Urol Res (2003) 31: 374–377
DOI 10.1007/s00240-003-0352-z

ORIGINAL PAPER

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GnRH-PAP hormonotoxin targets cytotoxicity to prostate cancer cell lines

Received: 15 May 2003 / Accepted: 16 July 2003 / Published online: 13 September 2003

Abstract Pokeweed antiviral protein (PAP) is a plant-derived, highly potent ribosome inactivating protein that causes inhibition of protein translation and rapid cell death. We have previously described potent cytotoxic activity of a GnRH (gonadotropin-releasing hormone) receptor-targeted conjugate protein (GnRH-PAP) and demonstrated that cytotoxicity depended on the number of GnRH receptors and the duration of exposure. Here, we demonstrate that the GnRH-PAP conjugate was cytotoxic to three different prostate cancer cell lines, supporting the feasibility of using such hormonotoxins as novel therapeutics for hormone-responsive cancers such as prostate cancer.

Keywords GnRH · PAP · Conjugate protein · Prostate cancer

Abbreviations *GnRH* Gonadotropin releasing hormone · *GnRHR* Gonadotropin releasing hormone receptor · *PAP* Pokeweed antiviral protein · *DT* Diphtheria toxin · *PE Pseudomonas* exotoxin

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Introduction

Hormonotoxins bind to a specific receptor on the cell surface, are internalized by receptor-mediated endocytosis, and mediate cytotoxicity upon delivery to the cytoplasm. Numerous reports have demonstrated that such toxins can be delivered to specific cells expressing the targeted receptor [1, 2, 3, 4, 5]. Toxin conjugates have been tested in dozens of human clinical studies and in animals (reviewed in [6]). Our own recent studies have focused on targeting toxicity of PAP via the GnRH receptor (GnRHR). While normal pituitary cells express high levels of GnRHR, most other normal cells express low to undetectable levels [7, 8, 9]. Malignant cells that express GnRHR include breast [10], endometrial [11, 12], ovarian [11, 13], and prostate [14]. Thus, these cancer types should be susceptible to specific cytotoxicity mediated by a GnRHR-targeted hormonotoxin such as GnRH-PAP.

Bacterial toxins that have been targeted to cancer cells include *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT) [15, 16, 17]. However, bacterial-derived toxins are very immunogenic [18]. Moreover, these toxins often display some degree of nonspecific toxicity because they are able to penetrate living cells via their cell recognition domain [18, 19]. Pokeweed antiviral protein (PAP) is produced by the plant *Phytolacca americana* and belongs to the ribosome-inactivating protein family [20]. This enzyme is an RNA N-glycosidase that specifically removes an adenine residue from a highly conserved and exposed surface region in the large ribosomal RNA of eukaryotic and prokaryotic ribosomes [21, 22, 23, 24], inducing a conformational change in the subunit. This irreversibly inactivates the ribosomal subunit and prevents the GTP-dependent binding of elongation factor-2 to the affected ribosome [25], thus inhibiting translation and blocking protein synthesis; this, in turn leads to cell death. PAP alone (which does not contain the cell-binding domain) is not able to penetrate living cells. Due to its specificity and extreme

toxicity, PAP is an ideal candidate for targeting cell death as the toxic moiety of a hormonotoxin.

Materials and methods

GnRH-PAP conjugate

Preparation of the GnRH-PAP conjugated protein, D-Lys^6-GnRH (a GnRH analog with enhanced receptor binding activity) has been described previously [5]. SDS-PAGE (12% reducing gel) analysis and mass spectrometry estimated unconjugated PAP in the final product to be at 25–35%; the remainder of the product was PAP with either one or two GnRH molecule(s) attached.

Cell culture

Chinese hamster ovary (CHO) control and CHO-GnRHR cells, a generous gift from Dr. Colin Clay (ARBL, CSU), were maintained as previously described [5]. CHO-GnRHR cells were generated following transfection with cDNA for the murine GnRH receptor fused to green fluorescence protein and yellow fluorescence protein, and express high levels of GnRHR [26]. Human prostate carcinoma (PCA) cell lines DU145 adenocarcinoma (androgen-insensitive and moderately metastatic), LNCaP (androgen-sensitive and non-metastatic) and PC3 (androgen-insensitive and highly metastatic) were maintained as previously described [5].

Expression of GnRHR message and protein

Expression of endogenous and transfected GnRHR was examined in CHO, CHO-GnRHR cells and PCA cell lines by RT-PCR and immunoprecipitation analysis. Total RNA was prepared from cellular extracts using Trizol^T (Gibco BRL/Invitrogen; Carlsbad, CA) and cDNA prepared using oligo dT primers and MMV reverse transcriptase. RT-PCR was performed using the intron spanning primers: 5′-GAC CTT GTC TGG AAA GAT CC −3′(nt 93–112)/ 5′-CAG GCT GAT CAC CAC CAT CA −3′(nt 392–412), based on the published sequence of human GnRHR (27). The amplified PCR products were separated on 1.5% agarose gels and visualized with ethidium bromide. Immunoprecipitation of GnRHR protein was carried out as previously described [28], using 1 μg GnRHR antibody (Lab Vision Corporation; Fremont, CA).

Toxicity assays

Two assays were employed to determine the effect of GnRH-PAP conjugate on cell lines. In the first assay, cell viability was determined using the MTT assay. A 96-well tissue culture plate (Nalge Nunc International; Rochester, NY) was seeded with 1,500 cells/well and incubated for 2 days. We added 100 μl of varying amounts of GnRH-PAP conjugate in culture medium to each well. After incubation for 5 additional days, viability was assessed. We added 20 µl MTT (Acros Organics; Morris Plains, NJ) from a 5 mg/ml stock solution to each well, and the plate was incubated at 37°C for 5 h. Media was removed and 100 μl of 75% isopropanol, 2% concentrated HCl was added to dissolve crystals. The plate was then read at absorbance of 490 nm using a Molecular Devices (Sunnyvale, CA) plate reader. In the second assay, clonogenic survival in colonies was measured following 6–7 days of exposure to varying amounts of GnRH-PAP conjugate as previously described. The number and area of colonies in treated cultures was expressed as a percentage of those in control cultures.

Cell viability and clonogenic survival experiments were conducted with triplicate samples, and values are expressed as mean ± standard deviation.

Results and discussion

Expression of GnRHR message and protein in CHO-GnRHR and PCA cell lines

The presence of GnRHR message, as detected by RT-PCR, is seen in Fig. 1, panel B. As expected, CHO parental control cells, while they expressed β-actin (panel A), did not express GnRHR message. However, all three PCA cell lines as well as CHO-GnRHR expressed detectable amounts of GnRHR message. Data from immunoprecipitation analysis with anti-GnRHR antibody (Fig. 1, panel C) were consistent with mRNA expression, showing the presence of GnRHR protein in CHO-GnRHR and all three PCA cell lines, but not in CHO control cells.

Toxicity of GnRH-PAP in CHO-GnRHR and PCA cell lines

Since all of the prostate cancer cell lines tested expressed GnRHR message and protein, we predicted they would be susceptible to cell killing by a GnRHR-targeted hormonotoxin such as GnRH-PAP. The cytotoxicity data confirming this are shown in Figs. 2–4. MTT assay showed 50% inhibition of cell viability at approximately 8×10^{-8} M GnRH-PAP (DU145 and PC3) and 2×10^{-7} M GnRH-PAP (LNCaP and CHO-GnRHR). In contrast, control CHO cells exhibited greater than 90% viability even at 3.0×10^{-7} M GnRH-PAP (Fig. 2). The difference in cell viability between CHO and all other cell lines was significant ($p < 0.003$ at 3.0×10^{-8} M GnRH-PAP). Stained plates from the clonogenic assay are shown in Fig. 3, and colony counts from these plates are graphed in Fig. 4 In control CHO cells, more than 90% clonogenic survival was seen at 1×10^{-7} M GnRH-PAP. In test cell lines, 50% inhibition of clonogenic survival was seen at approximately 4×10^{-9} M (DU145), 1×10^{-8} M (PC3 and CHO-GnRHR), and slightly greater than 1×10^{-7} M (LNCaP). Thus, greater sensitivity of the PCA cell lines to GnRH-PAP, compared to control CHO, ranged from 50% to more than two logs. As with the

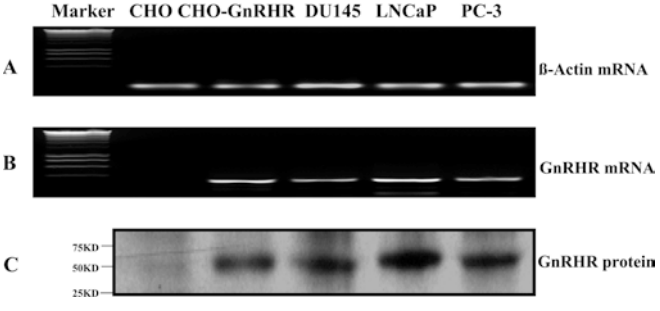

Fig. 1A–C Expression of GnRH mRNA and protein. Indicated cell lines were assayed for presence of control β-actin (A) and GnRHR (B) message by RT-PCR. Presence of GnRHR protein was assessed by immunoprecipitation (C)

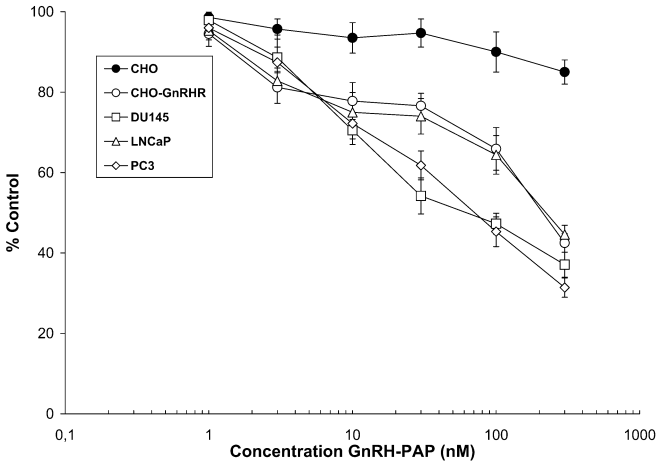

Fig. 2 Cytotoxicty of GnRH-PAP in PCA cell lines. MTT assay was used to measure cell viability in indicated cell lines exposed to increasing amounts of GnRH-PAP

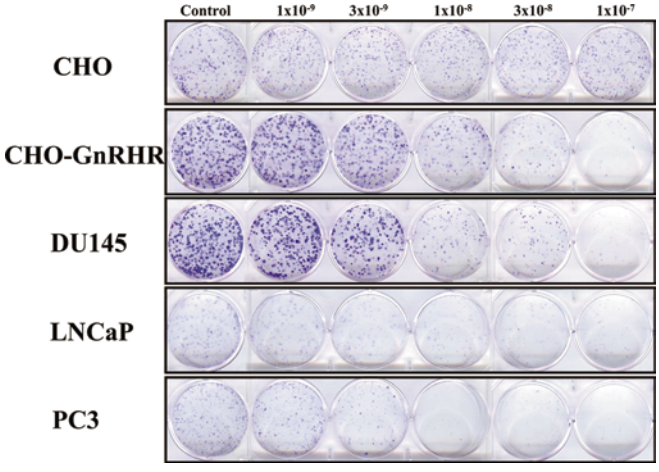

Fig. 3 GnRH-PAP inhibits survival of GnRH-PAP- exposed PCA cell lines. Clonogenic assay was used to measure survival in indicated cell lines exposed to increasing amounts of GnRH-PAP

viability assay, the difference in clonogenicity between CHO and all other cell lines was significant ($p < 0.0004$ at 3.0×10^{-8} M GnRH-PAP). Although the MTT and clonogenic survival assays measure different outcomes, the results seen in the different PCA cell lines were similar for both assays. Studies are underway to determine the mechanism of cell death.

We have previously shown that cytotoxicity from GnRH-PAP correlated with receptor number in various cell types [5]. However, another study demonstrated that cytotoxicity from recombinant toxins containing PE and DT in gastrointestinal and leukemic cancer cells did not correlate with the number of receptor sites [29]. Ultimately, the toxicity of such compounds depends not only on receptor number, but also on internalization and rate of delivery into the cytoplasm. These data demonstrate that prostate cancer cell lines express GnRHR message and protein, and that they are susceptible to cell toxicity mediated by a GnRH-PAP conjugate protein.

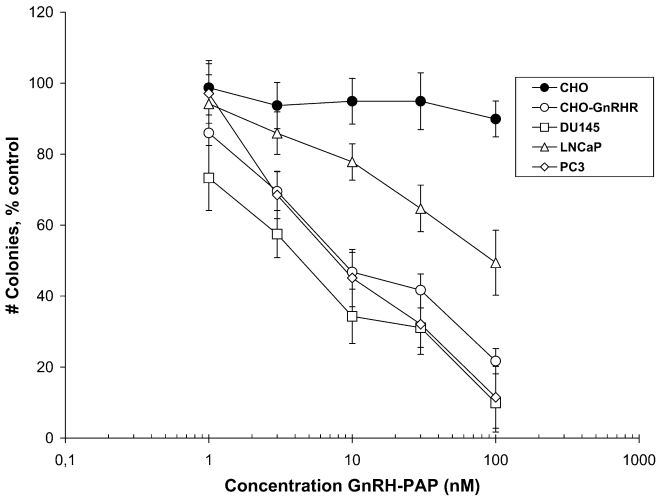

Fig. 4 Decrease in cell survival numbers in GnRH-PAP-exposed PCA cell lines. Colony counts from Fig. 3 show substantially decreased cell survival in indicated cell lines exposed to increasing amounts of GnRH-PAP

This is consistent with our previously reported finding of detectable levels of GnRH receptors on prostate cancer cell lines [5] and supports the feasibility of using hormotoxins to treat hormone responsive disease such as prostate, breast, endometrial and ovarian cancers in humans.

Acknowledgements This work was supported by NIH grant #R41 CA96049 to LM Glodé and TM Nett.

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