removed, the cells were washed with ice-cold PBS and proteins were extracted by lysing the cells with mammalian cell lysis reagents (Sigma). Lysates were scraped and passed through a 21-gauge needle to shear the DNA and centrifuged at 12,000 × g for 10 minutes at 4°C. Finally, supernatants were harvested and used for kinase analysis.

**Measurements of human IL-6 and TNF-α and phosphorylated ERK1/2 and p38 MAPKs by ELISA.** Human IL-6 and TNF-α in supernatants of 24-hour cultures of BMSCs with or without H929 multiple myeloma cells, in the presence of varying doses of reagents, were measured using a commercial human cytokine ELISA kit (Quantikine, R&D Systems, Minneapolis, MN). Cells were trypsinized and counted to express the remaining cells were used for the analyses of kinases. The effects of PACAP or dexamethasone on H929, IM-9, RPMI 8226, and U266 multiple myeloma cells cultured in BMSC-coated 12-well plates with serum containing fresh medium in the presence or absence of various concentrations of PACAP or dexamethasone as well as kinase inhibitors, transcription factor inhibitors, or receptor antagonists. After exposure to the corresponding test substances for 24 hours, culture supernatants were harvested and stored at −70°C for cytokine assays. After the media were removed, the cells were washed with ice-cold PBS and proteins were extracted by lysing the cells with mammalian cell lysis reagents (Sigma). Lysates were scraped and passed through a 21-gauge needle to shear the DNA and centrifuged at 12,000 × g for 10 minutes at 4°C. Finally, supernatants were harvested and used for kinase analysis.

**Nuclear extracts and determination of NF-κB activation.** Confluent monolayers of BMSCs were incubated with H929 multiple myeloma cells for 24 hours in the presence or absence of PACAP and/or kinase inhibitors, and nuclear extracts were prepared by using nuclear extract reagents (Active Motif, Carlsbad, CA). The activation of transcription factor NF-κB, p50 and p65 subunits, was determined with a specific ELISA-based assay (TransAM, Active Motif).

**mRNA analyses of TNF-α and PACAP receptors.** Quantification of gene-specific mRNA for TNF-α expression in H929 myeloma cells cultured in the presence of various treatments indicated was done by using...
Quantitative Human TNF-α mRNA probes and calibrator (R&D Systems). For the reverse transcription-PCR (RT-PCR) analysis of PACAP receptors, total RNA was isolated from the human BMSCs, H929 multiple myeloma cells, and peripheral blood mononuclear cells (PBMC) by extraction with the RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA (0.5 μg) from each sample was used for RT-PCR, which was done by SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA). The pairs of primers used for amplification of human PAC1-R (F1, 5'-TGTGTGTGTGACTAC-3' and B1, 5'-CTTTCTGCTGATATTCCGGG-3'), human VPAC1-R (F10, 5'-CCAGTCCGCCCTCGGCTAC-3' and B10, 5'-GGGAGACATGCTAGTCTG-3'), and human VPAC2-R (F2, 5'-CTGACGCC-CAATGAGCAGGATACCTG-3' and B3, 5'-CAGTAGTACGTTCTGTTGAGAC-3') were designed with the MacVector program (Accelrys, San Diego, CA) based on the reported sequences of human PAC1-R (Genbank accession no. NM_001118), human VPAC1-R (Genbank accession no. NM_004624), and human VPAC2-R (Genbank accession no. NM_003382). These pairs can specifically discriminate among these three PACAP/VIP receptors. The validity of the PCR products was verified by sequencing at SeqWright DNA Technology Services (Houston, TX).

Statistical analysis. The results were expressed as the mean ± SE. Multiple comparisons were made with an ANOVA and Tukey-Kramer or Dunnett's multiple comparison tests (InStat, GraphPad, San Diego, CA). Statistical analyses, curve fitting, and calculations were done using GraphPad Prism version 4.0c. The minimal level of significance was defined as P < 0.05 for all analyses.

Results

Effect of PACAP on cellular proliferation of human multiple myeloma cells. We first tested the effect of PACAP on established κ and λ light chain-secreting human myeloma-derived cell lines, H929, IM-9, RPMI 8226, and U266 and observed that PACAP indeed had an antiproliferative effect on these tumor cells (Fig. 1). Coculture of H929 myeloma cells with PACAP inhibited cell growth dose dependently and maximum inhibition was seen at 10⁻⁹ mol/L PACAP, which inhibited myeloma proliferation by 45% to 50% (Fig. 1A). PACAP showed significant cytotoxicity (40-55% growth inhibition; P < 0.01), with IC₅₀ from 0.57 to 1.90 nmol/mL in H929, IM-9, U266, and RPMI 8226 light chain-producing multiple myeloma cell lines (Fig. 1B). PACAP inhibited myeloma cell growth dose dependently, with a greater suppressive effect on the growth of κ light chain-secreting multiple myeloma cells, at subnanomolar concentrations of PACAP (Fig. 1B). Dexamethasone, a conventional cytotoxic chemotherapeutic, displayed a similar degree of suppression on the tumor cell growth (Fig. 1A). We further examined whether PACAP could augment cytotoxicity of dexamethasone in H929 cells. PACAP at 10⁻⁹ mol/L concentration significantly enhanced 10⁻⁷ mol/L dexamethasone-induced cytotoxicity in human myeloma H929 cells (Fig. 1A). Importantly, the addition of IL-6 (20 ng/mL) did not inhibit the suppressive effect of PACAP on the growth of H929 κ-light chain-secreting multiple myeloma cells, although IL-6 (20 ng/mL) alone triggered the growth of myeloma cells (Fig. 1A).

Effect of PACAP on IL-6 secretion in BMSCs triggered by adherence of human multiple myeloma cells to BMSCs. IL-6 is a major growth factor for human myeloma cells, and an increase in serum IL-6 could be responsible for the expansion of myeloma cells and the progression of myelomas (3-6). As shown in Fig. 2, human H929 myeloma cells cultured alone did not produce detectable levels of IL-6, whereas the normal human BMSCs alone produced low level of IL-6. PACAP (10⁻⁹ mol/L) did not significantly decrease the IL-6 production in BMSC cultures. The addition of myeloma cells further increased the expression of IL-6 by BMSCs, reaching a maximum level during 24 hours of incubation (Fig. 2A). The addition of PACAP or dexamethasone to the cocultures suppressed IL-6 secretion dose dependently, and maximum inhibition was seen at 10⁻⁹ mol/L PACAP, which inhibited IL-6 secretion by 65% to 70% (Fig. 2B). The suppressive potency of dexamethasone seemed to be comparable with that of PACAP. The growth of H929 myeloma cells in BMSC cocultures was also inhibited by 10⁻⁷ mol/L PACAP significantly (Fig. 2C and D), suggesting that PACAP can inhibit paracrine light chain-secreting multiple myeloma cell growth in the bone marrow milieu and overcome cell adhesion–related drug resistance.

Effect of PACAP on the expression of TNF-α mRNA in human multiple myeloma cells. Both patient myeloma cells and bone marrow mononuclear cells express the mRNA and protein for the proinflammatory cytokine TNF-α, and TNF-α secretion is significantly higher in those myeloma patients with bone disease (12, 33-35). Growth of human H929 myeloma cells in the bone marrow culture did not trigger TNF-α secretion. Neither PACAP nor dexamethasone affected TNF-α secretion in the bone marrow (data not shown). However, incubation of the H929 cells with
Figure 2. Effect of PACAP on IL-6 secretion in BMSCs triggered by adherence of human multiple myeloma cells (MMC) to BMSCs. A, the addition of H929 human light chain-secreting myeloma cells increased the expression of IL-6 by the BMSCs, reaching a maximum level during the 24-hour incubation. B, the addition of PACAP or dexamethasone to the cocultures suppressed IL-6 secretion dose dependently. The suppressive potency of dexamethasone seemed to be comparable with that of PACAP. Columns, mean of five replicates; bars, SE. *, *P < 0.05; **, **P < 0.01, compared with the multiple myeloma plus an untreated sample of BMSCs. C, the growth of human H929 myeloma cells was markedly suppressed by 10⁻⁹ mol/L PACAP in BMSC cocultures, without affecting their viability. Images were obtained by using a Nikon Diaphot inverted phase-contrast microscope (Nikon, Tokyo, Japan) with 10×/0.30 numerical aperture objective. Image were acquired using a Nikon Coolpix 995 digital camera and processed using Adobe Photoshop CS. D, the number of healthy H929 myeloma cells in culture alone or cocultured with BMSCs or in PACAP (10⁻⁹ mol/L) treatment group. Columns, mean of cell counts in 20 visual fields expressed in each group; bars, SE.

Figure 3. Effect of PACAP on TNF-α mRNA expression and protein secretion in human multiple myeloma cells. Human light chain-secreting myeloma cells express the proinflammatory cytokine TNF-α. Incubation of human H929 myeloma cells with varying doses of PACAP reduced TNF-α mRNA expression (A) and protein secretion (B) in the myeloma cell culture in a dose-dependent manner. Columns, mean of four replicates; bars, SE. **, **P < 0.01; *, P < 0.05, compared with the multiple myeloma cell cultured alone without treatment (none).
remarkably suppressed the phosphorylation of both ERK1/2 MAPK (Fig. 5A) and p38 MAPK (Fig. 5B) in response to the adhesion of H929 multiple myeloma cells to BMSCs. The use of selective inhibitors [PD98059, an inhibitor of MEK1; SB202190, an inhibitor of p38 MAPK; PDTC, a NF-κB inhibitor; H89, a cyclic AMP (cAMP)–dependent protein kinase inhibitor; and U73122, an inhibitor of phosphatidylinositol-specific PLC] for signal transduction and the quantification of the levels of phosphorylated ERK1/2 and p38 MAPK showed that PD98059 completely inhibited ERK1/2 MAPK activation (Fig. 5A), and SB202190, PDTC, or U73122 suppressed the activation of p38 MAPK (Fig. 5B). However, these two inhibitors, PD98059 and SB202190, had no effect on each other, and both PDTC and U73122 only suppressed the p38 MAPK activation, suggesting the activation of p38 MAPK was also modulated by NF-κB and/or PLC in BMSCs.

On the other hand, the adherence of human H929 myeloma cells to stromal cells greatly increases the secretion of IL-6 from the BMSCs. This increase in IL-6 production involves NF-κB activation in stromal cells by myeloma cell adhesion (8). PACAP at a concentration of 1 nmol/L significantly suppressed the multiple myeloma cell-BMSC interaction–induced phosphorylation of the p50 subunit of NF-κB (Fig. 5C). The p65 subunit of NF-κB was inactivated in the basal state in cultured BMSCs adhering with multiple myeloma cells (data not shown). Nevertheless, both PD98059 and SB202190 did not affect on the activation of the p50 subunit of NF-κB in the stromal cells in the presence of the myeloma cells (Fig. 5C). However, cAMP-dependent protein kinase inhibitor H89 significantly suppressed the activated NF-κB in BMSCs.

Effects of PACAP and signaling inhibitors on IL-6 secretion in the human multiple myeloma cells adherent to BMSCs. Figure 5D shows that multiple signaling pathways regulated NF-κB-dependent transcription of the IL-6 gene in BMSCs triggered by adherence of human light chain-secreting H929 multiple myeloma cells to BMSCs. PACAP markedly inhibits IL-6 secretion in BMSCs, in a time- and concentration-dependent manner, without affecting their viability (Fig. 2). The secretion of IL-6 in response to the adhesion of multiple myeloma cells to BMSCs was attenuated by PD98059 and SB202190, with SB202190 showing greater potency (Fig. 5D). Moreover, the secretion of IL-6 by BMSCs was remarkably inhibited by PDTC or U73122, whereas H89 also attenuated the production of IL-6 in response to the adhesion of H929 multiple myeloma cells to the BMSCs (Fig. 5D). These findings suggest that the response of BMSCs by adhering to multiple myeloma triggered the increased secretion of IL-6 in bone marrow, at least in part, independently mediated through multiple signaling pathway interactions in BMSCs. The use of inhibitors has been further examined by which the treatments of 10−9 mol/L PACAP combined with the kinase inhibitor(s) on IL-6 production in human H929 myeloma cell and BMSC cocultures (Fig. 6). Result shows that PACAP blocked the secretion of IL-6 induced by the adhesion of light chain-secreting multiple myeloma cells to BMSCs was further enhanced by the addition of PD98059, and these events remained unchanged after cotreated with H89 or SB202190. This suggests that the suppressive effect of PACAP does not associate with p42/p44 ERK MAPK. However, H89 plus PDTC slightly attenuated the activity of PACAP on the suppression of IL-6. H89 plus PDTC seems to have rather augmented the suppressive effect of PACAP, although statistically insignificant. These results therefore identify that p38 MAPK–mediated and p50 NF-κB–mediated signaling cascades are required for the inhibitory effect of PACAP on the adhesion-associated activation of IL-6 transcription and secretion in bone marrow milieu.

Discussion

Multiple myeloma is incurable with conventional and high-dose chemotherapies. Although novel biologically based treatment strategies targeting both multiple myeloma cells and the bone marrow microenvironment offer promise to improve multiple myeloma patient outcome, 65% of patients with relapsed and refractory disease do not respond. In the present study, we have shown that PACAP, a naturally occurring peptide, directly inhibits κ and λ light chain-secreting human multiple myeloma cell proliferation and TNF-α synthesis in myeloma cell cultures. Importantly, PACAP also indirectly inhibits tumor cell growth by suppressing proinflammatory cytokine IL-6 secretion in stromal cells triggered by adherence of human H929 myeloma cells to BMSCs. There is growing evidence that multiple myeloma patients with relapsed and refractory disease do not respond to thalidomide and other novel agents, including bortezomib (Velcade; ref. 39). PACAP might enhance sensitivity or overcome resistance to novel chemotherapeutic agents, thereby improving patient outcome in multiple myeloma. In the present study, we observed no cytotoxicity in PBMCs freshly obtained from bone marrow.
aspirates at PACAP concentrations of $10^{-15}$ to $10^{-5}$ mol/L (data not shown), suggesting potential selective cytotoxicity against the tumor cells and a clinically useful therapeutic index for PACAP in vivo. To determine whether PACAP enhances the cytotoxicity of a conventional therapeutic agent, we examined the effect of dexamethasone together with PACAP on the proliferation of H929 human multiple myeloma cells. Dexamethasone synergized with PACAP-induced cytotoxicity, suggesting differential apoptotic signaling cascades for PACAP versus dexamethasone (Fig. 1).

Dexamethasone induces caspase-9 activation via a cytochrome c–independent, second mitochondria-derived activator of caspases–dependent pathway (40), whether low concentrations of PACAP sensitize multiple myeloma cell lines and patient cells to DNA-damaging chemotherapeutic agents remains unknown.

Multiple myeloma cell growth, survival, drug resistance against conventional chemotherapies, and migration are mediated via cytokines in the bone marrow milieu (3–7). Importantly, adherence of multiple myeloma cells to BMSCs up-regulates cytokine secretion from BMSCs by triggering NF-κB-dependent transcription and secretion of IL-6, whereas inhibition of NF-κB activity abrogates this response (8). IL-6 triggers the proliferation of multiple myeloma cells by activation of the Ras/Raf/MEK/p42/44 ERK MAPK signaling cascade and survival of multiple myeloma cells via JAK2/STAT3 activation with downstream induction of Bcl-xl, inhibitor of apoptosis proteins, and Mcl-1 (2). Moreover, IL-6 protects against dexamethasone-induced apoptosis via PI3K/Akt signaling (4). We therefore examined whether exogenous IL-6 inhibited PACAP-induced cytotoxicity in human H929 myeloma cell. We found that IL-6 altered the action of PACAP-induced cytotoxicity (Fig. 1), suggesting that PACAP, in contrast to conventional therapies, can overcome the protective effects of these cytokines in the bone marrow milieu.

On the other hand, human multiple myeloma cells overproduced the proinflammatory cytokine TNF-α, which has been implicated in myeloma bone destruction (33). Our result showed that PACAP inhibits TNF-α synthesis in H929 multiple myeloma cells and IL-6 secretion in BMSCs triggered by adherence of myeloma cells to BMSCs (Figs. 2 and 3). This suggests that PACAP acts against multiple myeloma, at least in part, by inhibiting sequelae of these induced cytokines, and possibly also be beneficial for inhibiting the increased osteoclastic bone resorption in myeloma bone disease (41–43). Therefore, combining TNF-α inhibition with NF-κB blockade may enhance the cytotoxicity of multiple myeloma cells by PACAP.

We have shown previously that PACAP markedly suppressed renal proximal tubule epithelial cell injury caused by toxic effect of immunoglobulin κ light chains both in vitro and in vivo by suppressing light chain-stimulated p38 MAPK activation and NF-κB translocation (31). Because PACAP abrogated the effect of IL-6 on the growth of light chain-producing human H929 multiple myeloma, at least in part, by inhibiting sequelae of these induced cytokines, and possibly also be beneficial for inhibiting the increased osteoclastic bone resorption in myeloma bone disease (41–43). Therefore, combining TNF-α inhibition with NF-κB blockade may enhance the cytotoxicity of multiple myeloma cells by PACAP.

**Figure 5.** Effects of PACAP and signaling inhibitors on activation of p42/44 and p38 MAPKs and NF-κB as well as on IL-6 secretion from BMSCs adhering to human multiple myeloma cells. A and B, the adherence of human H929 multiple myeloma cells to BMSCs causes phosphorylation of both ERK1/2 MAPK (4) and p38 MAPK (B) in BMSCs. PD98059, an inhibitor of MEK1; SB202190, an inhibitor of p38 MAPK; PDTC, a NF-κB inhibitor; H89, a cAMP-dependent protein kinase inhibitor; and U73122, an inhibitor of phosphatidylinositol-specific PLC. The quantification of the levels of phosphorylated ERK1/2 and p38 MAPKs showed that $10^{-9}$ mol/L PACAP almost completely inhibited both ERK1/2 and p38 MAPK activations. The addition of 12.5 mmol/L PDTC or 10 μmol/L U73122 also suppressed the activation of p38 MAPK. C, PACAP ($10^{-9}$ mol/L) completely suppressed the H929 cell-BMSC interaction–induced phosphorylation of p50 subunit of NF-κB. However, neither 10 μmol/L PD98059 nor 10 μmol/L SB202190 affected the activated p50 NF-κB in the stromal cells in the presence of the myeloma cells, whereas 10 μmol/L of H89 significantly suppressed the activation of p50 NF-κB. D, the secretion of IL-6 in response to the adhesion of light chain-secreting myeloma cells to BMSCs was suppressed by $10^{-9}$ mol/L PACAP. The response of BMSCs by adhering to human multiple myeloma cells triggered the increased secretion of IL-6 in bone marrow attenuated by PD98059 or SB202190, with SB202190 showing the greater potency. H89 or U73122 partially inhibited IL-6 production in BMSCs, whereas the inhibition of NF-κB activity by PDTC remarkably eliminated this response. Columns, mean of four replicates per group; bars, SE. **, $P < 0.01$; *, $P < 0.05$, compared with the BMSCs adhering to human multiple myeloma bound in the absence of treatments (MMC + BMSC).
myeloma cells in bone marrow microenvironment, we next examined which signaling cascades triggered by the adhesion of human multiple myeloma cells to BMSCs to activate NF-κB regulated IL-6 expression were inhibited by this new agent. Our results showed that the adhesion of human multiple myeloma cells to BMSCs can activate several signaling pathways, including NF-κB-dependent transcription, p42/p44 ERK MAPK, and p38 MAPK activations, in BMSCs (Fig. 5A-C). The activation of p38 MAPK was also modulated by NF-κB and/or PLC in BMSCs. PACAP activated phosphoinositide PLC by stimulating intracellular IP3 accumulation dose dependently in BMSCs adhering with human multiple myeloma cells (data not shown). However, the role of PACAP on IP3 production in modulating IL-6 secretion in BMSCs is undefined. More importantly, PACAP remarkably suppressed the phosphorylation of both ERK and p38 MAPKs in response to the adhesion of multiple myeloma cells to BMSCs. The secretion of IL-6 by BMSCs in response to the adhesion of human light chain-secreting multiple myeloma cells to BMSCs, which significantly inhibited by 10^{-9} mol/L PACAP, was enhanced by the addition of PACAP combining with ERK1/2 MAPK inhibitor PD98059 (Fig. 6). These findings suggest that the increased expression of IL-6 was modulated with the activation of p38 MAPKs elicited by proliferation of multiple myeloma cells adherent to BMSCs. The suppressive effect of PACAP does not associate with p42/p44 MAPK that independently up-regulated the transcription and secretion of cytokines and adhesion molecules on both multiple myeloma cells and BMSCs. The effect of activating these pathways includes increased availability of the transcription factor NF-κB translocation resulting in up-regulation of NF-κB-dependent genes, including IL-6. The cAMP signaling may be the upstream event in NF-κB phosphorylation. But it is unlikely that the NF-κB-dependent cytokine inhibitory effect of PACAP is mediated through the stimulation of adenylate cyclase. The signaling cascades involved in the effect of PACAP inhibiting IL-6 paracrine myeloma cell growth mediates through the suppression of p38 MAPK as well as modulation of the activation of transcription factor, NF-κB, depending on the receptors with which it interacts.

Three PACAP receptors, PAC1, which is PACAP specific, and VPAC1 and VPAC2, which have similar affinities for PACAP and VIP, have been identified on almost every known type of tumor cell (32). PACAP actions are most relevant to the proliferation or survival of tumor cells (44). However, PACAP was also found to inhibit proliferation of certain types of neuroblastoma cells and small cell lung cancer cells (45). PACAP inhibits cell proliferation through a cAMP-dependent pathway by the expression of its short variant, whereas the growth-stimulatory action of PACAP is via a PLC-dependent mechanism and via a MAPK pathway through the SV2 subtype of human PAC1-R (32, 44, 45). At least six human PAC1-R variants derived from alternative splicing have been reported in different tissues with seven putative membrane-spanning domains and belong to a family of glycoprotein receptors, which are coupled to multiple signal transduction pathways (38). A report has suggested that the hop subtype of the rat PAC1-R (human PAC1-R SV2 variant) is a “mitogenic” variant (46). Transfection with this variant allowed host cells to proliferate in response to PACAP via a PLC-dependent mechanism, whereas expression of short variant maintained the inhibitory action of PACAP on neuroblast proliferation (46). We found that BMSCs express all three types of human PACAP receptors, including the PAC1-R short and SV1 and SV2 variants (Fig. 4). However, human light chain-secreting H929 myeloma cells expressed the PAC1-R short subtype and VPAC1-R, but not VPAC2-R, and PBMCs expressed the PAC1-R short subtype and VPAC2-R, but not VPAC1-R, implying that this complex situation might be linked to the cytogenetic heterogeneity of tumors, different origins of these cells, or the normal complement of subtypes of PACAP receptors expressed by these cells.

Chronic renal failure is often associated with human multiple myeloma and myeloma kidney and is a major contributing cause of morbidity and mortality in patients with multiple myeloma. These studies showed that excessive endocytosis and overloading with myeloma light chain proteins in renal proximal tubule cells produce proinflammatory cytokines (IL-6, IL-8, TNF-α, and monocyte chemoattractant protein-1) through p38, c-Jun NH2-terminal kinase, or ERK-type MAPK as well as modulation of activation of various transcription factors, including NF-κB and activator protein (29, 30). Despite an improved understanding of the pathophysiology, no effective treatment is known for myeloma kidney, except for the limited use of steroids. Our laboratory has shown recently renoprotection of myeloma kidney by PACAP and the marked inhibitory effects of PACAP on the production of proinflammatory cytokines stimulated by multiple myeloma light chains in human renal proximal tubule epithelial cells and in the kidneys of rats infused with multiple myeloma κ-light chains (31). Work from our laboratory has also shown that PACAP is capable of inhibiting multiple myeloma light chain-induced cytokine expression with a considerably greater potency than dexamethasone and has attenuated the resulting cell damage in the renal tubule cells (31). Thus, these studies have suggested that PACAP could be used...
induced cytotoxicity, we have shown that PACAP inhibits renal tubule injury in multiple myeloma. NF-
BMSCs adhering to human light chain-secreting multiple myeloma on multiple myeloma cells and BMSCs, and regulates cytokine transcription and secretion in BMSCs (7, 8). Our study showed that PACAP blocked phosphorylation of p50 NF-κB induced by the BMSCs adhering to human light chain-secreting multiple myeloma cells. Given that neither IL-6 nor TNF-α protects against PACAP-induced cytotoxicity, we have shown that PACAP inhibits the growth of human light chain-secreting multiple myeloma cells adherent to BMSCs, without cytotoxicity on PBMCs and BMSCs. Taken together, these findings promote PACAP38 as a promising candidate for an effective and safe renoprotective kidney and also as a new antitumor agent in multiple myeloma.

Acknowledgments


Grant support: Kaken American Foundation and the Multiple Myeloma Research Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Jerome L. Maderbrot for his editorial assistance on this article.

References


Pituitary Adenylate Cyclase-Activating Polypeptide Is a Potent Inhibitor of the Growth of Light Chain-Secreting Human Multiple Myeloma Cells

Min Li, Shirley Cortez, Tomoya Nakamachi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/17/8796

Cited articles
This article cites 45 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/17/8796.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/66/17/8796.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.