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DISSERTATION

QUANTITATIVE ASSESSMENT, STABILITY, AND CYTOTOXIC ACTIVITY OF GnRH-TOXIN CONJUGATES

Submitted by

Wei-Hsiung Yang

Department of Physiology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY WEI-HSIUNG YANG ENTITLED "QUANTITATIVE ASSESSMENT, STABILITY, AND CYTOTOXIC ACTIVITY OF GnRH-TOXIN CONJUGATES" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

DN Rao Veeramachaneni

Advisor

Department Head

ABSTRACT OF DISSERTATION

QUANTITATIVE ASSESSMENT, STABILITY, AND CYTOTOXIC ACTIVITY OF GnRH-TOXIN CONJUGATES

Hormone-dependent cancers such as breast, endometrial, ovarian, and prostate cancers remain major diseases in America and throughout world. These cancers can be treated by surgical castration, sex steroid antagonists, and GnRH agonists or antagonists. However, these treatments have side effects and disadvantages. In view of non-specific toxicity of most chemotherapeutic agents against many normal cells, the development of targeted chemotherapy is warranted. Efficient targeting of chemotherapeutic drugs to the cancerous area could be of great benefit for patients with advanced or metastatic tumors.

Pokeweed antiviral protein (PAP), a ribosome-inactivating protein, has potent antiviral activity or cytotoxic action against cells once inside the cytoplasm of both eukaryotes and prokaryotes. Ribonuclease A (RNase), a distributive endoribonuclease, possesses cytotoxic activity, appears to facilitate host defenses by killing pathogens, and plays a physiological role in cell death. Therefore, PAP and RNase are good candidates for use as the cytotoxic component of hormonotoxins. Recently, a series of cytotoxic conjugates containing a gonadotropin-releasing hormone (GnRH) analog and PAP or RNase was developed. D-Lys^6-GnRH-Pro^9-ethylamide (D-Lys^6-GnRH) was conjugated to PAP (GnRH-PAP) or RNase (GnRH-RNase) using a chemical cross-linking method.

The biological effectiveness of a conjugate is dependent on stability and half-life in the circulation until it enters cells as well as its cytotoxicity once it enters the target cell. Therefore, a sensitive, specific, and accurate assay was needed to evaluate stability in biological fluids and circulating half-life. This assay must be able to distinguish both the toxin and targeting moieties of the conjugate to ensure that the substance being measured is the intact conjugate. Therefore, a sensitive sandwich enzyme-linked immunoabsorbent assay (ELISA) for GnRH-toxin conjugates was developed, using anti-GnRH to capture the conjugate and anti-PAP or anti-RNase for quantification. The data showed that conjugates prepared with disulfide-bond linker are easily degraded in sheep serum. However, a maleimidobutyryl-group linker is very stable in sheep serum. Thus, a non-cleavable linker should be considered for conjugates designed for use *in vivo*.

The next objective was to determine if GnRH analog could be used as a cell-binding moiety to deliver PAP or RNase to cells expressing GnRH receptors. Chinese hamster ovary (CHO) cells that had been transfected with cDNA for the murine GnRH receptor to create a cell line expressing high levels of GnRH receptor and two mouse gonadotroph tumor cell lines that express endogenous GnRH receptors (αT3-1 and LβT2 cells) were used to evaluate the cytotoxic effects of GnRH-PAP and GnRH-RNase. Cytotoxicity of GnRH-PAP using human breast cancer, endometrial cancer, and prostate cancer cell lines was also examined. Treatment of GnRH receptor-positive cells with GnRH-PAP resulted in a dose-dependent inhibition of growth. Cytotoxic activity of GnRH-PAP was dependent on the number of GnRH receptors expressed on the surface of the cell lines (r^2 = 0.871). In contrast, GnRH-PAP did not alter growth of CHO cells which did not express GnRH receptors. Moreover, the cytotoxic activity of GnRH-PAP could be

partially inhibited by addition of excess GnRH analog. Neither free PAP nor GnRH analog alone affected proliferation of these cells. To determine the length of time that cells must be exposed to GnRH-PAP to completely inhibit proliferation, cells were treated at the beginning of a 7d incubation with GnRH-PAP for 1, 6, 24, 48, 72, or 96h. Growth was completely inhibited in cells exposed to GnRH-PAP for 72h or longer. These results suggest that GnRH or its analogs can be used to specifically deliver toxin molecules to cells that express GnRH receptors on their surfaces.

The result showed that the cytotoxicity of GnRH-RNase conjugates on cell lines expressing GnRH receptors was very low. This may be due to the presence of an endogenous RNase inhibitor in most cells.

Since the efficiency of induction of apoptosis may have direct effects on the therapeutic usefulness of GnRH-toxin conjugates, the ability of GnRH-PAP conjugates to induce apoptosis of αT3-1 cells was evaluated. The result showed for the first time that GnRH-PAP conjugates induced apoptosis after treatment of GnRH-PAP conjugates at 3h or longer. The result also showed that GnRH-PAP conjugates not only inhibit cell division but also induce cell death.

Thus, a new class of biomedicines that act as hormonotoxins against cells expressing GnRH receptors provides a novel approach for inhibiting reproduction and treating cancers which are dependent on reproductive hormones.

Wei-Hsiung Yang
Department of Physiology
Colorado State University
Fort Collins, Colorado 80523
Summer 2001

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CHAPTER ONE

REVIEW OF LITERATURE

I. Introduction

For decades, the use of anti-neoplastic agents has been the basis for treatment of various disseminated cancers. However, one of the limitations of chemotherapy is that the systemic administration of an effective dose of a wide variety of cytotoxic drugs is restricted by their nonselective toxic effect on cells other than cancerous ones. A more specific local delivery by targeting of highly active cytotoxic agents to tumor cells, based on Paul Ehrlich's idea of "magic bullets" (Ehrlich, 1956), offers an approach that might overcome these drawbacks. An immunotoxin (IT) or hormonotoxin (HT) is a cytotoxic agent that consists of a cell-binding moiety linked to a toxin moiety by chemical coupling or recombinant DNA technology (Pastan et al., 1986; Pastan and Fitzgerald, 1991). The cell-binding moiety can be an antibody, a growth factor, or a hormone that binds selectively to surface molecules on certain cell types.

Elimination of pituitary-gonadal function is one of several ways to inhibit the growth of sex steroid hormone-dependent tumors such as prostate cancer in males and breast, endometrial, and ovarian cancers in females. The glycoproteins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are secreted by gonadotropes in the anterior pituitary gland. Synthesis and secretion of FSH and LH are dependent on gonadotropin-releasing hormone (GnRH), a decapeptide released by the hypothalamus. Specific, high-affinity membrane receptors for GnRH have been identified in

gonadotropes and many sex steroid hormone-dependent tumors (i.e. breast, endometrial, ovarian, and prostate tumors). The information suggests that GnRH may be used as a cell-binding moiety to deliver toxin molecules into gonadotropes and tumors expressing GnRH receptors. Therefore, elimination of gonadal function and treatment of tumors harboring GnRH receptors can be achieved by the cytotoxin destroying gonadotropes and tumors, respectively. The purpose of this chapter is to review the information on immunotoxins and hormonotoxins, with emphasis on GnRH and the two toxins (pokeweed antiviral protein and ribonuclease A) used in this study.

II. Hypothalamic-Pituitary-Gonadal Axis

The regulation of normal mammalian reproductive function and sexual maturation requires the precision and integration of hormonal regulation at the hypothalamic, pituitary, and gonadal levels. This integration as a feedback loop (Pierce and Parsons, 1981) is termed the "hypothalamic-pituitary-gonadal axis". The center of this axis is the anterior pituitary gland. Primary anterior pituitary cells are comprised of a heterogeneous population of well differentiated, secretory cell types. These include corticotropes, which synthesize and secrete adrenocorticotropin (ACTH) as well as other hormones including lipotropins, endorphins, and enkephalin; lactotropes, which synthesize and secrete prolactin (PRL); somatotropes, which synthesize and secrete growth hormone (GH); thyrotropes, which synthesize and secrete thyroid-stimulating hormone (TSH); and gonadotropes, which synthesize and secrete LH and/or FSH (Daughadary, 1985; Marshall and Barkan, 1992). Somatotropes and lactotropes comprise 70% of the anterior pitutary cells (Reichlin, 1989). Corticotropes comprise 10% of the anterior pituitary cells

(Reichlin, 1989). Approximately 6-15% of the cells in the anterior pituitary gland of normal rats and mice are gonadotropes (Ibrahim et al., 1986; Gharib et al., 1990).

GnRH is a decapeptide synthesized in the neurosecretory cells in the preoptic area of the hypothalamus. GnRH is secreted into the hypophyseal portal circulation and is transported to the anterior pituitary gland, where it binds to receptors on gonadotropes to modulate the synthesis and secretion of the gonadotropins, FSH and LH. Gonadotropins, in turn, are secreted into the systemic circulation and act on the gonads to regulate gametogenesis and steroidogenesis (Desjardins et al., 1981; Gharib et al., 1990). FSH stimulates spermatogenesis in males and growth and maturation of ovarian follicles in females. In males, LH regulates the development and function of Leydig cells and stimulates androgen secretion. In females, LH regulates follicular development and promotes ovulation and corpus luteum formation. Gonadal steroids (i.e. androgens, estrogens, progesterone) and peptides (i.e. inhibin), in turn, are secreted into the systemic circulation and act to modulate hypothalamic and pituitary function in both positive and negative feedback loops (Pierce and Parsons, 1981; Conn et al., 1987; Gharib et al., 1990).

III. Gonadotropin-Releasing Hormone (GnRH)

Gonadotropin-releasing hormone (GnRH) is a small hormone composed of ten amino acids and stimulates synthesis and secretion of LH and, to a lesser extent, FSH from the anterior pituitary gland. GnRH is a decapeptide that is synthesized in the neurosecretory cells in the preoptic area of the hypothalamus and secreted into the hypophyseal portal circulation and transported to the anterior pituitary gland, where it binds to receptors on

gonadotropes to modulate the synthesis and secretion of FSH and LH. Determination of the structure and synthesis of GnRH was first accomplished in 1971 by Andrew Schally (Matsuo et al., 1971) and Roger Guillemen (Burgus et al., 1972), who eventually shared the Nobel Prize for this discovery. The molecular structure of GnRH was determined to be: pyro-Glu^{1}-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5}-Gly^{6}-Leu^{7}-Arg^{8}-Pro^{9}-Gly^{10}-NH_2.

GnRH has been studied in the past 30 years, and more than 3,000 GnRH analogs have been synthesized and characterized (Karten and Rivier, 1986; Vickery, 1986). Normally, GnRH has a very short half-life, about 2-4 min (Karten and Rivier, 1986; Lasdun et al., 1989; Lasdun and Orlowski, 1990). Thus, more potent and longer-lasting analogs were considered to be necessary for clinical applications. The studies on the relationship between structure and biological activity showed that histidine in position 2 and tryptophan in position 3 play a functional role in the biologic activity of GnRH, and simple substitutions or deletions in this active center decrease or abolish GnRH activity (Schally et al., 1976; Boepple et al., 1986; Karten and Rivier, 1986). Amino acids in positions 1 and 4-10 are essential for binding to the receptors (Schally et al., 1976; Boepple et al., 1986; Karten and Rivier, 1986). Chemical alterations of the amino acids at positions 6 and 10 produce superactive synthetic derivatives of GnRH that resist degradation by endopeptidases (Karten and Rivier, 1986) but retain a high affinity for the pituitary GnRH receptor. Endopeptidases cleave GnRH at the sixth position and are widely distributed in mammalian tissues. Substitutions of the various D-amino acids in the sixth position will yield agonists with 15-200 times the potency of native GnRH (Fujino et al., 1974; Coy et al., 1976; Boepple et al., 1986; Karten and Rivier, 1986; Vickery, 1986). Of these, the most important are: D-Trp^{6}-GnRH, D-Ala^{6}-GnRH, and D-

Lys^6-GnRH. These analogs have been proven valuable in the treatment of a wide variety of endocrinological disorders (Conn and Crowley, 1991; Barbieri, 1993).

In mammals, gonadotropin pulses result from pulses of GnRH released from hypothalamic neurosecretory cells into the pituitary portal circulation. The physiologic stimulation of secretion of gonadotropins requires intermittent GnRH release (Belchetz et al., 1978; Knobil, 1980). The binding of GnRH to specific receptors (60 kDa glycoprotein) distributed on the cell surface of the gonadotrope is the first and vital step in initiating of intracellular events (Kaiser et al., 1997) necessary for normal reproductive function. Coupling of GnRH to its receptor results in internalization and subsequent lysosomal degradation of the hormone-receptor complex (Hazum et al., 1980; Hazum et al., 1983). Liberated receptors are subsequently recycled to the cell surface, in part by up-regulation by GnRH with a concomittant increase in GnRH binding capacity (Vrecl et al., 1998). If the gonadotrope is exposed to GnRH for a prolonged period of time, down-regulation and desensitization occur and gonadotropin secretion is suppressed (Nett et al., 1981; Smith and Conn, 1984; Adams et al., 1986). Desensitization is the dissociation of GnRH-receptor binding from gonadotropin release. Down-regulation refers to a decreased number of receptors available for GnRH binding.

IV. Gonadotropin-Releasing Hormone Receptor (GnRHR)

GnRH and GnRHR are key molecules in regulating reproductive function in mammals. The greatest breakthrough for GnRHR was the initial cloning of a cDNA encoding the murine GnRHR (Tsutsumi et al., 1992). The GnRHR is a 327- to 328-amino acid protein (60 kDa glycoprotein) with seven putative membrane-spanning α helical

domains, characteristic of the family of G-protein-coupled receptors (Probst et al., 1992). More than 1000 different G protein-coupled receptors have been identified since the first receptors were cloned more than a decade ago (Kolakowski, 1994). Portions of the third intracellular loop and the sixth transmembrane region are thought to be important for G-protein coupling for most G-protein-coupled receptors (Gether, 2000) including the GnRH receptor (Schulz et al., 1999). It is believed that agonist binding to G-protein-coupled receptor results in a conformational change of the ligand-binding domain. Interestingly, the GnRHR lacks the typical intracellular carboxyl terminus, making it one of the smallest receptors with the seven-transmembrane segment motif. The GnRHR is the only G-protein-coupled receptor identified to date that activates phospholipase C and lacks a C-terminal tail. Several lines of evidence have demonstrated that this unique structural feature is associated with resistance to rapid desensitization of phosphoinositide signaling (Heding et al., 1998; Willars et al., 1999).

Just as changes in GnRH secretion from the hypothalamus affect FSH and LH synthesis and secretion, the number of GnRH receptors on the gonadotrope is also important for sensitivity of the pituitary to GnRH (Miller et al., 1990). Therefore, changes in FSH and LH synthesis and secretion are not only dependent on the amounts of GnRH secreted by the hypothalamus, but also on the numbers of GnRHR present on the cell membrane (Nett et al., 1981).

Specific, high-affinity membrane receptors for GnRH have been identified in gonadotropes and many immortalized cell lines, including αT3-1 and LβT2 cells. In αT3-1 cells, GnRH analog binds to receptors with a dissociation constant of 0.5 nM, similar to that measured in normal mouse (0.51 nM) anterior pituitary. The total number

of receptors for GnRH is estimated at 1.6 pmol/mg protein, about 5 times higher than in normal mouse (0.33 pmol/mg) (Horn et al., 1991). However, αT3-1 cells represent a homogenous cell population, whereas anterior pituitary cells are a heterogenous cell population, in which approximately 6-15% of the cells express the GnRH receptors. Thus, the estimated number of GnRHRs on αT3-1 cells is approximately 50% of the number on primary gonadotropes (Kaiser et al., 1997). The availability of immortalized cell models of gonadotropes has allowed considerable advance in the understanding of the structure and function of the GnRHR, the signal transduction pathways activated by GnRH, and molecular mechanisms of action of GnRH.

GnRHR mRNA is expressed in human pituitary gland, breast, breast tumor (Baumann et al., 1993), ovary, ovarian tumor (Ohno et al., 1993), prostate, prostate tumor, endometrium, and endometrial tumor (Imai et al., 1994). Human myometrium and uterine leiomyomata also possess specific binding sites for GnRH (Marinaccio et al., 1994). GnRHR genes were found in LNCaP (Limonta et al., 1993) and PC3 prostate, MDA-MB-231 (Harris et al., 1991) and MCF-7 breast (Kakar et al., 1994), Ishikawa endometrial (Srkalovich et al., 1990; Emons et al., 1993), and ovarian (Irmer et al., 1995) cancer cell lines. These cell lines contain high-affinity binding sites for GnRH (Vincze et al., 1991; Vincze et al., 1994). The high-affinity GnRH-binding sites are commonly regarded to be the same as the GnRH receptor of the pituitary gland. Therefore, it was proposed that the effects of GnRH analogs in these cancers are mediated by interactions with specific GnRH receptors. Because specific high-affinity binding sites for GnRH are present in about 50% of breast cancers (Fekete et al., 1989), in approximately 80% of endometrial and ovarian cancers (Emons and Schally, 1994), and in nearly 85% of prostate cancers

(Qayum et al., 1990), targeted chemotherapy based on cytotoxic GnRH analogs might be more efficacious and less toxic in these malignancies than conventional regimens of anti-neoplastic agents.

V. Immunotoxin and Hormonotoxin

In the past 25 years, with the advent of monoclonal antibody techniques, the antibody-toxin conjugate (immunotoxin) has been developed to kill targeted cells in the body. Immunotoxins consist of a monclonal antibody as the targeting moiety, a bacterial or plant toxin, and a linker that is stable in the systemic circulation but labile inside the cell (Pastan et al., 1992). Immunotoxins bind to the cell surface via the targeting moiety and are internalized by receptor-mediated endocytosis, a major pathway of internalization. The toxins are then delivered to either trans-Goligi/ER or acidified endosome where they are separated from the targeting moiety. Once in the cytoplasm, the toxins kill cells by inhibiting protein synthesis (Thrush et al., 1996) (Fig. 1). It is important to realize that only the combination of all three steps---binding, translocation, and catalytic function---leads to a potent toxin and that the three functions may not act independent of each other.

Most recently, the immunotoxin technique has been modified so that the hormones can be used for the targeting moiety (Schwartz et al., 1987; Singh et al., 1989; Singh and Sairam, 1990; Marcil et al, 1993). The application of the hormonotoxin technique makes it possible to develop a conjugate (i.e. GnRH-toxin conjugate) that has ability to eliminate gonadal function permanently. The technology is based on conjugation of a

toxic protein to a GnRH superagonist that will specifically deliver the toxin to gonadotropes in the anterior pituitary gland.

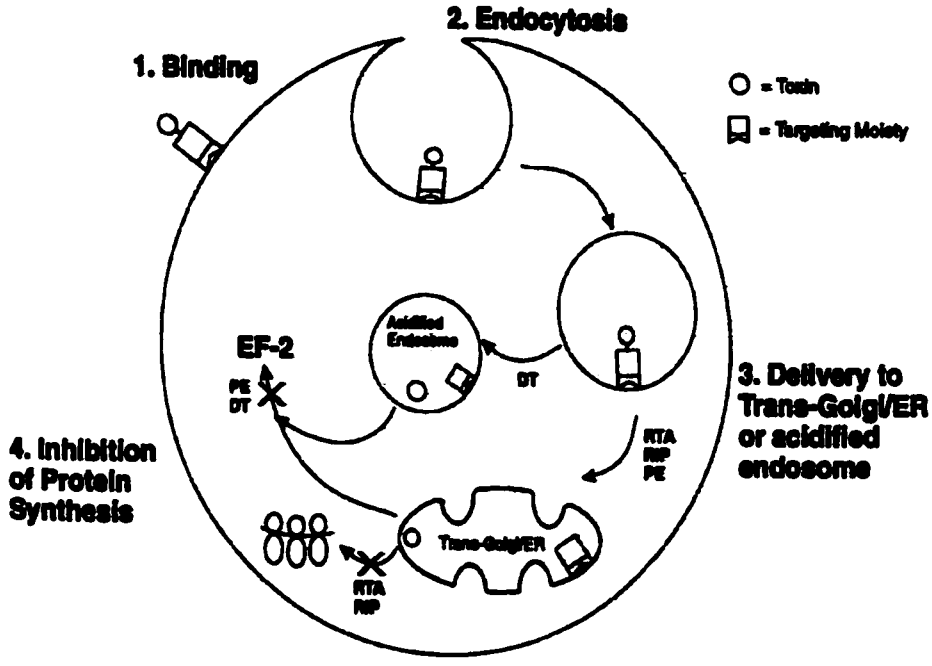

Fig. 1. Mode of action of immunotoxins (ITs) RTA, RIPs, PE, and DT. ITs bind to the cell surface via the targeting moiety and are internalized by receptor-mediated endocytosis. The plant toxins and PE are routed to the trans-Golgi network, and DT is routed to an acidified endosome. In these compartments, the toxins are separated from the targeting moiety. The toxins destroy cells by inhibiting protein synthesis; the plant toxins damage 28S rRNA, and the bacterial toxins inactivate EF-2. RTA, ricin toxin A-chain; RIP, ribosome-inactivating protein; PE, Pseudomonas exotoxin; DT, diphtheria toxin. (adapted and modified from Ann Rev Immunol 1996 14:49-71)

The most commonly used toxic moieties for making immunotoxins and hormonotoxins are derived from either plants or bacteria (Table 1). Both types of toxins destroy cells by inhibiting protein synthesis (Read and Stein, 1993). Some toxins, for example, diphtheria toxin, ricin, and abrin, consist of two peptide chains (Gill and Dinius,

1971). One of the chains (fragment A) contains the enzymatic activity that damages some essential function of the cell when it enters the cytoplasm. The other peptide chain (fragment B) consists of the targeting/binding activity that is involved in binding of the toxin to the cell and facilitating the transport of fragment A through cell membranes (Ogata et al., 1990). However, to target specific cells, fragment B must be removed or its activity blocked. More recently, several single chain toxins such as pokeweed antiviral protein (PAP), gelonin, or saporin (SAP) have been conjugated to immunoglobulins or hormones (Barbieri et al., 1993). These toxins are functionally analogous to fragment A of ricin or abrin in that they inhibit protein synthesis by inactivating the 28S rRNA.

Table 1. Toxins used for the preparation of immunotoxins and hormonotoxins

PLANT TOXINS (N-glycosidase for 28S rRNA)
Two chain toxins
1. Abrin
2. Ricin
3. Modeccin
Single chain toxins (RIPs)
1. Pokeweed antiviral protein (PAP)
2. Saporin (SAP)
3. Gelonin
4. Luffin
5. Momoridin
6. Trichosanthin
7. Trichokirin
BACTERIAL TOXINS (ADP Ribosylation of EF-2)
1. Diphtheria toxin (DT)
2. Pseudomonas exotoxin (PE)
3. Crossreacting material 107 (CRM-107)
FUNGAL TOXINS (single chain RIPs; ribonuclease of 28S rRNA)
1. α-sarin
2. restrictocin

(adapted and modified from Ann Rev Immunol 1996 14:49-71)

Like immunoglobulins, many hormones, growth factors, and neuropeptides/neurotransmitters such as corticotropin-releasing factor (Schwartz et al., 1987), thyrotropin-releasing hormone (Bacha and Reichlin, 1986), human placental lactogen (Chang et al., 1977), human choriogonadotropin (Oeltmann and Heath, 1979), insulin (Miskimins and Shimizu, 1979), luteinizing hormone (Myers and Villemez, 1989; Singh and Sairam, 1989; Singh et al., 1989; Singh and Sairam, 1990; Marcil et al., 1993; Singh et al., 1993), GnRH (Bajusz et al., 1989; Janaky et al., 1992; Janaky et al., 1992), and Substance P (Lappi and Wiley, 2000) have been conjugated to selective toxins. Recently, GnRH analogs have also been conjugated to doxorubicin and 2-pyrrolinodoxorubicin, a daunosamine-modified derivative of doxorubicin that is the most widely used anticancer drug, to form cytotoxic GnRH analogs AN-152 and AN-207, respectively (Nagy et al., 1996; Kovacs et al., 1997). Hybrid molecules AN-152 and AN-207 fully preserve the cytotoxic activity of their radicals, doxorubicin and 2-pyrrolinodoxorubicin, respectively, *in vitro*, and also retain the high binding affinity of the peptide hormone portion of the conjugates to rat pituitary receptors for GnRH (Nagy et al., 1996). The cytotoxic AN-207 conjugate is highly selective for the cells containing GnRH receptors, and less toxic to other cells such as somatotropes and thyrotropes, while 2-pyrrolinodoxorubicin nonselectively damages various cells of the pituitary gland (Kovacs et al., 1997). This approach, which remains to be tested clinically, could open a new area of cancer therapy because the cytotoxic analogs developed might have the potential to produce an eventual cure.

More recently, a recombinant GnRH-PAP fusion toxin has been developed (Schlick et al., 2000). The results showed that this recombinant GnRH-PAP fusion toxin

selectively inhibited the growth of the GnRH receptor-positive Ishikawa cell line (ID50 of 15 nM). However, several lines of evidence have shown that N-terminus and C-terminus of GnRH are essential for binding to the receptors (Schally et al., 1976; Boepple et al., 1986; Karten and Rivier, 1986). Therefore, fusion toxins in which either of the terminal amino acids of the GnRH molecule are altered, or attached to another moiety are very unlikely to have receptor binding activity. Thus, a recombinant GnRH-toxin conjugate is not a preferred approach. Therefore, whether recombinant GnRH-toxin conjugates have cytotoxic activity on GnRH-receptor-positive cells remains unclear.

VI. Pokeweed Antiviral Protein (PAP)

Many plant species synthesize toxic peptides called ribosome-inactivating proteins (RIPs). Pokeweed antiviral protein (PAP) produced by *Phytolacca americana* belongs to this peptide family (Irvin and Uckun, 1992). This enzyme is an RNA N-glycosidase which specifically removes an adenine residue from a highly conserved and exposed surface region in the large ribosomal RNA of eukaryotic and prokaryotic ribosomes (Endo et al., 1987; Endo and Tsurugi, 1987; Endo et al., 1988; Hartley et al., 1991), inducing a conformational change in the subunit. This irreversibly inactivates the ribosomal subunit and prevents the GTP-dependent binding of the elongation factor EF2 to the affected ribosome (Gessner and Irvin, 1980), thus inhibiting translation and blocking protein synthesis, leading to cell death. Recent evidence indicates that L3, a highly conserved ribosomal protein at the peptidyltransferase center, may provide a binding site for PAP, allowing depurination of the target adenine in its RNA subunit (Hudak et al., 1999)

PAP is a single-chain RIP (type I). In contrast, type II RIPs are composed of two peptides (Stirpe et al., 1992), one of them, designated the A chain, containing the N-glycosidase activity and the other, designated the B chain, containing the binding moiety. PAP has been classified as an antiviral agent because it reduces the infectivity of many plant viruses when co-inoculated with a virus on the leaves of susceptible species (Tomlinson et al., 1974). PAP has also been shown to be highly toxic *in vitro* to cells infected with different animal viruses including the human immunodeficiency virus (HIV) (Aron and Irvin, 1980; Olson et al., 1991; Rajamohan et al., 1999), poliovirus (Ussery et al., 1977), cytomegalovirus, influenza virus (Irvin and Uckun, 1992), and herpes simplex virus (Aron and Irvin, 1980). Unlike type II RIPs (e.g. ricin) or bacterial toxins (which are able to penetrate living cells through their cell recognition domain), PAP alone (which does not contain the cell-binding domain) is not able to penerate living cells. PAP, which contains four cysteine residues involved in two intramolecular disulfide bonds (Cys-34 to Cys-258 and Cys-84 to Cys-105), is not glycosylated. It has been shown that glycosylated proteins (i.e. ricin) increase lectin-dependent cytotoxic activity (Hubbard et al., 1986; Li et al., 1992). These results suggest that PAP alone is not cytotoxic. PAP has been reported to have minimal toxic effects upon cultured cells (Ussery et al., 1977; Masuho et al., 1982). Recently, it was found that PAP was nontoxic to human sperm and female genital tract epithelial cells even at a concentration 2,000 times higher than its IC(50) value against HIV-1 (D'Cruz and Uckun, 2001). Consequently, PAP represents an excellent candidate for the toxic moiety of an immunotoxin or hormonotoxin. PAP has been chemically linked to monoclonal antibodies to make immunotoxins because of its toxic activity and also because the

protein lacks carbohydrate residues reducing nonspecific reactions (Hubbard et al., 1986). When targeted to CD4+ cells, PAP inhibits HIV replication (Zarling et al., 1990). When targeted to human B cells, PAP eradicates leukemic progenitor cells obtained from patients with common-B-lineage acute lymphoblastic leukemia (Uckun et al., 1986; Myers et al., 1991; Jansen et al., 1992). Thus, PAP may provide useful tools when linked to a targeting protein, such as an antibody or GnRH, to kill cancerous cells and gonadotropes. Therefore PAP is a good candidate to be used as an immunotoxin or hormonotoxin.

VII. Ribonuclease A (RNase)

Bovine pancreatic ribonuclease A (RNase) is composed of 124 amino acid residues with a molecular weight of 13,683, and contains four disulfide bonds at positions 26-84, 40-95, 58-110, and 65-72. This enzyme is a distributive endoribonuclease that catalyzes the cleavage of the P-$O^{5'}$ bond of RNA on the 3' side of pyrimidine residues (i.e. cleavage of uridylyl (3'→ 5') adenosine (UpA)) (Thomson et al., 1995).

Endogenous RNases possess cytotoxic activities (Leone et al., 1973; Vescia et al., 1980; Fredens et al., 1982; Molina and Kierszenbaum, 1988; Frigas et al., 1991), appear to facilitate host defense by killing pathogens (Herriott et al., 1961; Thompson and Shively, 1966; Demain, 1967; McCormick et al., 1974; McLaren et al., 1984; Ackerman et al., 1985; Molina et al., 1988), and play physiological roles in cell death (McClure et al., 1990). RNase has also been shown to be as potent as ricin at abolishing protein synthesis on injection into oocytes (Saxena et al., 1991; Zewe et al., 1997). These interesting properties have led to using RNase in several therapeutic trials in human and

animal models (Matousek, 1973; Glukhov et al., 1976; Mikulski et al., 1990; Ardelt et al., 1991). To explore the cytotoxic potential of RNase toward mammalian cells, bovine pancreatic ribonuclease A was coupled via a disulfide bond to human transferrin or antibodies to the transferrin receptor (Rybak et al., 1991). The RNase hybrid proteins were cytotoxic to K562 human erythroleukemia cells *in vitro* with an IC50 around 10^{-7} M (Rybak et al., 1991). It was also found that when angiogenin (a 14.4-kDa human plasma protein with 65% homology to RNase A and a member of the human ribonuclease A superfamily) was fused to the transferrin or anti-transferrin receptor monoclonal antibody by chemical cross-linking or recombinant methods, these immunotoxins exhibit cell type-specific cytotoxic activity (Rybak et al., 1992; Newton et al., 1996; Zewe et al., 1997). In contrast, angiogenin alone does not have any cytotoxicity at physiological concentrations. Thus, it appears that RNase is a good candidate for use as an immunotoxin or hormonotoxin.

VIII. Sex Steroid Hormone-Dependent Cancers

Hormone-dependent cancers such as prostate cancers in men, and breast, endometrial, and ovarian cancers in women remain a leading cause of death throughout the world.

Breast cancer is the most common malignancy in women. More than 500,000 new cases of breast cancer are reported worldwide each year, and the annual mortality due to this malignancy is about 45,000 in the United States alone (Schally and Comaru-Schally, 1997). About 30% of women with breast cancers have estrogen-dependent tumors and can be treated with some success by hormonal manipulations such as Tamoxifen (Santen et al., 1990; Emons and Schally, 1994; Schally and Comaru-Schally, 1997). Experimental

and clinical studies showed that agonists of GnRH might be useful for treatment of estrogen-dependent breast cancer (Schally, 1989; Schally et al., 1989). Several studies showed GnRH receptors on human breast cancer specimens and in breast cancer cell lines (Miller et al., 1985; Eidne et al., 1987; Fekete et al., 1989; Emons and Schally, 1994). Approximately 50% of a large series of breast cancer biopsy samples (n = 500) were found to be positive for GnRH receptors (Fekete et al., 1989). It has been shown that cytotoxic analogs of GnRH bind with high affinity to human breast cancers (Halmos et al., 1999). Experimental results showed that analog AN-207 and anlog AN-152 given intraperitonally produced 90% inhibition of MXT mouse mammary tumor growth. One injection of AN-207 caused a complete regression of MX-1 hormone-independent doxorubicin-resistant human breast cancers in nude mice, which remained tumor-free for at least 50 days after treatment (Kahan et al., 1999). These results suggest that targeted cytotoxic GnRH analogs could be considered for treatment of GnRH receptor-positive breast cancers in women.

Endometrial cancer is the second most common gynecologic cancer in the western world, ranking behind breast cancer (Emons and Schally, 1994). Surgery or radiotherapy is successful in 70% of cases, but new methods are needed for relapsed cancers (Emons and Schally, 1994). Endometrial cancer is estrogen-dependent and high affinity receptors for GnRH are present on membranes of 80% of human endometrial cancers and human HEC-1A and Ishikawa endometrial cancer cell lines (Srkalovic et al., 1990; Emons et al., 1993; Emons and Schally, 1994). Experimental studies showed that agonist D-Trp^{6}-GnRH and antagonist Cetrorelix inhibited the growth of Ishikawa human endometrial cell lines *in vitro* (Emons et al., 1993). In view of the presence of GnRH receptors on

endometrial cancers, targeted cytotoxic analogs are being investigated. Results indicate that cytotoxic analog AN-207 inhibits proliferation of HEC-1A endometrial cancers xenografted into nude mice (Schally and Nagy, 1999).

Ovarian cancer is the fourth most frequent cause of cancer-related deaths in women (Schally and Comaru-Schally, 1997). Treatment based on chemotherapy or surgery is not very effective, and mortality rates are increasing (Schally and Comaru-Schally, 1997). Experimental studies showed that specific receptors for GnRH have been found in 75% of surgically removed human ovarian carcinomas (Emons et al., 1989; Emons and Schally, 1994; Srkalovic et al., 1998) and in EFO-21, EFO-27, and OV-1063 human ovarian cancer cell lines (Emons et al., 1993; Yano et al., 1994). In view of the presence of receptors for GnRH on ovarian cancers, the effects of targeted cytotoxic GnRH analogs on growth of ovarian cancers were evaluated (Miyazaki et al., 1997; Miyazaki et al., 1999). Experimental results showed that cytotoxic analog AN-152 inhibited significantly the growth of GnRH receptor-positive OV-1063 ovarian tumors in nude mice. AN-152 did not inhibit the growth of GnRH receptor-negative UCI-107 human ovarian carcinoma in nude mice (Miyazaki et al., 1997). Also, the results showed that the growth of OV-1063 ovarian cancers could be suppressed by administration of AN-207 (Miyazaki et al., 1999). These studies suggest that targeted chemotherapy based on GnRH analogs may improve the management of ovarian cancer.

Carcinoma of the prostate represents the most common malignancy in the American male and is the second leading cause of cancer-related deaths among adult men (Crawford, 1990; Sharifi and Soloway, 1990). Approximately 70% of human prostate cancers are testosterone-dependent (Crawford, 1990; Sharifi and Soloway, 1990). The

treatment of advanced prostate cancer is usually based on androgen dependence of the tumor (Crawford, 1990; Sharifi and Soloway, 1990). Receptors for GnRH have been found in human prostate cancer samples (Qayum et al., 1990), in androgen-sensitive LNCaP and androgen-independent DU-145 human prostate cancer cell lines (Qayum et al., 1990; Limonta et al., 1992; Dondi et al., 1994). Recent experiments of a large number of specimens of human prostate adenocarcinomas showed that 85% of cancers exhibited high affinity binding for GnRH and expressed mRNA for GnRH receptors (Halmos et al., 2000). The expression of specific GnRH receptor in a high percentage of human prostate cancers (Halmos et al., 2000) provides a rationale for the development of methods for therapy of this malignancy based on targeted cytotoxic GnRH analogs.

To eliminate gonadal function to ameliorate the sex steroid hormone-dependent tumors can be achieved by surgical castration, injection of GnRH superagonists or antagonists, or hormonotoxins (i.e. GnRH-toxin conjugates). Surgical castration is directed but has side effects such as negative emotion (i.e. pain, depression) and hospitalization. Injection of GnRH superagonists is very expensive, with average wholesale price for a 3 month infusion pump costing in excess of $2,000 (http://www.fertilitext.org/gonadotr.html). Most commonly, the injections are given monthly for six months or longer. Considering that most patients with breast cancer survive for 3 years after diagnosis of metastases, this means the annual cost for the patients choosing this therapeutic approach is $8,000. Also, side effects may include infection, pain, and swelling at the injection site. Continuous stimulation of the pituitary by chronic administration of GnRH or its superactive agonists produces inhibition of hypophyseal-gonadal axis through the process of down-regulation of pituitary receptors

for GnRH, decrease in expression of GnRH receptor gene, desensitization of the pituitary gonadotropes, and a suppression of circulating levels of LH and sex steroids (Naor, 1990; Conn and Crawley, 1991; Emons and Schally, 1994). Since GnRH agonists have these disadvantages, the use of GnRH antagonists which cause an immediate and dose-related inhibition of LH and competitive blockade of the receptors should be much more advantageous. However, many investigators have recently demonstrated that chronic administration of GnRH antagonist Cetrorelix to rats also produces desensitization of gonadotropes, down-regulation of pituitary GnRH receptors, and a decrease in the levels of mRNA for GnRH receptors (Pinski et al., 1996). Moreover, hormone-independent breast, endometrial, ovarian, and prostate cancers can still grow and proliferate after these types of combinational treatments have been applied. Thus, a new approach for cancer treatment is required. Hormonotoxins are cytotoxic agents designed to selectively kill populations of cells that display specific cell surface receptors or binding sites. Thus, it appears that hormonotoxin is a good method to treat hormone-dependent cancers. An ideal hormonotoxin should be very active so that only small amounts need to be given to cause target cell regressions, stable so it remains functional to reach the interior of target tissues, and nonimmunogenic so it can be given repeatedly. Thus, the concept of developing GnRH-toxin conjugates may be able to directly suppress hormone-dependent tumor growth.

IX. Summary and Goals of This Research

As indicated previously, GnRH receptors have been identified on gonadotropes and tumor cells from endometrial, mammary, ovarian, and prostatic tissues as well as on

tumor cell lines (Eidne et al., 1987; Quayum et al., 1990; Emons et al., 1993; Imai et al., 1994). For these reasons, small size (a decapeptide), highly potent analogs, and specific high-affinity binding sites make GnRH an excellent choice to develop a hormonotoxin that can be used to eliminate gonadotrope function and treat cancers harboring GnRH receptors.

Recently, a series of cytotoxic conjugates containing GnRH analog and PAP or RNase was developed in our lab. Here we have used *in vitro* methods to examine the usefulness and application of GnRH-toxin conjugates. We anticipate the results from these experiments may lead to the development of a new method for treatment of steroid-dependent cancers.

For *in vivo* therapy, the toxic moiety of the hormonotoxin and immunotoxin must be coupled to the targeting ligand so that it remains stable in the systemic circulation and tissues but is labile within the target cell. Therefore, the biological effectiveness of a conjugate is dependent on stability and half-life in the circulation as well as its cytotoxicity once it enters the target cell. Thus, linkers should be considered in conjugate design. Therefore, to assess stability and half-life we need a sensitive, specific, and accurate assay to determine the effectiveness of a conjugate. This assay must be able to distinguish both moieties (binding and enzymatic moieties) and not recognize components of conjugate when linkage is broken. Therefore, a sandwich enzyme-linked immunoabsorbent assay (ELISA) for GnRH-toxin conjugate was developed (Chapter Two). The second aim of Chapter Two was to determine the stability of GnRH-toxin conjugates in sheep serum in *in vitro* model.

Based on previous findings, we are interested in developing an *in vitro* model to determine the effect of GnRH-toxin conjugates on growth and proliferation of cells harboring GnRH receptors (Chapter Three). The influence on antiproliferative activity of toxin modification and conjugation of GnRH analogs was also determined in Chapter Three.

Cell death is a critical factor limiting the productivity of animal cells. Apoptosis (programmed cell death) was described as a particular set of transformation at the microscopic level associated with cell death. Since the efficiency of induction of apoptosis may have direct effects on the therapeutic usefulness of GnRH-toxin conjugates, we have determined if GnRH-toxin conjugates induce apoptosis of GnRH receptor-positive cell lines (αT3-1 cells) in Chapter Four.

CHAPTER TWO

ESTABLISHMENT OF A SANDWICH ENZYME-LINKED IMMUNOABSORBENT ASSAY FOR MEASUREMENT OF GnRH-TOXIN CONJUGATES AND STABILITY OF GnRH-TOXIN CONJUGATES IN SHEEP SERUM IN VITRO

I. Introduction

Hormones and growth factors exhibit high specificity in their interaction with receptors on the cell surface. Thus these hormones and factors can be used as ligands to couple to cytotoxic agents for the purpose of destroying the receptor bearing cells. These hybrids have been termed hormonotoxins and many examples of such conjugates have been reported (Chang et al., 1977; Miskimins and Shimizu, 1979; Oeltmann and Heath, 1979; Bacha and Reichlin, 1986; Schwarz et al., 1987; Myers and Villemez, 1989; Singh and Sairam, 1989; Singh et al., 1989; Singh and Sairam, 1990; Marcil et al., 1993; Singh et al., 1993), including GnRH (Bajusz et al., 1989; Janaky et al., 1992; Janaky et al., 1992). Any malignant cell dependent on the respective hormones or factors can become a target for these specific hormonotoxins.

For *in vivo* therapy, the toxic moiety of the hormonotoxin and immunotoxin must be coupled to the targeting ligand so that it remains stable in the systemic circulation and tissues but is labile within the target cell. Therefore, the biological effectiveness of a conjugate is dependent on stability and half-life ($t_{1/2}$) in the circulation until it enters cells

as well as its cytotoxicity once it enters the target cell. Thus, type of linking agent should be considered in conjugate design. A variety of cross-linkers have been developed based on whether the holotoxin or the active domain of the toxin is used (Thorpe et al., 1987). Disulfide-bond linkers have been used most frequently in conjugate design. However, several lines of evidence suggest that disulfide-bond linkages are not stable or long lived *in vivo* (Blakey et al., 1987; Thorpe et al., 1987; Fulton et al., 1988). A sensitive, specific, and accurate assay was needed to evaluate the stability of conjugates in biological fluids. This assay must be able to distinguish both moieties (binding and enzymatic moieties) and not recognize components of conjugate when linkage is broken. Thus, sandwich (two-side) enzyme-linked immunoabsorbent assay (ELISA) is a good choice.

Recently, a series of cytotoxic conjugates containing GnRH and PAP or RNase was developed in our lab. In order to measure these conjugates in tissue extracts, in body fluid and in serum, the first aim of this study was to develop a sensitive, specific, and accurate sandwich ELISA. The second aim of this study was to determine the stability of GnRH-toxin conjugates in sheep serum in an *in vitro* model. The use of this model may permit a better understanding of the *in vivo* action of GnRH-toxin conjugates.

II. Materials and Methods

A. Conjugates

The toxins used in this study are PAP and RNase. PAP was purified from Pokeweed (*Phytolacca americana*) leaves as described previously (Irvin, 1983) with minor modifications. Briefly, Pokeweed leaves were homogenized in 5 mM sodium phosphate, pH 6.5 in a Waring blender. The extract containing PAP was filtered through a strainer

and centrifuged at 10,000 ×g. PAP was then purified by ammonium sulfate precipitation (40-100% saturation), Fractogel EMD-CM (EM Science) ion-exchange chromatography and DEAE Sepharose CL-6B (Sigma) chromatography. The purified protein was dialyzed against water and lyophilized. Purity of PAP was assessed by SDS-PAGE (12% reducing gel). Bovine pancreatic ribonuclease A (RNase) was obtained from Sigma.

The noncleavable conjugates of D-Lys^6-GnRH with PAP (ncGnRH-PAP) were prepared basically as below. (1) Thiolation of D-Lys^6-GnRH. D-Lys^6-GnRH was reacted with 2-iminothiolane to produce SH-GnRH. The yield of the SH-GnRH analog was 60-70%. The progress of the reaction was monitored by C_{18}-HPLC. The final product was also analyzed by mass spectroscopy. (2) Introduction of a maleimidobutyryl group into PAP. PAP was reacted with Sulfo-GMBS (Pierce, Rockford, IL) for 60 min at room temperature. (3) Reaction of thiolated D-Lys^6-GnRH with maleimidobutyryl-PAP. After incubation for 40 min at room temperature, Cys-SH was added to block any residual maleimide groups. Therefore, PAP was conjugated to D-Lys^6-GnRH with a maleimidobutyryl crosslinker. SDS-PAGE (12% reducing gel) analysis and mass spectrometry showed that the final product (as expected) was heterogenous and contained three major fractions: PAP conjugates with one GnRH molecule attached, conjugate with two GnRH molecules, and unconjugated PAP. The unconjugated PAP content in the final product was estimated to be in the range of 25-35%. The noncleavable conjugates of D-Lys^6-GnRH with RNase (ncGnRH-RNase) were prepared by a similar method.

The cleavable conjugates of D-Lys^6-GnRH with PAP (cGnRH-PAP) were prepared basically as below. (1) Introduction of 2-pyridyldithio propionate (PDP) group into D-Lys^6-GnRH using N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP). After 40-min

incubation at room temperature, the reaction was applied to Sephadex LH-20 column. The product (PDP-D-Lys^6-GnRH) was analyzed by C_{18}-HPLC and mass spectroscopy. (2) Simultaneous reaction of PAP with 2-iminothiolane (2-IT) and PDP-D-Lys^6-GnRH. Therefore, PAP was conjugated to D-Lys^6-GnRH via disulfide-bond linkage. The conjugate was analyzed by non-reducing SDS-PAGE and (as expected) the final product was found to be heterogenous. The unconjugated PAP in the final product was estimated to be in the range of 40-60%. The cleavable conjugates of D-Lys^6-GnRH with RNase (cGnRH-RNase) were prepared using similar methodology.

B. Generation and purification of polyclonal antibodies and reagents

Protocols involving animal use in the development and experimental application of this assay were approved by the Colorado State University Committee on the Use and Care of Animals.

A 4-kg rabbit was immunized with PAP followed by a booster 4 weeks later. The first blood sample (~ 50ml) was collected after an additional 2 weeks from the marginal ear vein under gentle vaccuum, allowed to clot and centrifuged at 5000 ×g for 10 min. Serum was heat-inactivated at 56 C for 30 min. The titer for rabbit anti-PAP antibody was determined by radioimmunoassay (RIA).

To produce an anti-D-Leu^6-GnRH antiserum, 1 mg of a D-Leu^6-GnRH (Leuprolide)-Keyhole Limpet Hemocyanin (KLH) conjugate was prepared as described previously (Adams and Adams, 1986). After conjugation, the conjugate was emulsified with Freund's complete adjuvant. Two rabbits were immunized at multiple sites intradermally followed by a boost about 4 weeks later. The first blood sample was collected after an

additional 2 weeks from the marginal ear vein under gentle vaccuum, allowed to clot and centrifuged at 5000 ×g for 10 min. Serum was heat-inactivated at 56 C for 30 min. Titer of the rabbit anti-D-Leu^6-GnRH antibody was determined by radioimmunoassay (RIA).

Antiserum to RNase was obtained from CHEMICON International, Inc (Temecula, CA).

Immunoglobin fractions with high titers were purified from heat-inactivated serum using a DEAE Affi-Gel Blue column (Bio-Rad Laboratories, Hercules, CA). DEAE Affi-Gel Blue gel column (working pH: 2-10) contains crosslinked agarose and is used to purify protease-free IgG from serum (0.14 ml serum/ml gel). Eluted fractions (~1.5 ml/tube) were collected and their absorbances were measured at 280 nm on a spectrophotometer (Beckman DU 640, Fullerton, CA). Those fractions representing the highest concentration of immunoglobulin were concentrated using a 100,000-MW cut-off Centriprep Concentrator (Amicon, Beverly, MA) and resuspended in PBS. Purity was verified by visualization of the immunoglobulin bands on a Coomassie-stained 12.5% SDS-polyacrylamide gel. The purified anti-GnRH fraction was used as a capture antibody. The purified anti-PAP and anti-RNase antibody fractions were biotinylated using EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) and used as the detection antibodies. The biotinylated IgG was prepared by dissolving 2-10 mg of anti-PAP or anti-RNase IgG in 1 ml of PBS solution. Add 2 mg of Sulfo-NHS-Biotin to 100 µl of distilled water. Add sufficient volume of concentrated Sulfo-NHS-Biotin to yield 12-fold molar excess of Sulfo-NHS-Biotin to a 10 mg/ml IgG solution or a 20-fold molar excess of reagent to a 2 mg/ml solution. Place the test tube in ice and incubate for 2 hours. Then, use dialysis to remove unreacted biotin. Store bioitinylated IgG at 4 C until ready to use.

C. Optimization of antibody reagents

Optimal concentrations of antibodies for the GnRH-PAP and GnRH-RNase in the ELISAs were determined using criss-cross serial-dilution analysis as described previously (Granger et al., 1999). Stock capture antibody (2.4 mg/ml) was prepared in coating buffer (0.05% Thimerosal in PBS) starting at 1:100 and diluted 1:2 in series across the columns of the 96-well plate to a final dilution of 1:12,800. The plate was coated overnight at 4 C and washed three times with 200 µl of wash buffer (PBS/0.05% Tween-20). Blocking (preventing nonspecific adsorption) was achieved with BLOTTO (Pierce, Rockford, IL) at 150 µl/well for 1 h at room temperature (24 C) with gentle shaking. GnRH-PAP or GnRH-RNase samples of 200 ng/ml, 1 ng/ml, and 0 ng/ml were prepared in a dilution buffer (10% BLOTTO/PBS/0.05% Tween-20). Samples were added at 100 µl/well for 1 h. Dilutions of stock biotinylated antibody (anti-PAP: 3.5 mg/ml, anti-RNase: 3.1 mg/ml) were prepared in dilution buffer starting at 1:100 and diluted 1:2 in series down the rows of the 96-well plate to a final dilution of 1:12,800. The plates were incubated with shaking for 1 h, washed, and 100 µl of a 1:5,000 dilution of peroxidase-conjugated streptavidin (Jackson Immuno-Research) was added for 30 min. Following a final wash, 150 µl tetramethylbenzidine (TMB) (Pierce, Rockford, IL) was added to each well for color development. The reaction was stopped after 15 min with 100 µl of 1.5 M H_2SO_4. Absorbances at 450 and 595 nm were measured. The diluted pair of capture and biotinylated antibodies that gave the best signal-to-noise ratio was selected for use in the subsequent GnRH-PAP or GnRH-RNase ELISA.

D. Procedure for GnRH-toxin ELISA

The procedure for the GnRH-toxin ELISA was based on a previously published method (Shinohara et al., 1995). The capture antibody (rabbit anti-GnRH) was diluted to 1:200 in coating buffer (PBS/0.05% Thimerosal). A 100-µl aliquot of this diluted coating antibody was added to a 96-well microtiter plate overnight at 4 C. The plate was washed three times with 200 µl of wash buffer and blocked with BLOTTO (Pierce) at 150 µl/well for 1 h. Samples were prepared in a dilution buffer (10% BLOTTO/PBS/0.05% Tween-20) and added to the washed plate in duplicate wells at 100 µl/well for 1 h. After washing, 100 µl/well of the diluted biotinylated antibody (anti-PAP or anti-RNase) at 1:800 was added for 1 h. Peroxidase-conjugated streptavidin was diluted at 1:5,000 and 100 µl/well added to the washed plate for 30 min. TMB was added at 150 µl/well to the washed plate for approximately 15 min and the color reaction was stopped with 1.5 M H_2SO_4 (100 µl/well). Absorbances were quantified and standard curves were calculated.

E. Optimization of ELISA

Reagents used in the assay were optimized to give the greatest signal-to-noise ratio. Two blocking buffers (BLOTTO (Pierce), and 2% BSA/PBS solution) were tested for the selection of an appropriate blocking buffer. After the coating step, 150 µl of each buffer was used to block for 1 h. Samples for the standard curves were prepared in the respective dilution buffers for each type of blocker: 10% BLOTTO/0.05% Tween-20/PBS, 2% BSA/PBS/0.05% Tween-20. The samples were added to the plate and the remainder of the GnRH-toxin ELISA protocol followed as described in section D. Streptavidin concentration and development time for the TMB chromogen was

determined through comparison of GnRH-toxin standard curves under several reaction conditions. Streptavidin (0.5 mg/ml) was diluted in dilution buffer at 1:2,500, 1:5,000, and 1:10,000. Standard curves of GnRH-toxin were run at each of these concentrations with color development times of 10, 15, and 20 min.

F. Specificity

Assay specificity was examined by determining the ability of D-Lys^6-GnRH, PAP or RNase, ovine LH (oLH), and ovine FSH (oFSH) to interact with the antiserum. Standard curves with a maximal concentration of 400 ng/ml were constructed for each hormone or protein. These were run in the ELISA protocol as described and compared with a standard curve prepared using GnRH-toxin.

G. Intra- and inter-assay variation

Intra-assay variation was tested by running GnRH-PAP or GnRH-RNase at 100, 6.25, and 0.39 ng/ml in quadruplicate within one assay. Inter-assay variation was determined by testing 100, 6.25, 0.39 ng/ml samples in duplicate in three separate assays.

H. Effect of normal sheep serum

The effect of serum on the assay was examined by constructing GnRH-PAP or GnRH-RNase standard curves in the presence of normal sheep serum. GnRH-PAP or GnRH-RNase standard curves were prepared in the presence of 2%, 5%, 10%, and 20% pooled normal sheep serum and compared to a standard curve without serum. This was done in three separate assays with samples tested in duplicate in each assay.

I. Stability study of GnRH-toxin conjugates

Blood samples were obtained from healthy female sheep. The serum was collected and stored frozen at –20 C until use. The hydrolysis of ncGnRH-PAP, cGnRH-PAP, ncGnRH-RNase, and cGnRH-RNase in sheep serum was determined by dissolving 1 μg of each conjugate in 100 μl of sheep serum. Since 10% fetal bovine serum (FBS) (Gibco) was added in cell culture medium, a study on the hydrolysis of cGnRH-PAP in 10% FBS was carried out at a substrate concentration of 1 μg in 100 μl. The samples were incubated for 2, 5, 10, 20, 30, 60, 90, and 120 min at 37 C. At the end of the incubation, the samples were put onto ice immediately to stop the hydrolysis. The levels of intact conjugates were measured by a sandwich ELISA.

The percentage of intact GnRH-toxin conjugate at 2, 5, 10, 20, 30, 60, 90, and 120 min was used to calculate the $t_{1/2}$ of the conjugate in sheep serum and 10% FBS by linear regression analysis of the concentration vs. time. The area under the curve (AUC) for the concentration of intact conjugates in sheep serum and 10% FBS was calculated by using the trapezoidal rule between 0 and 120 min. Statistical analyses were performed by using Student's *t* test.

J. GnRH-toxin levels in sheep serum

The GnRH-toxin ELISA was used to examine the rate of clearance of GnRH-PAP or GnRH-RNase in sheep serum after administration of GnRH-PAP or GnRH-RNase. GnRH-PAP (ncGnRH-PAP: 2.25 mg/sheep; cGnRH-PAP: 2.16 mg/sheep) or ncGnRH-RNase (1.34 mg/sheep) were injected intravenously into sheep and blood was collected every 30 min after injection for 4 h. Serum was harvested and stored frozen at –20 C until

use. The GnRH-PAP or GnRH-RNase levels in sheep serum over time were run in the ELISA protocol as described above.

III. Results

A. Optimization of GnRH-toxin ELISA

Reagents, buffers, and reaction times were optimized for this assay. The criss-cross serial dilution analysis of antibody reagents yielded an optimal concentration of 1:200 (12.0 μg/ml) for capture antibody and 1:800 (4.4 μg/ml for anti-PAP; 3.9 μg/ml for anti-RNase) for biotinylated antibody which were used in the assay. The BLOTTO buffer was selected for use as a blocking reagent as well as a dilution buffer (10% BLOTTO/0.05% Tween-20/PBS). This buffer gave the highest signal-to-noise ratio of 9.414 compared to 2% BSA (Table 2). The optimal streptavidin concentration was a 1:5,000 dilution of prepared stock. The best color development was achieved at 15 min (data not shown).

Table 2. Standard non-cleavable GnRH-PAP curves were constructed to test two types of blocking buffer

Blocking buffer	Absorbance at 200 ng/ml GnRH-PAP	Absorbance at 0 ng/ml GnRH-PAP	Signal-to-noise ratio (max/min)
BLOTTO	1.205	0.128	9.414
2% BSA	1.258	0.632	1.991

The highest signal-to-noise ratio (maximum/minimum absorbance) was obtained with BLOTTO buffer. BLOTTO contains nonfat dry milk in TBS, pH 7.4, with 0.01% antifoam A, and 600ppm Kathon CG/ICP as a preservative. A 10% BLOTTO/0.05% Tween-20/PBS solution was used for the dilution buffer.

Upon establishment of optimal reaction conditions, a standard curve was constructed for GnRH-PAP or GnRH-RNase. On both GnRH-toxin standard curves, the lower limit of detection was approximately 0.39 ng/ml in multiple trials (n = 11), with the curve extending to achieve a maximal concentration at 400 ng/ml (Fig. 2 and 3).

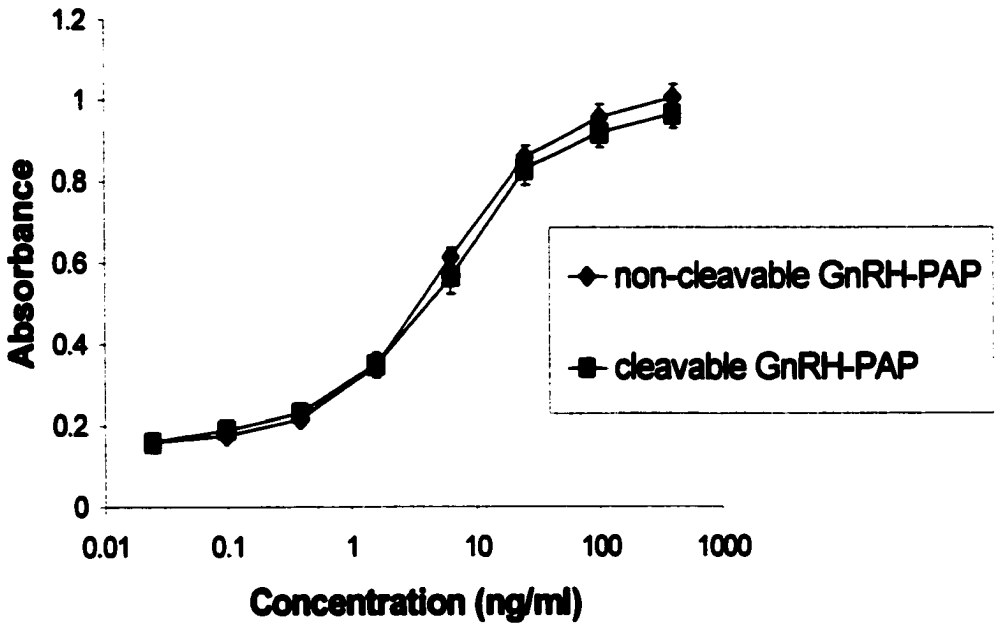

Fig. 2. Standard curves for non-cleavable GnRH-PAP (ncGnRH-PAP) and cleavable GnRH-PAP (cGnRH-PAP) as determined by a sandwich ELISA. Triplicates samples were prepared for each point. Results are mean +/- S.E.M.

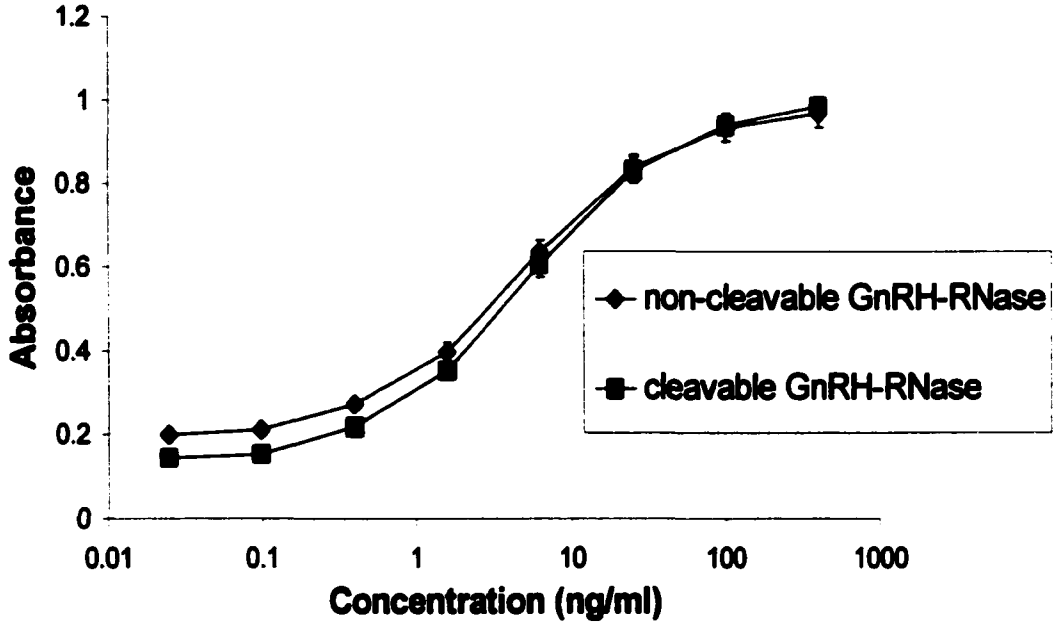

Fig. 3. Standard curves for non-cleavable GnRH-RNase (ncGnRH-RNase) and cleavable GnRH-RNase (cGnRH-RNase) as determined by a sandwich ELISA. Triplicate samples were prepared for each point. Results are mean +/- S.E.M.

B. Verification of specificity

The specificity of the GnRH-toxin ELISA against other hormones and proteins (D-Lys^6-GnRH, oLH, oFSH, PAP or RNase) was tested in three separate trials. The specificity for GnRH-PAP and GnRH-RNase is shown in Fig. 4 and Fig. 5, respectively, with no cross-reactivity observed within the normal working limits of the assay. The assay was deemed specific for GnRH-PAP or GnRH-RNase.

C. Intra- and inter-assay variation

Assay variation is shown in Table 3. Intra-assay variation of GnRH-PAP and GnRH-RNase from quadruplicate samples within the same assay was found to range from 5.22 to 23.51% and from 4.30 to 15.76% coefficient of variation (CV), respectively. Inter-assay variation of GnRH-PAP and GnRH-RNase from three separate assays was found to range from 8.73 to 19.17% and from 4.62 to 15.66% CV, respectively.

D. Serum effects

As we intend to use this assay to determine GnRH-PAP or GnRH-RNase levels in serum, it was necessary to determine the possible effects of serum on the assay system. Fig. 6 and Fig. 7 for GnRH-PAP and GnRH-RNase, respectively, demonstrate a reproducible, slight suppressive effect that is proportional to the concentration of serum. However, no significant difference occurs even in the presence of 20% serum.

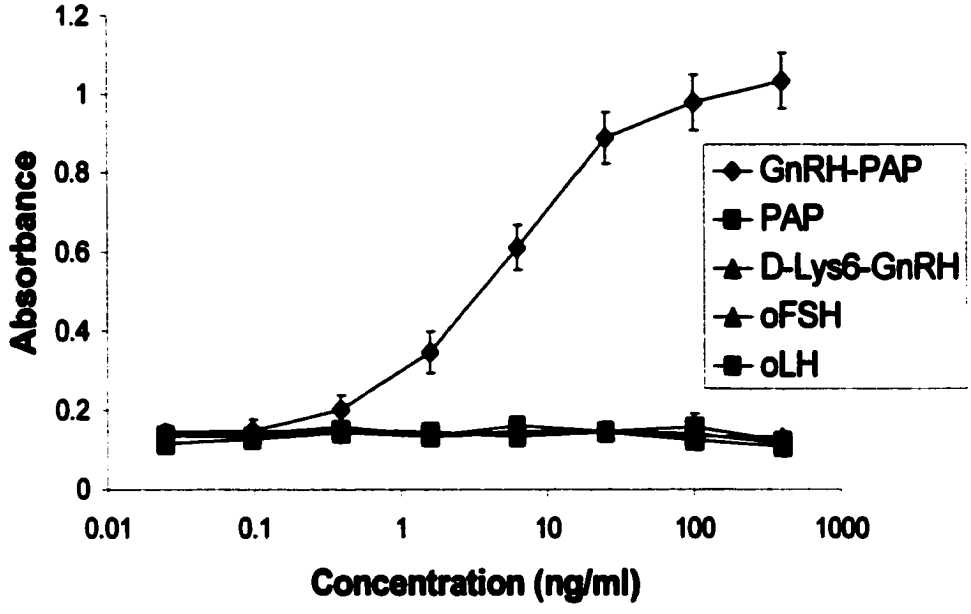

Fig. 4. There was no cross-reaction of PAP, D-Lys^6-GnRH, ovine FSH, and ovine LH observed within the accurate detection range of the GnRH-PAP ELISA assay (0.039-100 ng/ml). Triplicate samples were prepared for each point. Results are mean +/- S.E.M.

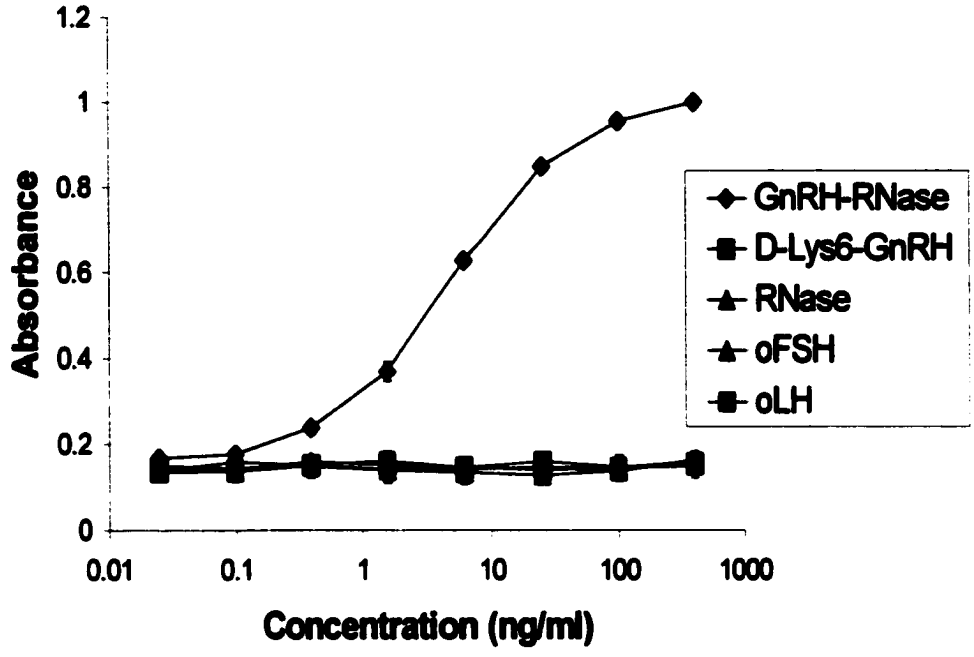

Fig. 5. There was no cross-reaction of RNase, D-Lys^6-GnRH, ovine FSH, and ovine LH observed within the accurate detection range of the GnRH-RNase ELISA assay (0.039-100 ng/ml). Triplicate samples were prepared for each point. Results are mean +/- S.E.M.

Table 3. Intra- and inter-assay variance expressed as coefficient of variance (CV)

Variation	ncGnRH-PAP (ng/ml)	n	Absorbance	SD	CV
Intra-assay	100	3	0.971	0.145	14.96
	6.25	3	0.605	0.032	5.22
	0.39	3	0.217	0.034	15.61
Inter-assay	100	6	0.967	0.084	8.73
	6.25	6	0.659	0.058	8.85
	0.39	6	0.250	0.032	12.94

Variation	cGnRH-PAP (ng/ml)	n	Absorbance	SD	CV
Intra-assay	100	3	0.937	0.126	13.47
	6.25	3	0.596	0.140	23.51
	0.39	3	0.216	0.024	11.18
Inter-assay	100	6	0.966	0.111	11.46
	6.25	6	0.622	0.119	19.17
	0.39	6	0.250	0.033	13.37

Variation	ncGnRH-RNase (ng/ml)	n	Absorbance	SD	CV
Intra-assay	100	3	0.890	0.094	10.58
	6.25	3	0.614	0.097	15.76
	0.39	3	0.278	0.024	8.58
Inter-assay	100	6	0.956	0.096	10.03
	6.25	6	0.652	0.079	12.18
	0.39	6	0.270	0.042	15.66

Variation	cGnRH-RNase (ng/ml)	n	Absorbance	SD	CV
Intra-assay	100	3	0.940	0.040	4.30
	6.25	3	0.604	0.068	11.23
	0.39	3	0.219	0.012	5.49
Inter-assay	100	3	0.941	0.061	6.47
	6.25	3	0.597	0.091	15.22
	0.39	3	0.212	0.010	4.62

Variation tested at concentrations of 100, 6.25, and 0.39 ng/ml GnRH-toxin conjugates.

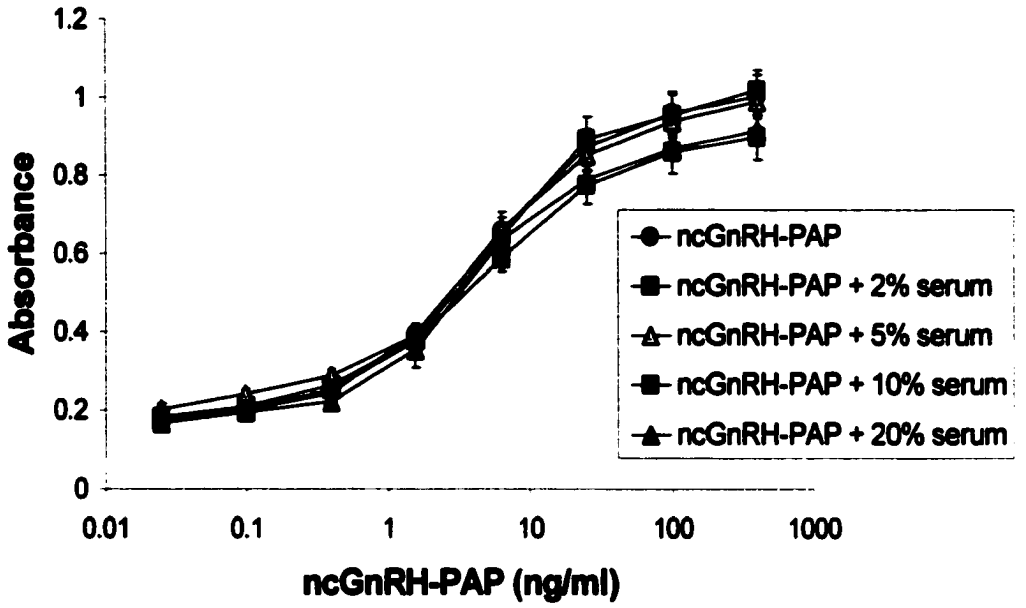

Fig. 6. Representative results from three trials exploring the effect of pooled normal sheep serum on the non-cleavable GnRH-PAP (ncGnRH-PAP) curve. Triplicate samples were prepared for each point. Results are mean +/- S.E.M.

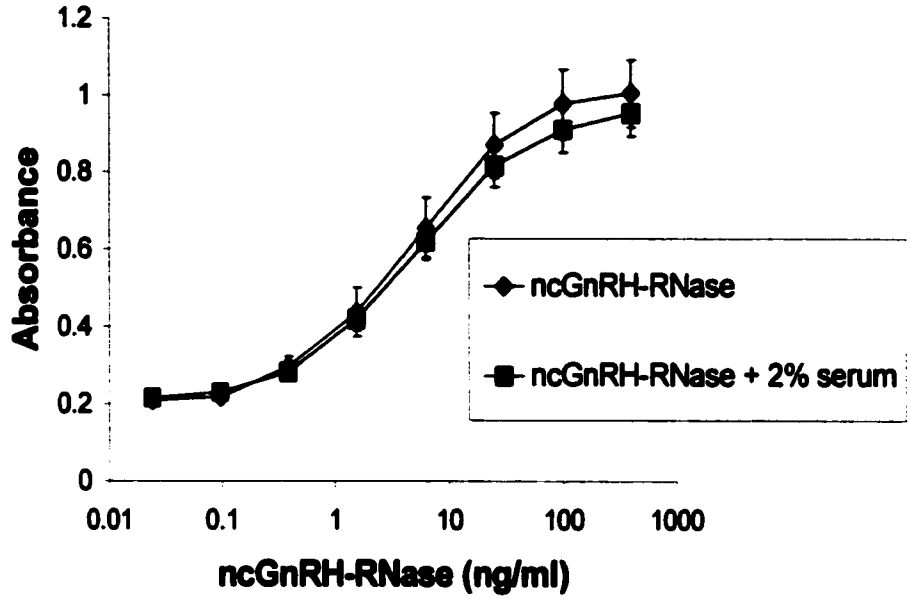

Fig. 7. Representative results from three trials exploring the effect of pooled normal sheep serum on the non-cleavable GnRH-RNase (ncGnRH-RNase) curve. Triplicate samples were prepared for each point. Results are mean +/- S.E.M.

E. Stability study of GnRH-toxin conjugates

The rate of deconjugation of ncGnRH-PAP and cGnRH-PAP in sheep serum or 10% FBS is shown in Fig. 8. The $t_{1/2}$ of cGnRH-PAP in sheep serum was calculated to be 6.17 +/- 0.82 min. Only a slight deconjugation of ncGnRH-PAP occurred in sheep serum within the 2h time period examined. Interestingly, we found the rate of deconjugation of cGnRH-PAP in 10% FBS was very slow.

The rate of deconjugation of ncGnRH-RNase and cGnRH-RNase in sheep serum is shown in Fig. 9. The $t_{1/2}$ of cGnRH-RNase in sheep serum was calculated to be 13.66 +/- 4.41 min. Only a slight deconjugation of ncGnRH-RNase occurred in sheep serum within the 2h time period examined.

F. GnRH-toxin levels in sheep serum

The experimental application of the assay was explored by measuring the levels of GnRH-PAP and GnRH-RNase in sheep serum after single intravenous injection of GnRH-PAP and GnRH-RNase, respectively. The level of GnRH-PAP and GnRH-RNase in the circulation is shown in Fig. 10 and Fig. 11, respectively. In both cases, the levels of non-cleavable conjugates were detectable by using sandwich ELISA at 30 min after injection. However, levels of cleavable GnRH-PAP conjugates were very low in sheep serum after administration of cGnRH-PAP (Fig. 10).

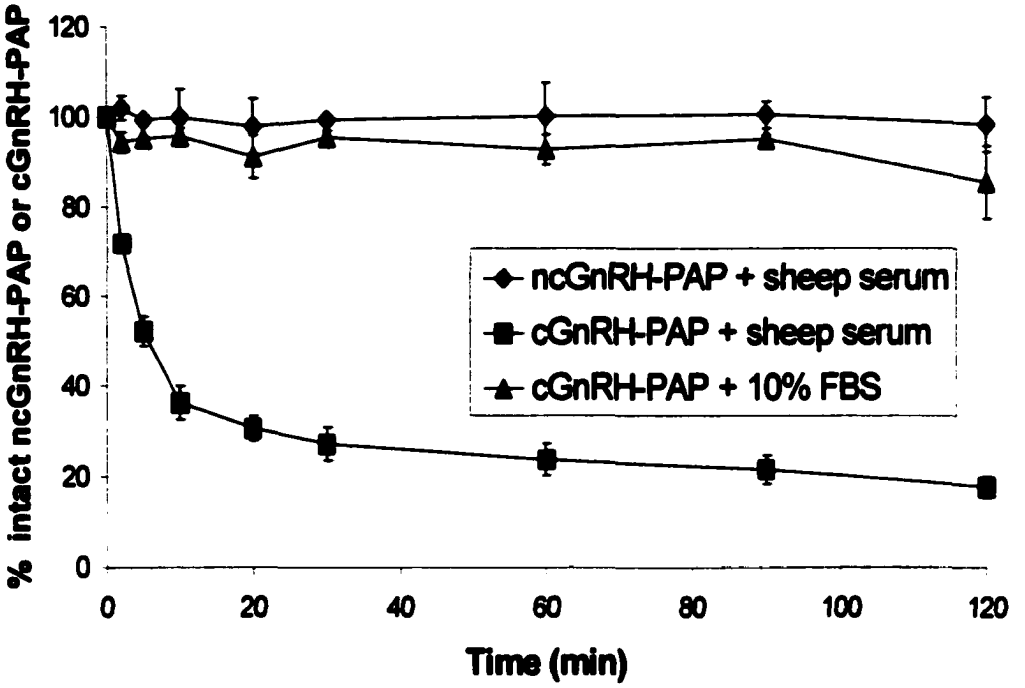

Fig. 8. Hydrolysis of ncGnRH-PAP and cGnRH-PAP after incubation with pooled normal sheep serum or 10% FBS at 37 C in a 95% air/5% CO2 atmosphere. The percentage of intact ncGnRH-PAP or cGnRH-PAP was determined by sandwich ELISA as described in Materials and Methods. Duplicate samples were prepared for each point. Results are mean +/- S.E.M. Half life of cGnRH-PAP in serum is 6.17 +/- 0.82 min.

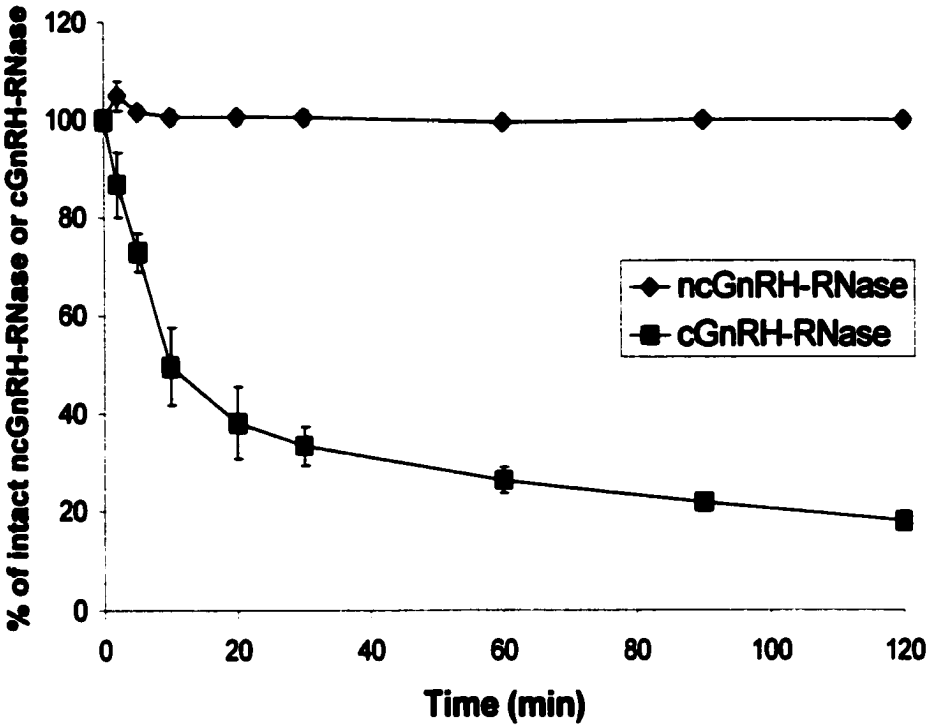

Fig. 9. Hydrolysis of ncGnRH-RNase and cGnRH-RNase after incubation with pooled normal sheep serum at 37 C in a 95% air/5% CO2 atmosphere. The percentage of intact ncGnRH-RNase or cGnRH-RNase was determined by sandwich ELISA as described in Materials and Methods. Duplicate samples were prepared for each point. Results are mean +/- S.E.M. Half life of cGnRH-RNase in serum is 13.66 +/- 4.41 min.

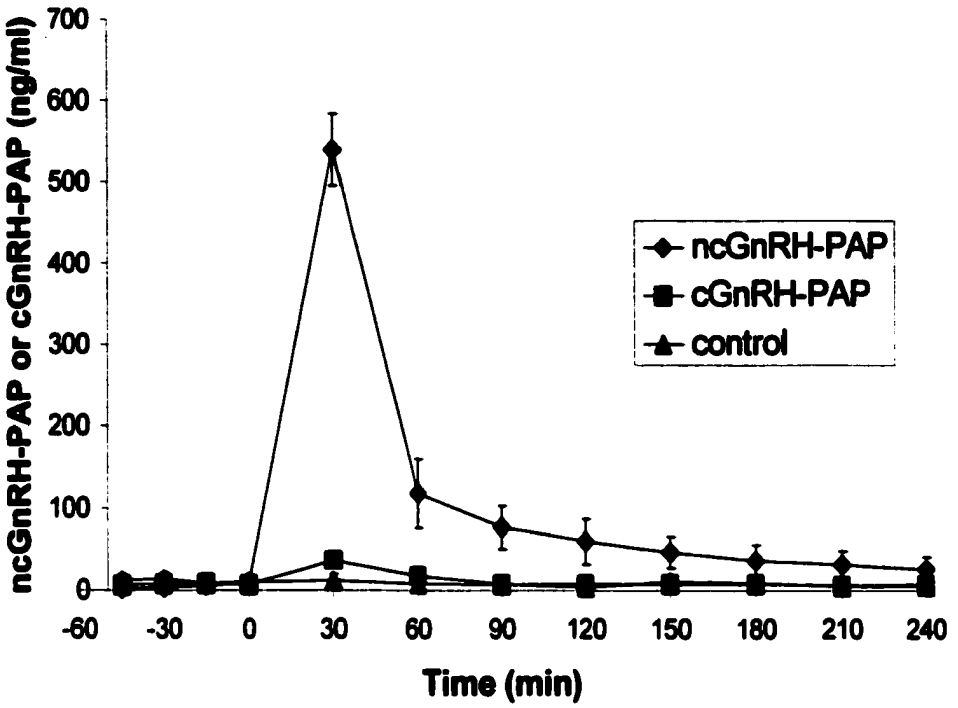

Fig. 10. Time course of non-cleavable GnRH-PAP and cleavable GnRH-PAP levels in the serum following non-cleavable GnRH-PAP and cleavable GnRH-PAP, respectively, injection at 0 min. Control group of sheep received GnRH injection at 0 min. Triplicate samples were prepared for each point. Results are mean +/- S.E.M.

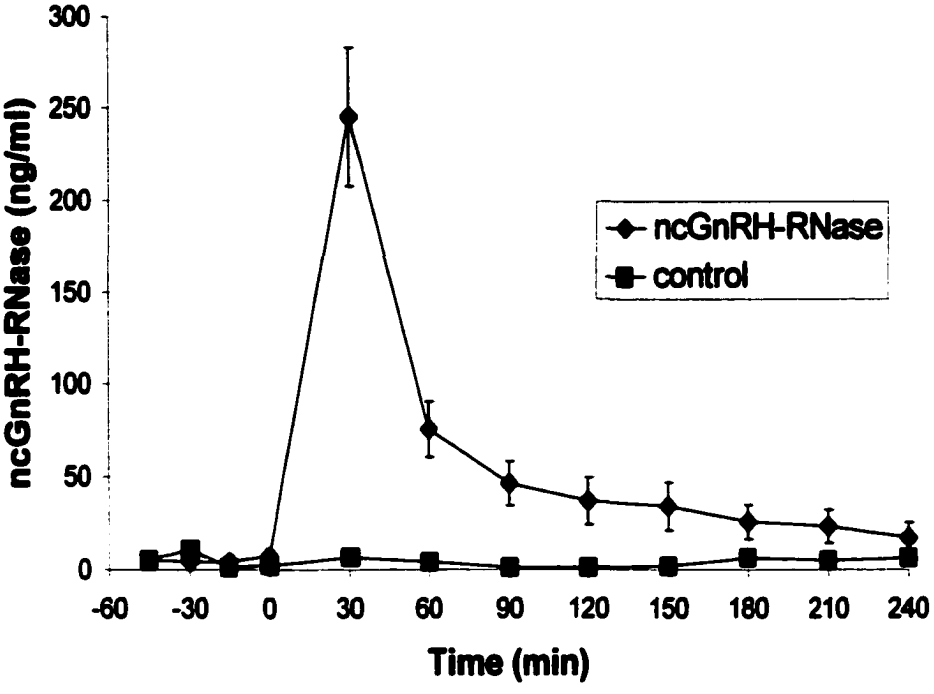

Fig. 11. Time course of ncGnRH-RNase levels in the serum following ncGnRH-RNase injection at 0 min. Control group of sheep received GnRH injection at 0 min. Triplicate samples were prepared for each point. Results are mean +/- S.E.M.

IV. Discussion

This report describes the development of a GnRH-toxin conjugate sandwich ELISA that will assist in our understanding on the stability and half-life of GnRH-toxin conjugates. To our knowledge, this report is the first to demonstrate the sandwich ELISA for measurement of GnRH-toxin conjugates.

In order to be a valuable research tool in this endeavor, an ELISA assay must meet several essential requirements. It must be specific for the substance to be measured and display sensitivity over the detection concentration range. Measurement of a sample should be accurate and consistent throughout multiple trials. The assay must be practical and flexible to accommodate a variety of sample types.

We have shown that this assay is specific for GnRH-toxin conjugates under optimized reaction conditions. Many factors may be present in the sheep and/or human body fluid and tissue samples tested that could cross-react with the polyclonal antibodies used in this assay. A lack of significant cross-reactivity was found to the D-Lys^6-GnRH, and PAP or RNase, as well as two gonadotropins, oFSH, and oLH.

This assay is sensitive and consistent in its measurement of GnRH-toxin conjugates. The sensitivity of the assay (0.39 ng/ml) is appropriate for measurement of conjugates. The accuracy of measurement is relatively consistent, particularly within the detection limits of the assay (from 0.39 ng/ml up to 100 ng/ml).

Flexibility of the assay to measure GnRH-toxin conjugates has been demonstrated in several types of samples. BLOTTO, BSA (2%), Casein, and Superblock are commonly used blocking buffers in ELISA (Granger et al., 1999; Hamai et al., 1999; Yee and Brown, 1999). Usually, BSA (2%) is commonly used. However, we found the signal-to-

noise ratio of 2% BSA is lower than that of BLOTTO buffer. Granger et al. (1999) showed a similar result in their ELISA test. Therefore, the BLOTTO buffer was selected for use as a blocking reagent as well as a dilution buffer (10% BLOTTO/0.05% Tween-20/PBS) in our sandwich ELISA system.

As we intend to use this ELISA to determine GnRH-PAP or GnRH-RNase levels in serum, it was necessary to determine the possible effects of serum on the assay system. It has been shown that plasma or serum demonstrates a suppressive effect that is proportional to the concentration of plasma or serum in an ELISA for human M-CSF (Hanamura et al., 1988) and for human GM-CSF (Sallerfors and Olofsson, 1991). Though sheep normal serum does not display a concentration-dependent suppression in our study, most samples (including standard samples) are diluted in 2% or 5% serum where this effect becomes minimal.

The linker is extremely important when constructing immunotoxins and hormonotoxins. In this study, we determined *in vitro* the rate of hydrolysis of GnRH-toxin conjugates in sheep serum. Our results showed that disulfide-bond linker of cleavable conjugate is easily degraded by enzymes in sheep serum because $t_{1/2}$ of cGnRH-PAP and cGnRH-RNase is 6.17 +/- 0.82 min and 13.66 +/- 4.41 min, respectively. However, maleimidobutyryl-group linker of non-cleavable conjugate is very stable in sheep serum even at 37 C for 120 min. Improvement of the stability is important for the preparation of effective and stable immunotoxin and hormonotoxin conjugates. Evidence is mounting that stable linkers should be considered in conjugate design, including hindered disulfide-bond linkers, modified short-chain (Gly-Phe-Leu-Gly) linker, and poly(ethylene glycol)-dipeptidyl linkers (Dosio et al., 1998; Palyi et al., 1999;

Woo et al., 1999; Suzawa et al., 2000). For instance, Cumber et al. (1992) and Woo et al. (1999) showed that hindered disulfide-bond linkers on immunotoxins showed enhanced stability (3-fold more stable) over non-hindered disulfide-bond linkers. Thus, this protection reduces proteolysis, prolongs half-life in blood stream, and improves therapeutic potential.

Interestingly, we found that disulfide-bond linker is quite stable after incubation with 10% FBS at 37 C for 120 min (Fig. 8). This is probably due to heat-inactivation of commercially available FBS or due to diluted concentrations of enzymes in solution. Thus, cleavable GnRH-toxin conjugates may be appropriate for *in vitro* study but not *in vivo* study.

We have explored the practical application of this assay to measure concentrations of GnRH-toxin in sheep serum after administration of GnRH-toxin conjugates. Levels of non-cleavable conjugates were detectable (ncGnRH-PAP: ~550 ng/ml; ncGnRH-RNase: ~250ng/ml) by using sandwich ELISA at 30 min after injection (Figs. 10 and 11). However, the levels of cleavable GnRH-PAP conjugates were very low in sheep serum after administration of cGnRH-PAP. This result indicates that cGnRH-PAP is easily degraded in sheep serum. This result is correlated with the *in vitro* stability study (Fig. 8). In order to examine the rate of blood clearance of GnRH-toxin conjugates, in the near future, we need to collect blood samples more frequently after administration of conjugates.

In conclusion, we have developed an assay that meets the criteria for the accurate and reliable measurement of GnRH-toxin conjugates in a variety of sample types. This new assay will provide a valuable research tool in discerning the role of GnRH-toxin

conjugate in *in vivo* and *in vitro* systems and increase understanding of the basic biology of these conjugates. In this study we demonstrate that disulfide linkage is not stable in systemic circulation and use of a non-cleavable linker should be considered in conjugate design. The *in vitro* characteristics of GnRH-toxin conjugates composed of maleimidobutyryl-group linker suggest clinical usefulness. These observations will allow for better understanding of the hormonotoxin or immunotoxin design, which is a subject of ongoing research aimed at improving present methods for cancer therapy and chemical castration.

CHAPTER THREE

CYTOTOXIC ACTIVITY OF GnRH-TOXIN CONJUGATES IN CELL LINES EXPRESSING GnRH RECEPTORS

I. Introduction

Immunotoxins and hormonotoxins are cytotoxic agents designed to selectively destroy targeted populations of cells that display specific surface antigens or receptors. Most immunotoxins and hormonotoxins have been developed for cancer treatment (Pastan and Fitzgerald, 1991; Pincus, 1996). Immunotoxins and hormonotoxins contain a ligand such as a hormone (or growth factor), monoclonal antibody, or fragment of an antibody which is linked to a protein toxin from bacteria or plants. After the ligand subunit binds to the surface of the target cell, the complex molecule (ligand, toxin, and receptor) internalizes and the toxin kills the cell. Bacterial toxins which have been targeted to cancer cells include *Pseudomonas* exotoxin and diphtheria toxin (Chang et al., 1977; Myers and Villemez, 1989; Pai et al., 1992). However, these toxins derived from bacteria are very immunogenic and cannot be humanized by standard techniques (Brinkmann and Pastan, 1994). Moreover, these toxins display some degree of nonspecific toxicity because these toxins are able to penetrate living cells through their cell recognition domain (Godal et al., 1983; Brinkmann and Pastan, 1994). The use of a member of the type-I ribosome-inactivating proteins (i.e. pokeweed antiviral protein) or ribonuclease A superfamily (i.e. bovine pancreatic ribonuclease A or angiogenin) may

reduce the problems associated with immunogenicity and nonspecific uptake by most cells (Bond, 1988; Denis and Mahler, 1990; D'Cruz and Uckun, 2001).

GnRH was originally identified and has long been recognized as the central regulator of the hypothalamic-pituitary-gonadal axis. Potent GnRH agonists and antagonists have been used to treat sex steroid hormone-dependent cancers of the breast, endometrium, ovary, and prostate (Isaacs and Coffey, 1981; Vickery, 1986; Hoffken, 1992; Weinbauer and Nieschlag, 1992). The suppression of gonadal steroid secretion by hormonal therapy with these agonists and antagonists reduces the growth of these hormone-dependent tumors. Moreover, many human breast, endometrial, ovarian, and prostate cancer tissues and/or cell lines have GnRH receptors on their surface even after they have lost their dependency on sex steroid hormones for growth (Miller et al., 1985; Eidne et al., 1987; Jennes et al., 1988; Quayum et al., 1990; Emons et al., 1993; Imai et al., 1994). This suggests that target chemotherapy for cancer treatment is required. Recently, highly potent GnRH analog conjugates showed selective cytotoxicity against human breast and prostate cancer cell lines *in vitro* (Pai et al., 1992; Palyi et al., 1999). GnRH analogs conjugated to cytotoxic agents may be useful for the treatment of human breast, endometrial, ovarian, and prostate cancers, especially for the sex steroid hormone-independent (androgen-independent and estrogen-independent) tumors because these tumors continue to proliferate after the hormonal therapy has been applied (Pai et al., 1992). For these reasons, GnRH can be used as a cell-binding moiety to deliver toxin molecules such as PAP or RNase into tissues and cell lines expressing GnRH receptors.

Recently, a series of cytotoxic conjugates containing GnRH and PAP or RNase was developed in our lab. These conjugates were constructed using chemical cross-linking

methods. The present work with gonadotrope-derived cell lines and cancer cell lines that have GnRH receptors was undertaken to examine the effects of GnRH-toxin conjugates on *in vitro* suppression of growth and proliferation of these cell lines.

II. Materials and Methods

A. Conjugates

The toxins used in this study were PAP and RNase. PAP was purified from Pokeweed (*Phytolacca americana*) leaves as described previously (Irvin, 1983) with minor modifications. The conjugation procedure was described previously (Chapter Two).

B. Cell Culture

Chinese hamster ovary (CHO) cells were transfected with cDNA for the murine GnRH receptor fused to green fluorescense protein and yellow fluorescense protein (GFP-YFP) to create a cell line expressing high levels of GnRH receptor (CHO-GnRHR) (Nelson et al., 1999). These CHO-GnRHR cells were a generous gift from Dr. CM Clay (ARBL, CSU). CHO-GnRHR cells were maintained in Dulbecco's modified Eagle Minimal Essential Medium (Sigma) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum, and 1% nonessential amino acids.

αT3-1 cells (a mouse gonadotrope tumor cell line that expresses endogenous GnRH receptors) (Kaiser et al., 1997) were maintained in Dulbecco's modified Eagle Minimal Essential Medium (Sigma) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 5% fetal bovine serum, and 5% horse serum.

LβT2 cells (a mouse clonal gonadotrope tumor cell line that expresses endogenous GnRH receptors) (Kaiser et al., 1997) were maintained in Dulbecco's modified Eagle Minimal Essential Medium (Sigma) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum.

Du145 human prostate adenocarcinoma (androgen-insensitive and moderately metastatic) cells, Ishikawa human endometrial adenocarcinoma cells, LNCaP human prostate adenocarcinoma (androgen-sensitive and non-metastatic) cells, MCF-7 human breat adenocarcinoma cells, PC3 human prostate adenocarcinoma (androgen-insensitive and highly metastatic) cells, and ppC1 human prostate adenocarcinoma cells were a generous gift from Dr. M Glode (University of Colorado Health Science Center, Denver, CO). These cells were maintained in RPMI 1640 medium (Sigma) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum.

All cell lines were cultured in a humidified environment with 5% CO_2 at 37 C and the medium was replaced every 2-3 days.

C. Clonogenic assay

Cell survival was assessed by colony formation as described previously (Palyi et al., 1996). Briefly, 800 cells were put into 100-mm diameter Petri dishes in 6 ml medium. Two days later, varying amounts of the test compounds were each added to these dishes which were then maintained for 6-7 days in a CO_2 incubator. The cells were fixed with methanol-acetic acid (3:1) solution and then stained with crystal violet. The number of colonies, containing a minimum of approximately 50 cells, was counted using a

dissection microscope. The number and area of colonies in treated cultures was expressed as a percentage of those in control cultures.

A time course of the action of GnRH-toxin conjugates against CHO-GnRHR and αT3-1 cells was investigated as follows. Cells were placed in a 100-mm Petri dish at 800 cells per dish and incubated for 2 days (CHO-GnRHR cells) or 7 days (αT3-1 cells). The cells were grown for 1, 6, 24, 48, 72, or 96 additional hours in the presence of 1 μM ncGnRH-PAP conjugates. After removing the medium containing 1 μM of ncGnRH-PAP conjugates at each time point, the cells were incubated until 7 days from the first addition of ncGnRH-PAP with normal culture medium. The cells were fixed with methanol-acetic acid (3:1) solution and then stained with crystal violet. The number of colonies, containing a minimum of 50 cells, was counted using a dissection microscope. The number and area of colonies in treated cultures was expressed as a percentage of those in control cultures.

D. Cell proliferation assay

Each well of a cell culture plate (96 well, Nunc) was plated with 2,000 cells. After incubation at 37 C for 2 days, 100 μl of varying amounts of GnRH-PAP (or GnRH-RNase), D-Lys^6-GnRH, or PAP (or RNase) in culture medium was added to each well. After incubation for two additional days, viability was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacture's instructions. The CellTiter 96 Aqueous One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. The MTS

tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Assays are performed by adding this reagent directly to culture wells, incubating for 1 hour and then recording absorbance at 492nm with a 96-well plate reader. Each experiment was performed in triplicate.

A time course of the action of ncGnRH-PAP against αT3-1 cells was investigated as follows. Cells were placed in a 96-well cell culture plate at 2,000 cells per well and incubated for 2 days. The cells were grown for 10, 20, 30, 60, 90, 120, 150, 180, 360, and 1440 additional minutes in the presence of 1 μM of ncGnRH-PAP. After removing the medium containing 1 μM ncGnRH-PAP conjugates at each time point, the cells were grown for 2 days in normal culture medium. Then, viability was determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacture's instructions. Each experiment was performed in triplicate.

E. Receptor binding assay

Approximately 500,000 cells/tube from the αT3-1, CHO-GnRHR, LβT2, DU145, Ishikawa, LNCaP, MCF-7, PC3, and ppC1 cell lines were put in plastic assay tube. Concentrations of freshly prepared [^{125}I]D-Ala^{6}-GnRH-des-Gly^{10}-Pro^{9}-N-ethyl amide between 20 pM and 4.5 nM in 200 μl ice-cold complete medium were then added to each tube in the presence or absence of 450 nM of unlabeled D-Ala^{6}-GnRH-des-Gly^{10}-Pro^{9}-N-ethyl amide. Cells were incubated on ice for 4-5 hr. Then 3 ml ice-cold binding assay buffer was added to each tube before centrifugation (16,000 rpm, 20 min, 4 C).

Radioactivity in the pellet was quantified using an Apex Automatic γ-counter (Micromedic Systems, Inc., Horsham, PA). Specific counts were determined as total counts per min bound less the counts per min bound in the presence of 450 nM of unlabeled D-Ala^{6}-GnRH-des-Gly^{10}-Pro^{9}-N-ethyl amide. At least three independent experiments were conducted, and the pooled data were analyzed by a nonlinear regression using GraphPad prism Software (GraphPad Software, Inc., San Diego, CA). A one-site model provided the best fit of the data. Statistical analyses were performed by using Student's *t* test.

III. Results

A. Cytotoxic activity of GnRH-PAP conjugates on CHO-GnRHR and αT3-1 cells

a. Clonogenic assay

To investigate whether GnRH-PAP conjugates can inhibit growth of the cells that express GnRH receptors on their surface, CHO-GnRHR cells were treated with varying amounts of ncGnRH-PAP. Representative results with CHO-GnRHR cells in the clonogenic assay are depicted in Fig. 12 and Fig.13. GnRH-PAP conjugate was able to induce cell death of CHO-GnRHR cells, whereas PAP alone did not decrease the cell viability. As shown in Fig. 14, GnRH-PAP conjugates inhibited the growth of CHO-GnRHR cells in a concentration-dependent manner, and the concentrations of conjugates giving a 50% inhibition of cell growth (ID50) were found to be ~0.3 μM and ~0.2 μM for ncGnRH-PAP and cGnRH-PAP, respectively. However, GnRH and PAP alone did not affect the growth of CHO-GnRHR cells.

Two different experiments were performed to demonstrate that the cytotoxicity was due to the binding of GnRH-PAP conjugates to GnRH receptors. First, the cytotoxic activity of GnRH-PAP against CHO-GnRHR cells was investigated in the presence of varying concentrations of GnRH. Excess GnRH effectively reduced the cytotoxicity of GnRH-PAP conjugates against CHO-GnRHR cells (Fig. 15). Secondly, we investigated the effect of GnRH-PAP conjugates on CHO cells that did not express GnRH receptors on their surface. The conjugates had no cytotoxic activities against GnRH-receptor-negative CHO cells (Fig. 16).

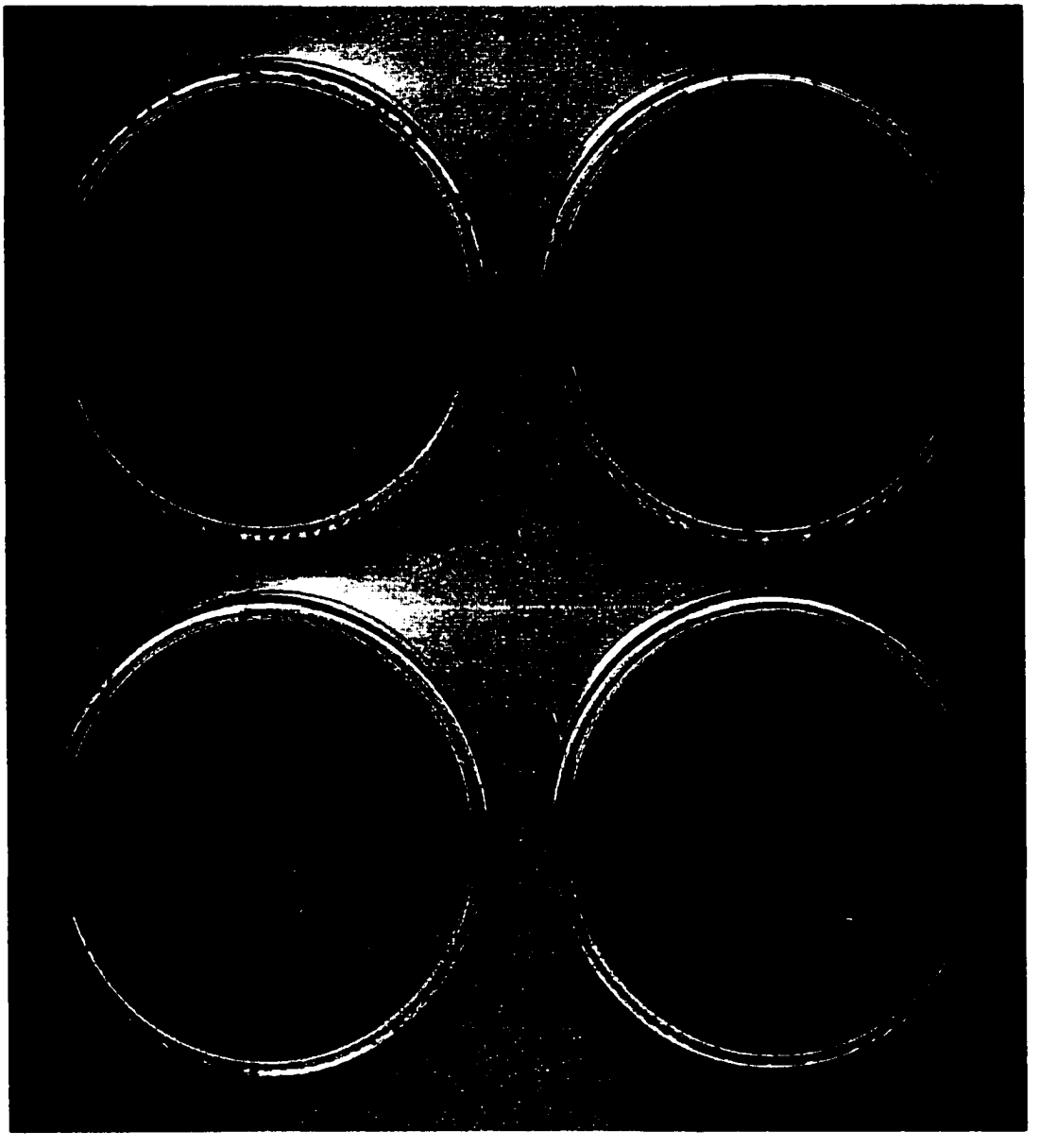

Fig. 12. Effects of ncGnRH-PAP on CHO-GnRHR cells. Cells were untreated (A) or were treated with PAP at 10^{-6} M (B), ncGnRH-PAP at 10^{-7} M (C), or ncGnRH-PAP at 10^{-6} M (D) for 6 days.

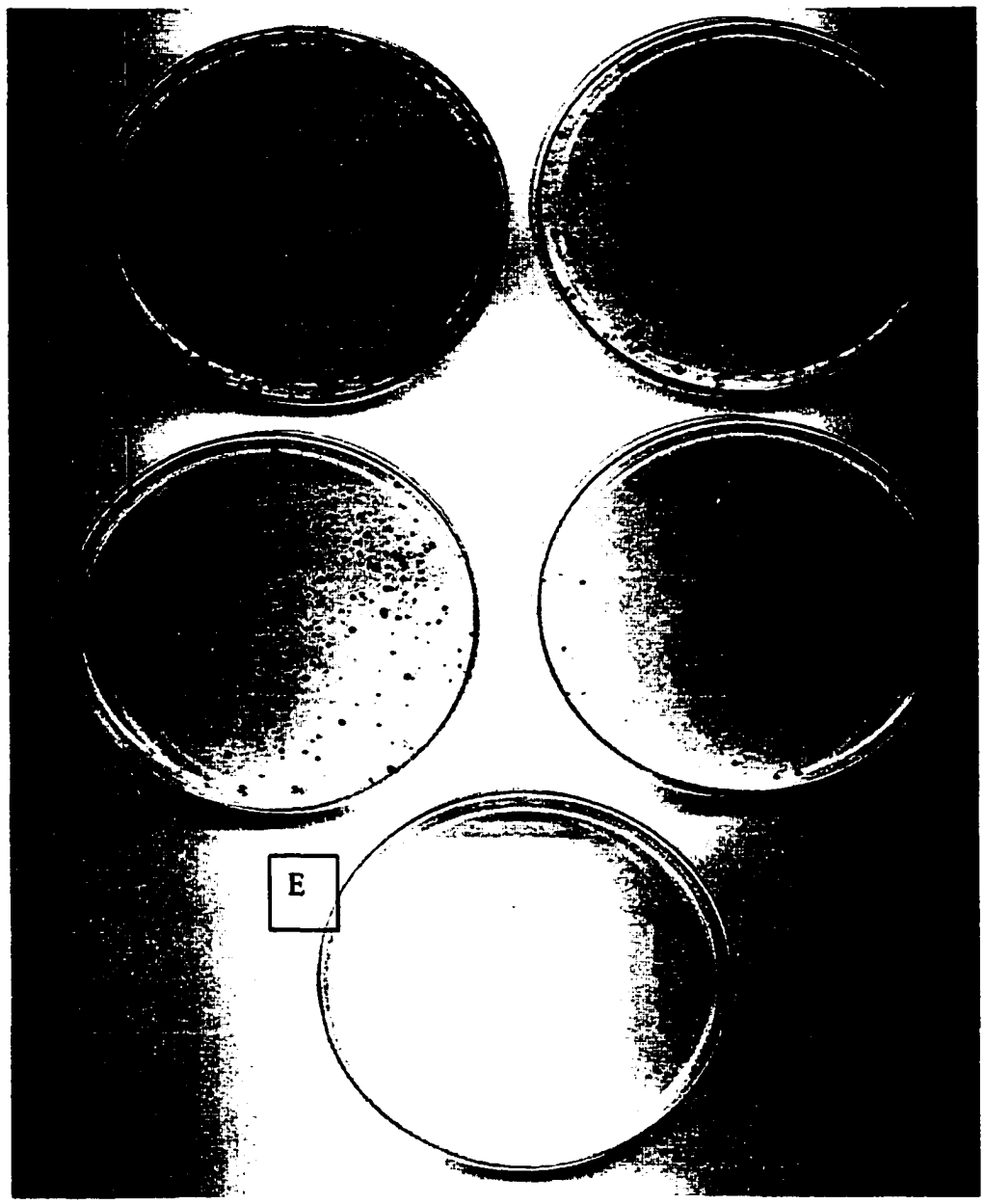

Fig. 13. Effects of ncGnRH-PAP on CHO-GnRHR cells. Cells were untreated (A) or were treated with ncGnRH-PAP at 10^{-7} M (B), ncGnRH-PAP at 3.33×10^{-7} M (C), ncGnRH-PAP at 6.67×10^{-7} M (D), or ncGnRH-PAP at 10^{-6} M (E) for 6 days.

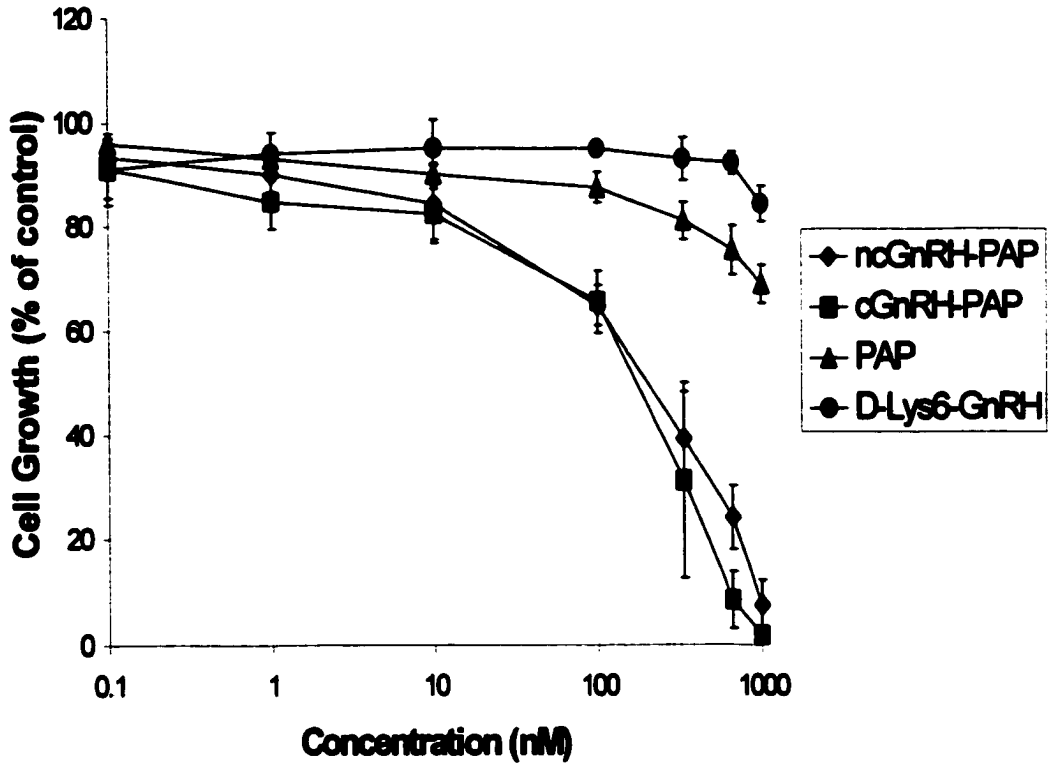

Fig. 14. Cytotoxicity of GnRH-PAP against CHO-GnRHR cells in the clonogenic assay. CHO-GnRHR cells were seeded in 100-mm diameter Petri dishes and maintained in complete medium at 37 C for 2 days. Then, various concentrations of either GnRH-PAP, D-Lys^{6}-GnRH or PAP were added. After 6 days of incubation, cell viability was estimated with crystal violet staining.

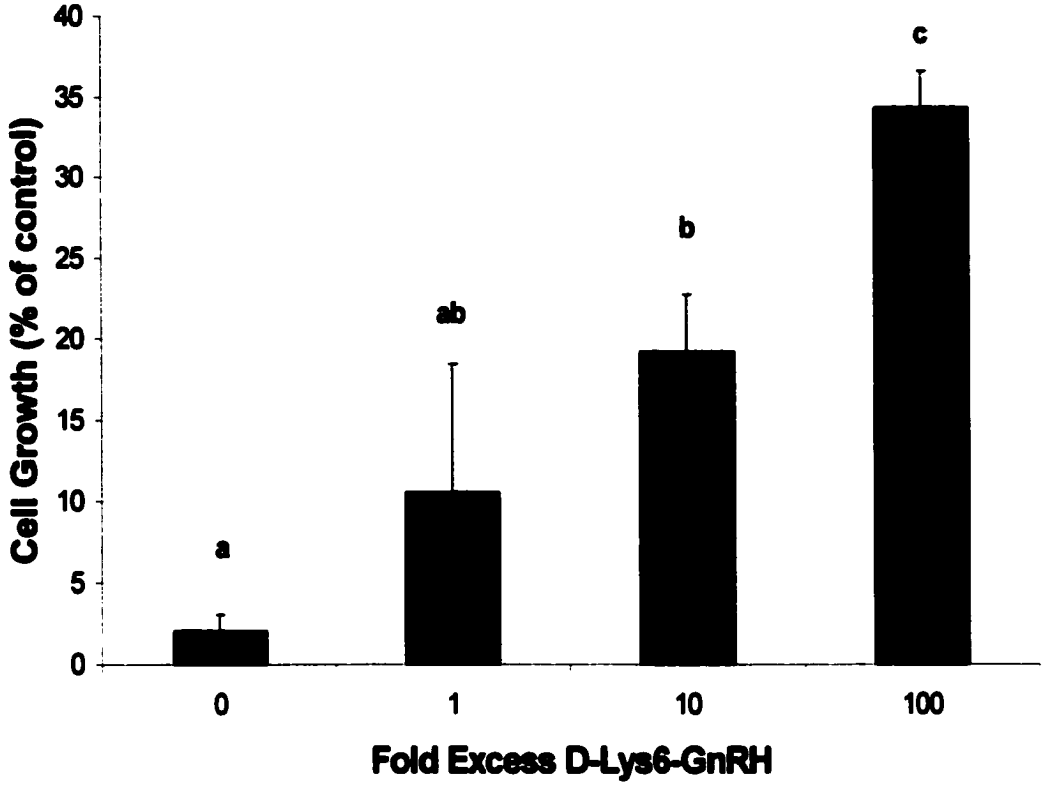

Fig. 15. Inhibition of the cytotoxic effects of GnRH-PAP by increasing doses of GnRH. CHO-GnRHR cells were seeded in 100-mm diameter Petri dishes and maintained in complete medium at 37 C for 2 days. Then, 1 μM ncGnRH-PAP was added in the presence of varying concentrations of D-Lys^{6}-GnRH. After 6 days of incubation, cell viability was estimated with crystal violet staining. The number of colonies in treated cultures was expressed as a percentage of those in control cultures. The data are presented as mean +/- SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey test for comparisons of multiple groups. Means with the same letter are not significantly different.

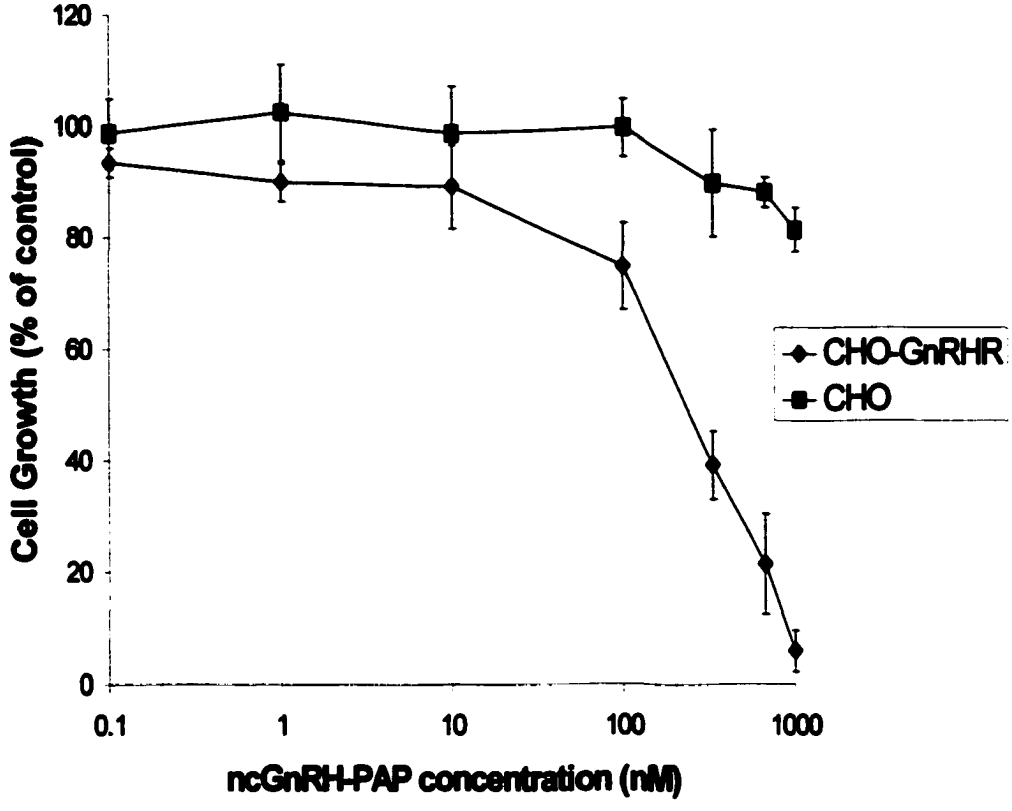

Fig. 16. Growth of CHO cells without GnRH receptors are not affected by GnRH-PAP. CHO-GnRHR and CHO cells were seeded in 100-mm diameter Petri dishes and maintained in complete medium at 37 C for 2 days. Then, various concentrations of ncGnRH-PAP were added. After 6 days of incubation, cell viability was estimated with crystal violet staining.

b. Cell proliferation assay

To determine if GnRH-PAP conjugates can inhibit proliferation of cells expressing GnRH receptors on their surface, CHO-GnRHR cells and αT3-1 cells were treated with varying amounts of conjugates for 2 days. As shown in Figs. 17 and 18, the conjugates inhibited the proliferation of these cells in a concentration-dependent manner, and the concentrations of conjugates giving a 50% inhibition of cell proliferation (ID50) were ~0.8 μM and 0.4 μM for CHO-GnRHR and αT3-1 cells, respectively. However, neither GnRH nor PAP alone affected proliferation of either cell types. Only at concentration of 5 μM, GnRH and PAP slightly inhibited the proliferation of αT3-1 cells.

c. Time course

We evaluated the time course of the cytotoxic activity of GnRH-PAP conjugates against CHO-GnRHR and αT3-1 cells by growing the cells for 1, 6, 24, 48, 72, and 96 hours in the presence of conjugates. Exposure of each cell type to GnRH-PAP for 72 hrs or longer was required to achieve complete inhibition of cell growth (Figs. 19 and 20).

B. PAP vs. RNase

To investigate whether GnRH-RNase conjugates could inhibit growth of the cells expressing GnRH receptors on their surface, CHO-GnRHR and αT3-1 cells were treated with varying amounts of GnRH-RNase conjugates. As shown in Figs. 21, 22, and 23, neither the GnRH-RNase conjugate nor RNase alone could inhibit the growth of both cells at concentrations up to 5 μM. However, as previously described, GnRH-PAP conjugates were able to inhibit growth of both cell types in a dose-dependent manner.

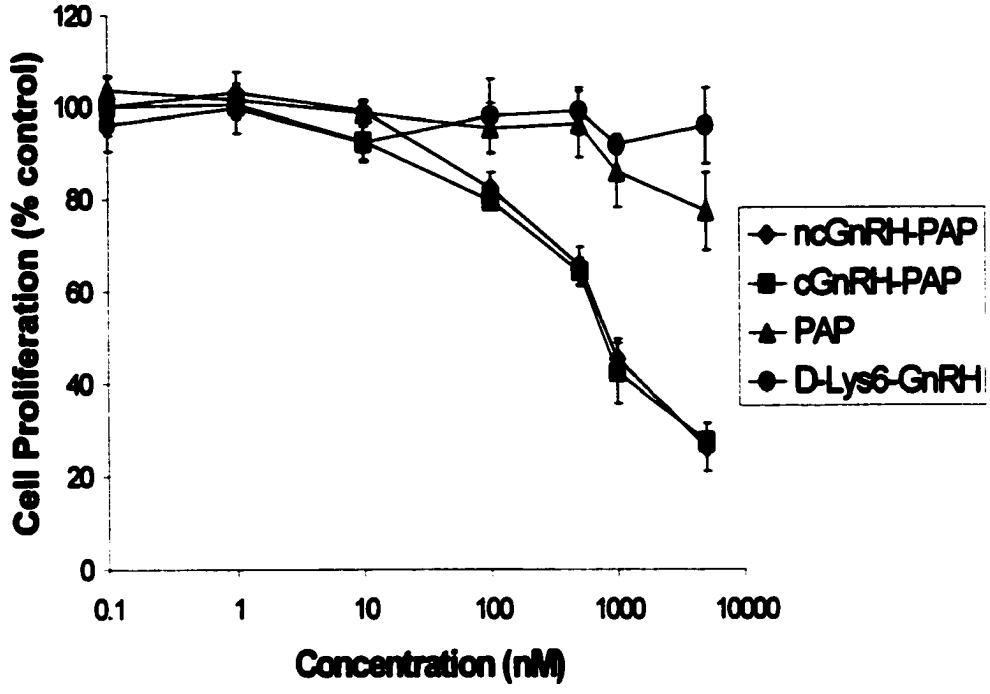

Fig. 17. Cytotoxicity of GnRH-PAP against CHO-GnRHR cells in the cell proliferation assay. CHO-GnRHR cells were seeded in 96-well cell culture plate and maintained in complete medium at 37 C for 2 days. Then, various concentrations of either GnRH-PAP, D-Lys^6-GnRH or PAP were added. After 2 days of incubation, cell proliferation was measured.

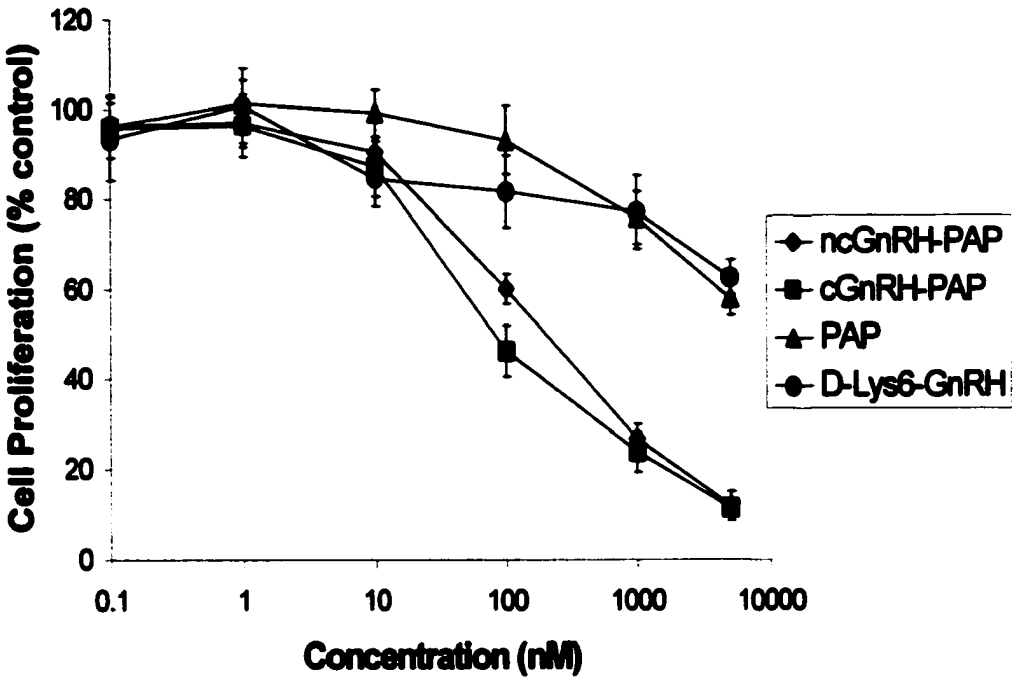

Fig. 18. Cytotoxicity of GnRH-PAP against αT3-1 cells in the cell proliferation assay. αT3-1 cells were seeded in 96-well cell culture plate and maintained in complete medium at 37 C for 2 days. Then, various concentrations of either GnRH-PAP, D-Lys^6-GnRH or PAP were added. After 2 days of incubation, cell proliferation was measured.

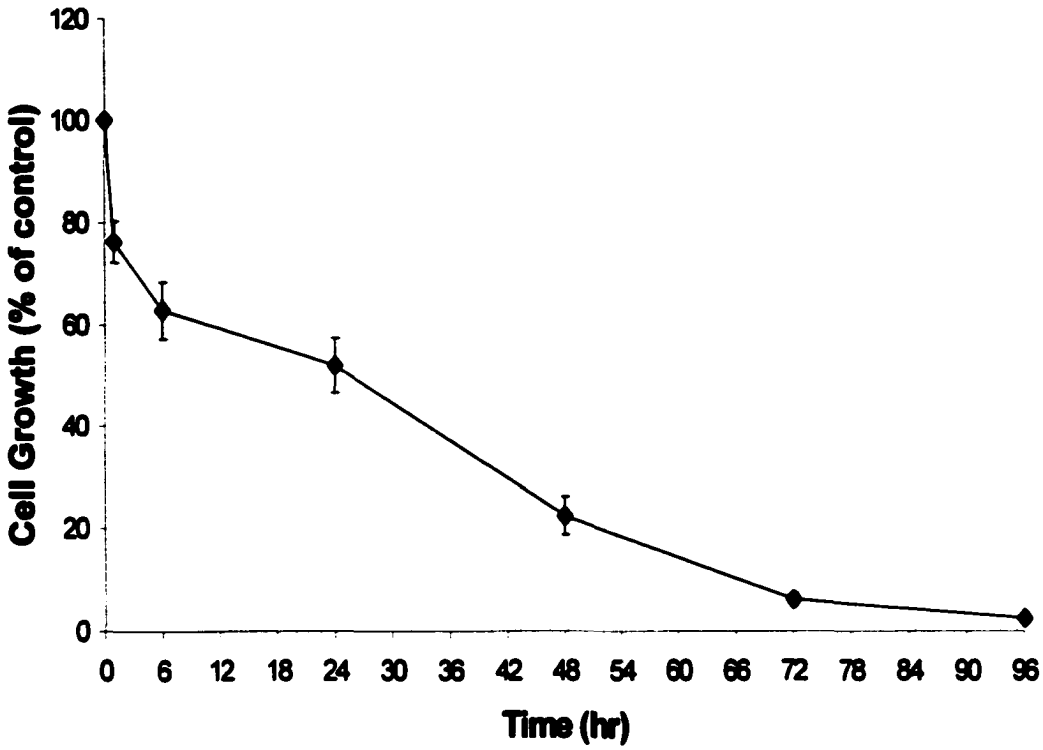

Fig. 19. Effect of time of exposure of CHO-GnRHR cells to GnRH-PAP on cellular growth rate. Cells were placed in a 100-mm Petri dish at 800 cells per dish and incubated for 2 days. The cells were grown for 1, 6, 24, 48, 72, or 96 additional hours in the presence of 1 μM ncGnRH-PAP. After removing the medium containing 1 μM of ncGnRH-PAP at each time point, the cells were incubated until 7 days from the first addition of ncGnRH-PAP with normal culture medium.

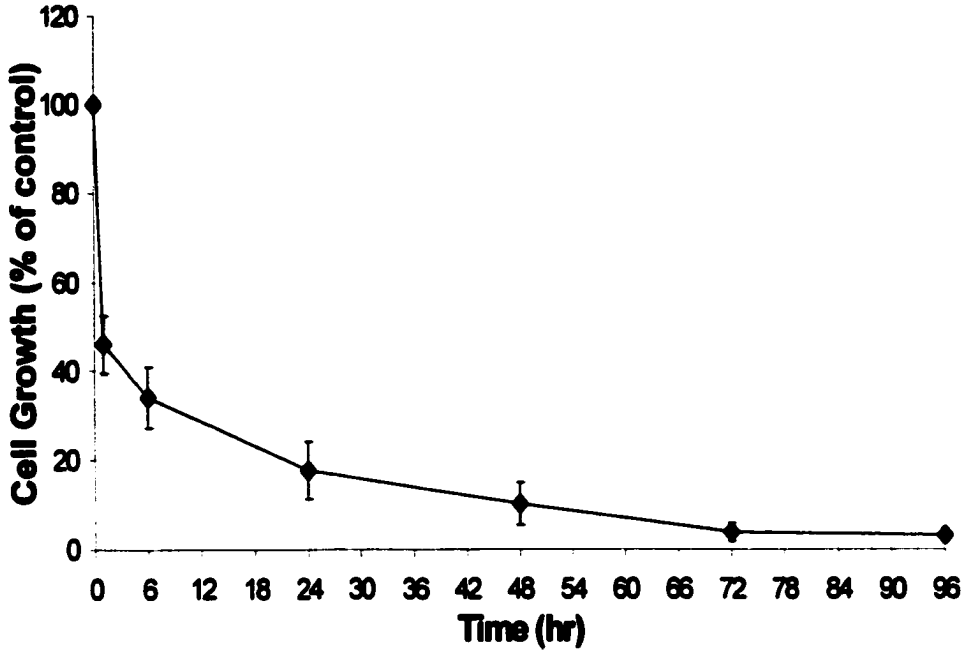

Fig. 20. Effect of time of exposure of αT3-1 cells to GnRH-PAP on cellular growth rate. Cells were placed in a 100-mm Petri dish at 800 cells per dish and incubated for 7 days. The cells were grown for 1, 6, 24, 48, 72, or 96 additional hours in the presence of 1 μM ncGnRH-PAP. After removing the medium containing 1 μM of ncGnRH-PAP at each time point, the cells were incubated until 7 days from the first addition of ncGnRH-PAP with normal culture medium.

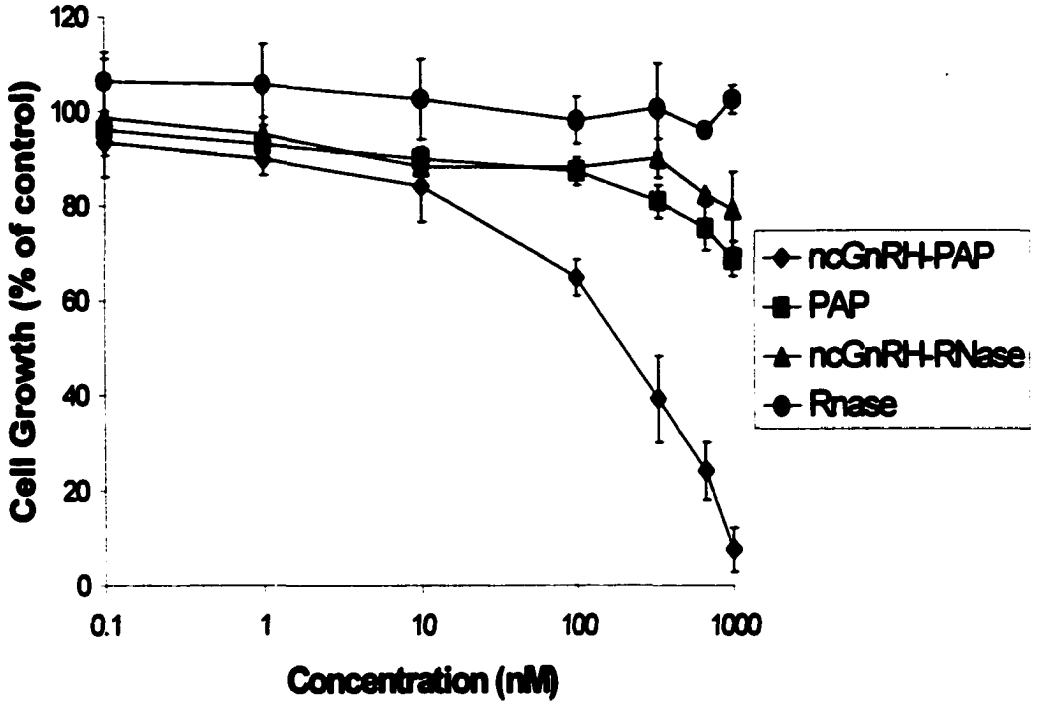

Fig. 21. Cytotoxicity of GnRH-PAP and GnRH-RNase against CHO-GnRHR cells in the clonogenic assay. CHO-GnRHR cells were seeded in 100-mm diameter Petri dishes and maintained in complete medium at 37 C for 2 days. Then, various concentrations of either GnRH-PAP, GnRH-RNase, PAP, or RNase were added. After 6 days of incubation, cell viability was estimated with crystal violet staining.

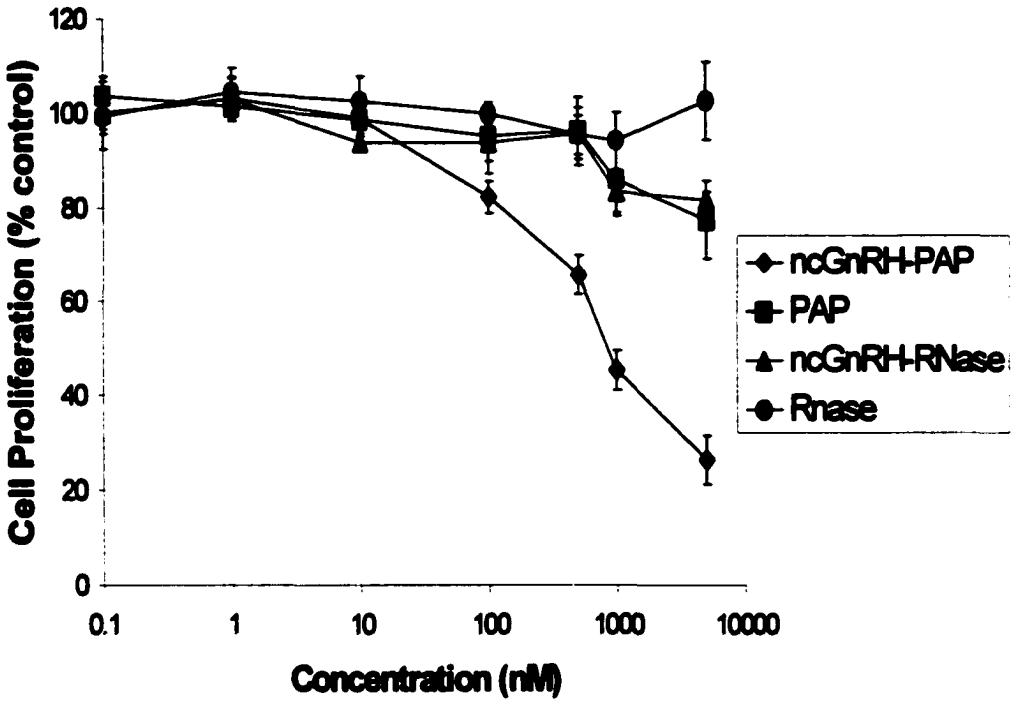

Fig. 22. Cytotoxicity of GnRH-PAP and GnRH-RNase against CHO-GnRHR cells in the cell proliferation assay. CHO-GnRHR cells were seeded in 96-well cell culture plate and maintained in complete medium at 37 C for 2 days. Then, various concentrations of either GnRH-PAP, GnRH-RNase, PAP, or RNase were added. After 2 days of incubation, cell proliferation was measured.

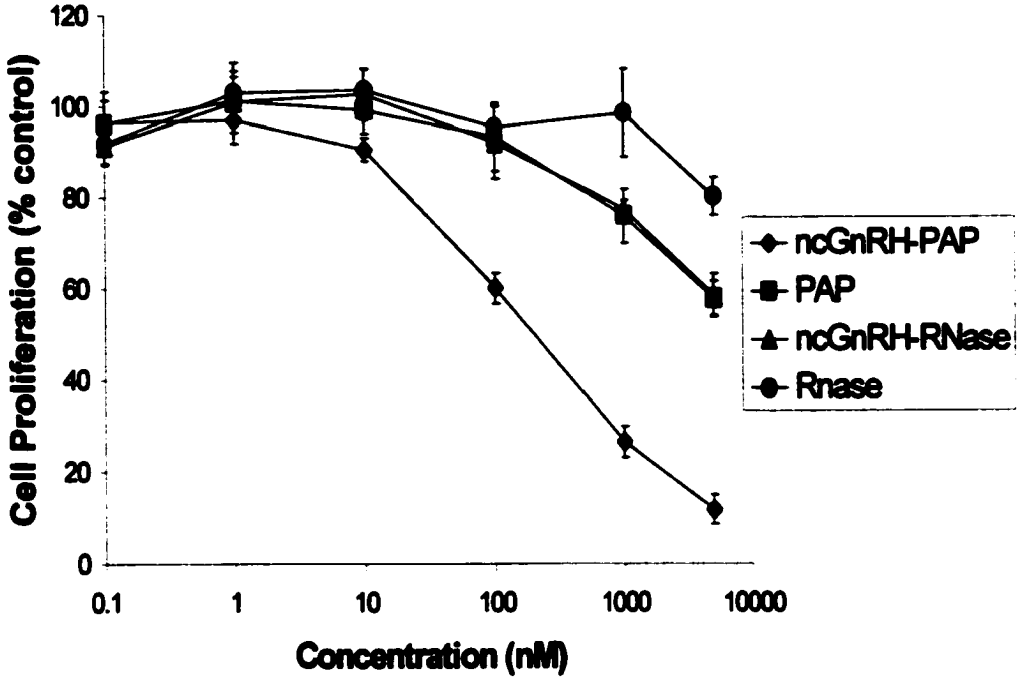

Fig. 23. Cytotoxicity of GnRH-PAP and GnRH-RNase against αT3-1 cells in the cell proliferation assay. αT3-1 cells were seeded in 96-well cell culture plate and maintained in complete medium at 37 C for 2 days. Then, various concentrations of either GnRH-PAP, GnRH-RNase, PAP, or RNase were added. After 2 days of incubation, cell proliferation was measured.

C. Cytotoxic activity of GnRH-toxin conjugates on human tumor cell lines

To determine if the human cancer cells had GnRH receptors, binding studies were performed on six human cancer cell lines, CHO-GnRHR cells, αT3-1 cells, and LβT2 cells. Scatchard analysis of GnRH receptors (Table 4) indicated that high-affinity, low-capacity binding sites for GnRH were found in αT3-1, CHO-GnRHR, and LβT2 cells. However, the concentration of GnRH receptors in human cancer cell lines was lower ($p < 0.05$) than that in αT3-1, CHO-GnRHR, and LβT2 cells.

Table 4. Scatchard analysis of GnRH receptors on cell lines

Cell line	Bmax (fmol/10^6 cells)	Kd (pM)
αT3-1	231.92 +/- 53.72 a	867.55 +/- 269.81 a
CHO-GnRHR	204.84 +/- 44.66 a	480.29 +/- 78.67 ab
LβT2	120.24 +/- 43.97 a	354.78 +/- 125.11 ab
PC3	8.61 +/- 3.31 b	185.75 +/- 85.71 ab
Ishikawa	7.60 +/- 3.03 b	150.53 +/- 39.96 b
Du145	6.17 +/- 2.45 b	180.02 +/- 18.75 ab
MCF-7	5.46 +/- 3.81 b	109.13 +/- 60.35 b
LNCaP	2.89 +/- 1.46 b	32.26 +/- 19.42 b
ppC1	1.26 +/- 0.29 b	116.34 +/- 14.30 b

Means with the same letter are not significantly different.

The representative results of cytotoxicity of the ncGnRH-PAP and cGnRH-PAP conjugates against αT3-1, CHO-GnRHR, LβT2, Du145, and ppC1 cells are shown in Figs. 24 and 25, respectively. At 1 μM ncGnRH-PAP, the GnRH-PAP conjugate inhibited proliferation of αT3-1 cells by about 75%, CHO-GnRHR cells by 55%, LβT2 cells by 38%, Du145 prostate cancer cells by 21%, and ppC1 prostate cancer cells by 22% (Fig. 24). Similar results were obtained using cGnRH-PAP (Fig. 25).

The relationship between cell viability and number of GnRH receptors on each of the cell lines is shown in Table 5 and Fig. 26. There was a linear (r^2 = 0.871) relationship between the cytotoxicity of ncPc and numbers of GnRH receptors in the cell lines (Fig. 26).

Table 5. The relationship between cell viability and GnRH receptors on cell lines

Cell line	Bmax (fmol/10^6 cells)	Cell Viability ***
αT3-1	231.92 +/- 53.72 a	26.60 +/- 3.40 a
CHO-GnRHR	204.84 +/- 44.66 a	45.38 +/- 4.23 b
LβT2	120.24 +/- 43.97 a	61.75 +/- 2.30 c
PC3	8.61 +/- 3.31 b	65.77 +/- 2.70 c
Ishikawa	7.60 +/- 3.03 b	70.25 +/- 3.13 cd
Du145	6.17 +/- 2.45 b	78.79 +/- 4.21 d
MCF-7	5.46 +/- 3.81 b	83.66 +/- 3.30 d
ppC1	1.26 +/- 0.29 b	77.51 +/- 1.59 d

*** Cell viability (% of control) in the presence of 10^{-6}M ncGnRH-PAP
Means with the same letter are not significantly different

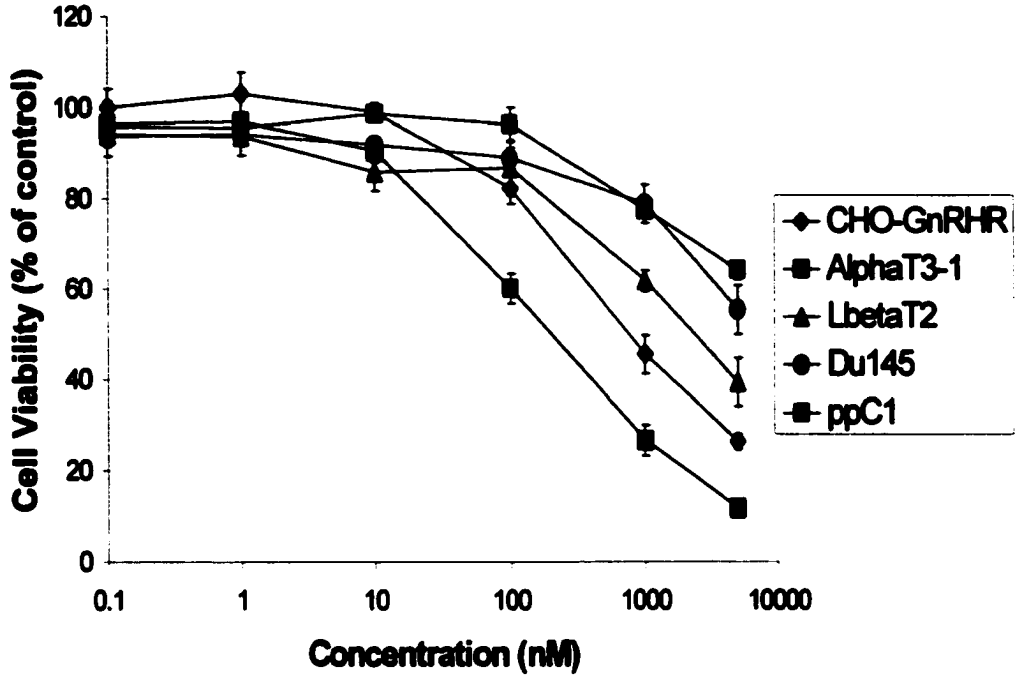

Fig. 24. Representative results of cytotoxicity of the ncGnRH-PAP conjugates against αT3-1, CHO-GnRHR, LβT2, Du145, and ppC1 cells by cell proliferation assay.

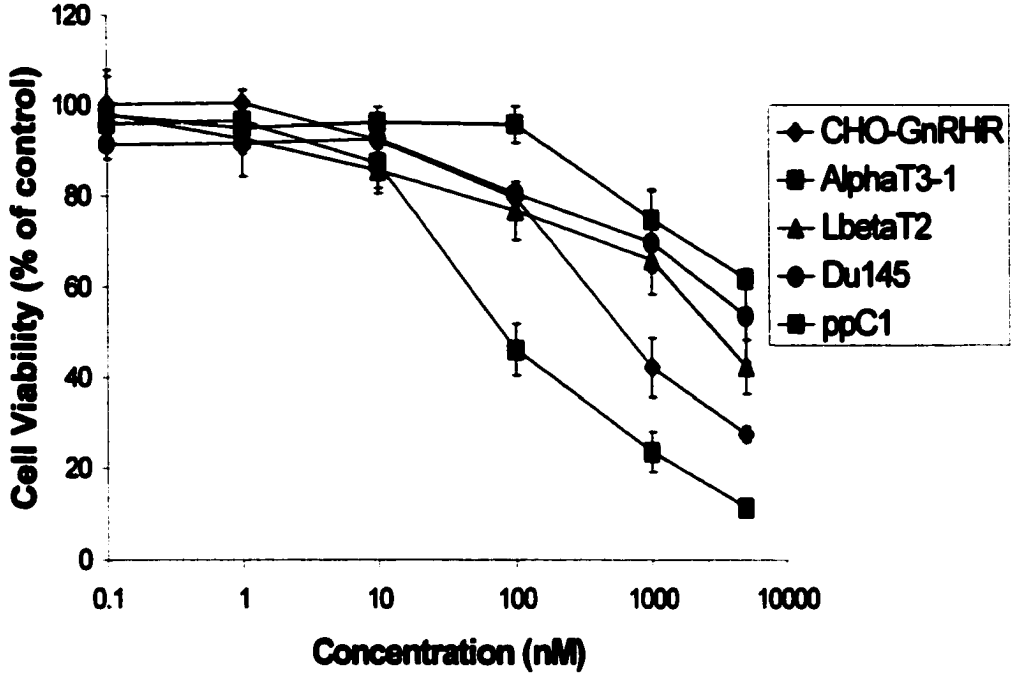

Fig. 25. Representative results of cytotoxicity of the cGnRH-PAP conjugates against αT3-1, CHO-GnRHR, LβT2, Du145, and ppC1 cells by cell proliferation assay.

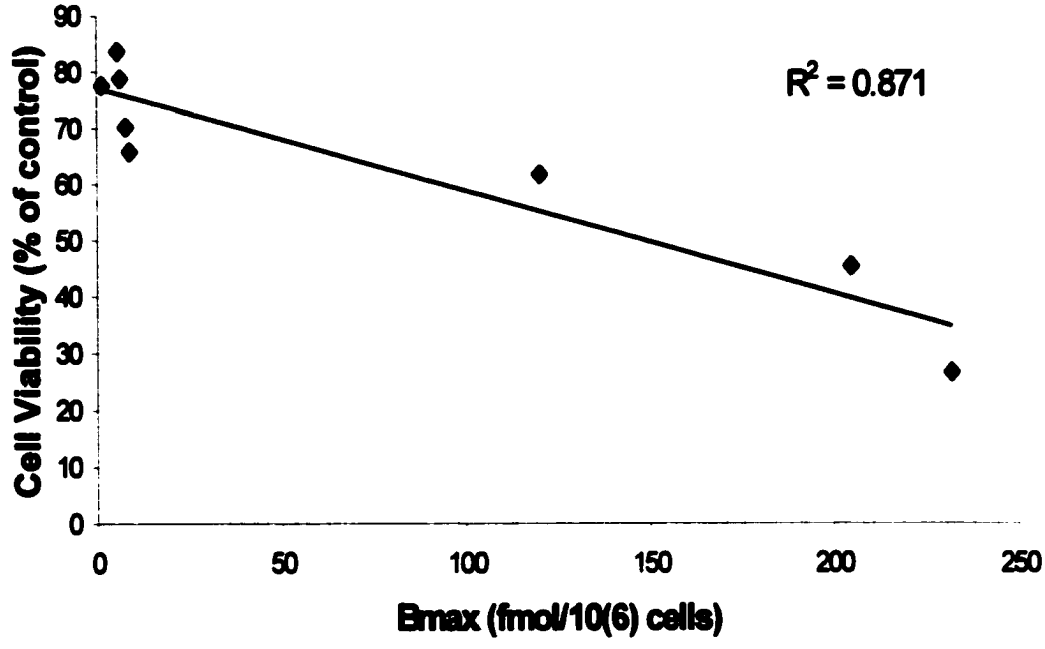

Fig. 26. Relationship between cell viability and number of GnRH receptors in the cell lines. The number of GnRH receptors on eight cell lines (αT3-1, CHO-GnRHR, LβT2, Du145, Ishikawa, LNCaP, MCF-7, PC3, and ppC1 cells) was determined by receptor binding assay. The cell viability of eight cell lines was evaluated by cell proliferation assay.

IV. Discussion

The present experiments demonstrated that GnRH-PAP but not GnRH-RNase conjugates reduced cloning efficiency and proliferation of cell lines expressing GnRH receptors. The cell lines used proved to be suitable for studying specific cytotoxic effects of GnRH-toxin conjugates because these lines have GnRH receptors. All the effects observed here were under specific *in vitro* conditions.

Our preliminary data showed that GnRH-PAP and GnRH-RNase conjugates were able to inhibit *in vitro* protein synthesis with a relatively good efficiency but to a slightly lesser extent than the purified native PAP and RNase, respectively (data not shown). Several other investigators have obtained similar results (Rajamohan et al., 1999; Schlick et al., 2000). This slightly reduced activity of the cytotoxins may be due to conformational changes induced by conjugation to partially block the catalytic site of toxins.

Prior to cytotoxicity experiments, our preliminary results showed that GnRH-PAP and GnRH-RNase conjugates were able to bind to GnRH receptors in bovine pituitary membrane preparation, albeit with slightly less efficiency than GnRH alone (data not shown). This slightly reduced activity of the cytotoxins may be due to restricted movement in solution making interaction with receptor more difficult.

Cytotoxicity of GnRH-PAP was tested on cell lines expressing GnRH receptors. The GnRH-PAP showed significant cytotoxicity on these GnRH-receptor-positive cell lines, whereas the conjugate had no effect on GnRH-receptor-negative cell lines (i.e. CHO cells). Extracellular PAP or GnRH alone was not cytotoxic toward cell lines harboring GnRH receptors. Also, the cytotoxic activities of GnRH-PAP conjugates could be

effectively reduced by addition of exogenous GnRH. These results clearly indicate that GnRH-PAP conjugates can destroy cell types that express GnRH receptors.

The lower inhibitory values obtained in the cell proliferation experiments may be due to the higher initial number of cells than the cells in the clonogenic assay. The time period of clonogenic assay is longer (6-7 days) than that of the cell proliferation experiments (2 days). The antigrowth effect is dose- and time- dependent: the longer the exposure time, the higher the rate of inhibition.

When compared to a GnRH-PAP conjugate constructed by using recombinant DNA technology (Schlick et al., 2000), the cytotoxicity of our GnRH-PAP conjugate appears lower. Cytotoxicity of GnRH-PAP conjugates is highly dependent on the targeting moiety and the number of ligand receptors on the cell surface. Evidence is mounting that both ends of GnRH structure are required for receptor binding (Spona, 1975; Schally et al., 1976; Boepple et al., 1986; Karten and Rivier, 1986). Therefore, fusion toxins in which either of the terminal amino acids of the GnRH molecule are altered, or attached to another moiety are very unlikely to have receptor binding activity. Thus, it is unclear that recombinant GnRH-toxin conjugates (or fusion toxins) would retain binding and cytotoxic activity on GnRH-receptor-positive cells. Based on these, more experimentation on GnRH conjugates constructed by using recombinant DNA technology method is needed.

The internalization and the rate of delivery of the toxin into the cytoplasm are also important factors. For a GnRH-toxin conjugate to cause death of a gonadotrope or a tumor cell expressing GnRH receptor, the first event is the conjugate must bind to receptor and be internalized by the cell. This occurs by receptor-mediated endocytosis.

Once GnRH binds to receptor, it is internalized by gonadotropes and sequestered in endosomes (Conn and Hazum, 1981; Duello et al., 1983). It has been found that GnRH apprears to be internalized when conjugated to small molecule (Szoke et al., 1994) or larger molecule (Hopkins and Gregory, 1977). However, the rate of internalization of GnRH-toxin conjugates compared to that of GnRH alone is not clear.

In this study, we also compared the cytotoxicity of both GnRH-PAP conjugates (cGnRH-PAP and ncGnRH-PAP). The results showed that the cytotoxic activity of both conjugates is similar. This indicates that the linker of the conjugate does not have any influence on cytotoxic activity of the conjugate once the conjugate enters the cells. Ramakrishnan and Houston (1984) showed the similar result that both noncleavable (maleimido ester cross-link) and cleavable (disulfide bond) PAP-containing immunotoxins were cytotoxic. However, they found ricin A chain linked to antibody by a noncleavable cross-link was not cytotoxic. In the Chapter Two, we showed that ncGnRH-PAP is stable in *in vitro* and *in vivo* system, however, cGnRH-PAP is not stable in *in vivo* system. Therefore, we should use ncGnRH-PAP for further *in vivo* study in the future.

We found that the cytotoxicity of GnRH-RNase conjugates on cell lines expressing GnRH receptors was very low. This low cytotoxic activity may be due to the presence of endogenous RNase inhibitor. RNase inhibitor is a 50-KDa protein that constitutes ~ 0.01% of the protein in mammalian cells (Lee and Vallee, 1993). However, Gho and Chae (1999) showed that their GnRH-RNase conjugates could specifically inhibit the proliferation of GnRH-receptor-positive human prostate and breast tumor cells. The reason for the discrepancy between their findings and the studies employed herein is

unknown. Thus, more experimentation is needed to clarify potential use of GnRH-RNase conjugates.

Many investigators have shown that specific high-affinity binding sites for GnRH are present in about 50% of breast cancers (Fekete et al., 1989), in approximately 80% of endometrial and ovarian cancers (Emons and Schally, 1994), and in nearly 85% of prostate cancers (Qayum et al., 1990). Our results clearly indicate that GnRH-PAP conjugates can kill the specific cell lines that express the GnRH receptors. Therefore, GnRH-PAP conjugates provide a potential treatment for cancers. Moreover, GnRH-PAP conjugates may have dual effect if cancers are sex steroid hormone-dependent and GnRH-receptor-positive. This approach, which remains to be tested clinically, could open up a new area of cancer therapy because the cytotoxic analogs developed might have the potential to produce an eventual cure.

In conclusion, Since GnRH-PAP appears to destroy cells harboring GnRH receptors, it should be possible to use it to kill gonadotropin-secreting cells in the pituitary gland and thereby prevent reproduction (i.e. chemical castration) and destroy tumor cells that harbor GnRH receptors (i.e. treat breast, endometrial, ovarian, and prostate cancers).

CHAPTER FOUR

GnRH-TOXIN CONJUGATES ON CELL APOPTOSIS STUDY

I. Introduction

Cell death may follow two distinct patterns: apoptosis and necrosis. Apoptosis is a term used to describe the terminal morphological and biochemical events seen in programmed cell death (Wyllie, 1992). Apoptosis has been the subject of great interest because it was clearly demonstrated to mediate cell death, not only during development but also in response to chemotherapy and radiation therapy for treatment of neoplasia (Eastman, 1990; Hickman, 1992). The events of apoptosis are very different from those seen in necrosis (Majno and Joris, 1995). Necrosis is the death of cells as a result of direct injury, usually beginning at the cell surface. Necrotic cells exhibit early lysis of the plasma membrane before any significant alterations of nuclear morphology are detected. Necrotic cells initially swell before lysis, whereas apoptotic cells show cell shrinkage. Necrotic cells eventually exhibit swelling of the nucleus, whereas apoptotic cells exhibit characteristic nuclear morphological changes, including chromatin condensation and hypersegmentation of nuclear chromatin of irregular size. These hypersegmented nuclear structures may then bud from the rapidly blebbing cell surface to form "apoptotic bodies".

A hallmark of apoptosis is that the nuclear DNA extracted from apoptotic cells is often degraded in an internucleosomal pattern (Arends et al., 1990; Compton, 1992). That

is, DNA cleavage during apoptosis occurred at sites between nucleosomes. This DNA fragmentation is analyzed using agarose gel electrophoresis to demonstrate a laddering pattern at ~200bp intervals. It has been suggested that these nuclear changes are due to the activation of Ca^{2+}- and Mg^{2+}-dependent endonucleases.

Immunotoxins and hormonotoxins are cytotoxic agents composed of a binding moiety and a toxin. The binding moiety directs the toxin to a target cell, and then the toxin causes cell death. A variety of plant and bacterial toxins have been used to make immunotoxins and hormonotoxins. Recently, it has been shown that these toxins and conjugates induced apoptosis in various cell lines (Hughes et al., 1996; Oda et al., 1997; Williams et al., 1997; Keppler-Hafkemeyer et al., 1998; Komatsu et al., 1998; Komatsu et al., 2000). For instance, recent studies have demonstrated that ricin induces cell lysis and DNA fragmentation in a process reminiscent of programmed cell death or apoptosis (Sandvig and van Deurs, 1992; Oda et al., 1997; Oda et al., 1998). Recently, a series of cytotoxic conjugates containing GnRH and PAP or RNase was developed in our lab. However, the incidence of apoptosis or necrosis in cell lines expressing GnRH receptors after treatment of GnRH-toxin conjugates has not yet been elucidated. It is important to understand that the efficiency of induction of apoptosis and the apoptotic pathway may have direct effects on the therapeutic usefulness of GnRH-toxin conjugates. Here we describe for the first time that the incidence of apoptosis in αT3-1 cells after treatment with GnRH-PAP conjugates.

II. Materials and Methods

A. Conjugates

The toxin used in this study was PAP. PAP was purified from Pokeweed (*Phytolacca americana*) leaves as described previously (Irvin, 1983) with minor modifications. The conjugation was described previously (Chapter Two).

B. Cell culture

αT3-1 cells (a mouse gonadotrope tumor cell line that expresses endogenous GnRH receptors) were maintained in Dulbecco's modified Eagle Minimal Essential Medium (Sigma) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 5% fetal bovine serum, and 5% horse serum. All cells were cultured in a humidified environment with 5% CO_2 at 37 C and the medium was replaced every 2-3 days.

C. Cell counting for antiproliferation

Proliferation of αT3-1 cells in control and treated cultures were determined as described (Palyi et al., 1996). Briefly, 500,000 cells were plated into 100-mm diameter plastic Petri dishes. After 2 days, the cells were treated with different concentrations of the ncGnRH-PAP for additional 2 days. Cells were then trypsinized and counted in a Neubauer-type haemocytometer and viability was assessed by trypan blue exclusion. Two to three dishes were used in each group. The ratio of live to dead cells in each group was determined. The data are presented as mean +/- SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey test for comparisons of multiple groups. Values at $P < 0.05$ were considered to be significant.

D. DNA fragmentation analysis

Culture of αT3-1 cells with ncGnRH-PAP was performed as described previously (Chapter Three). Briefly, 50,000 cells were plated into 100-mm diameter plastic Petri dishes. After 2 days, the cells were grown for 1, 3, 6, 12, 24, and 48 additional hours in the presence of 1 μM of ncGnRH-PAP. At each time point, cells were washed once with PBS and then harvested and centrifuged (1,000 g, 10 min, 4 C). The pelleted cells were kept at –70 C until extraction of DNA. The cells were homogenized in lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.5% SDS) as described previously (Oda et al., 1997). The lysate was incubated with 250 μg/ml of proteinase K for 15 h at 55 C. The solution was then extracted with an equal volume of phenol (buffer saturated)/chloroform/isoamyl alcohol, 25:24:1. After centrifugation (1500 g, 5 min), the aqueous (top) phase was transferred to a new tube. Nucleic acids were precipitated with 2.5 volumes of 100% ethanol for 1 h at –20 C. The DNA pellet was washed in ethanol (70%), air-dried, dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and stored at 4 C until assayed. After spectrophotometric quantification of DNA concentration, the DNA samples and a 1-kilobase DNA ladder molecular weight marker were electrophoresed in a 1.5% agarose gel with TAE buffer (0.04 M Tris-acetate and 0.002 M EDTA) at 110 V for 1.5 h. DNA was visualized by ethidium bromide staining. Photographs were taken with Polaroid film (Polaroid, Cambridge, MA). This experiment was performed in minumum of five times.

III. Results

A. Antiproliferation assay

To determine the precentage of cell death in cells treated with GnRH-PAP conjugates, αT3-1 cells were treated with varying amounts of GnRH-PAP conjugates (ranging from 10^{-6} M to 10^{-10} M) for 2 days. Fig. 27 shows that GnRH-PAP conjugates induced cell death at the concentration of 10^{-7} M or higher. At 10^{-7} M of GnRH-PAP conjugates, 20% of the cells were dead; at 10^{-6} M, 68% of cells were dead. GnRH-PAP conjugates caused a decrease in number of total (live plus dead) cells in a dose-dependent manner. The data indicates that GnRH-PAP conjugates not only block cell division but also inhibit protein synthesis and induce cell death.

B. DNA fragmentation analysis

DNA fragmentation is generally considered to be characteristic apoptotic changes. In this study, we found that incubating αT3-1 cells for 3 h or longer with 1 μM GnRH-PAP resulted in DNA ladder formation. A representative result is shown in Fig. 28.

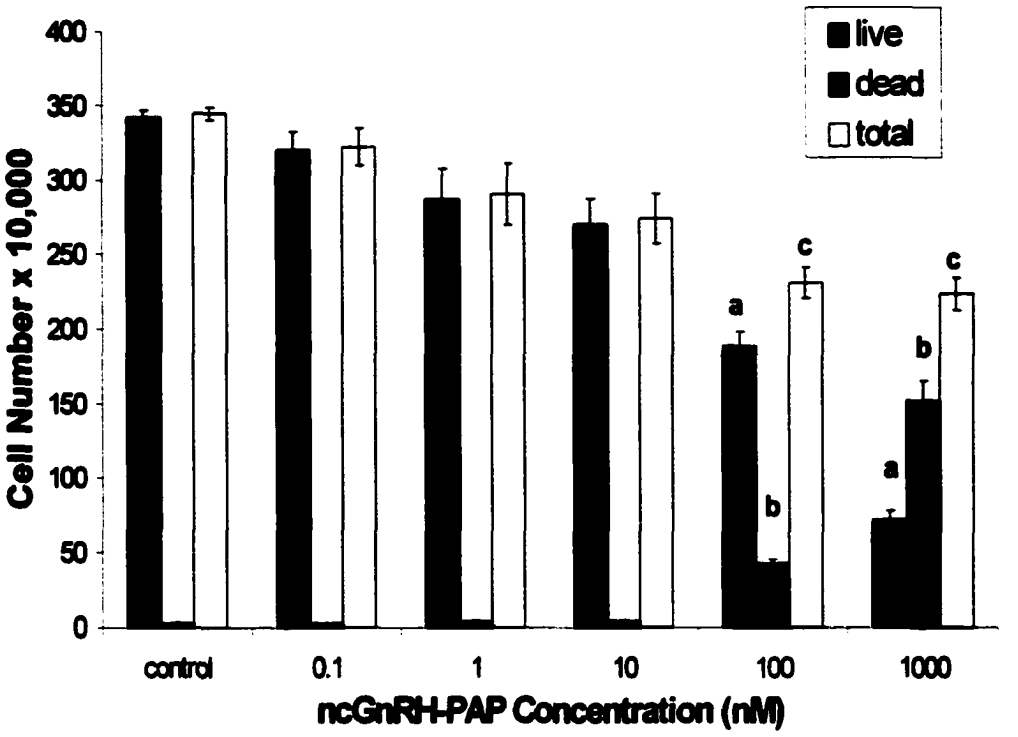

Fig. 27. Effect of GnRH-PAP on the proliferation and viability of αT3-1 cells. Seeding concentration was 500,000 cells per 100-mm diameter Petri dish. After 2 days, the cells were treated with different concentrations of ncGnRH-PAP for additional 2 days. Cells were then trypsinized and counted in a Neubauer-type haemocytometer and viability was assessed by trypan blue exclusion. a: $P < 0.05$ vs control; b: $P < 0.05$ vs control; c: $P < 0.05$ vs control.

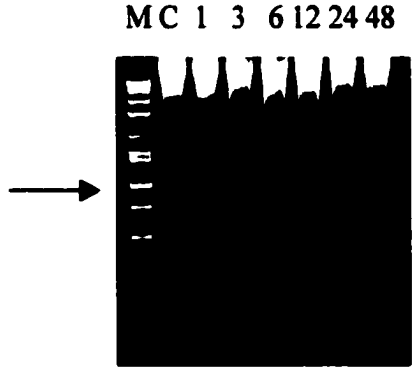

Fig. 28. Representative GnRH-PAP-induced DNA fragmentation in αT3-1 cells. Cells were incubated in the presence or absence of 1 μM GnRH-PAP in DMEM medium for 1, 3, 6, 12, 24, and 48 h at 37 C. DNA was extracted and analyzed in a 1.5% agarose gel as described under "Materials and Methods". M, molecular size standards; C, control; arrow indicates 1000 bp standard marker.

IV. Discussion

In this study, we investigated death of αT3-1 cells and apoptosis induced by exposure to the GnRH-PAP conjugates. After incubation for 3 h or longer GnRH-PAP conjugates induced cell death and induced apoptosis. It has been shown that ricin (Komatsu et al., 2000), as well as other protein toxins such as diphtheria toxin and *Pseudomonas* toxin, can cause apoptotic cell death as judged by DNA fragmentation and target cell lysis (Komatsu et al., 1998). These data indicate that plant or bacterial toxins are inducers of apoptosis. Recently, Danila et al. (1999) demonstrated that the receptor-specific cytotoxic effect of 2-pyrrolinodoxorubicin conjugated to D-Lys^6-GnRH exerted through induction of apoptosis and DNA fragmentation.

Apoptosis is an integral biologic mechanism of many developmental and pathophysiological states and reflects multiple biochemical events that ultimately lead to cell death via chromatin condensation, DNA fragmentation, cell shrinkage, and disassembly into membrane-enclosed vesicles (apoptotic bodies) (Thornberry and Lazebnik, 1998). Recent studies suggest that apoptotic pathway is in part modulated by a net equilibrium of proapoptotic Bax and antiapoptotic Bcl-2 regulatory proteins (Adams and Cory, 1998). Repression or down-regulation of steady-state protein levels of the antiapoptotic Bcl-2 with concurrent increases in Bax levels is a common mechanism for apoptotic induction. Caspase 3 activity can be induced by proapoptotic Bax and inhibited by the antiapoptotic Bcl-2. Activation of caspase family enzymes results in cleavage of cytoskeletal and nuclear proteins, and nucleosomal fragmentation of DNA (Tilly and Hsueh, 1993). Convergent events in apoptosis center on mitochondria. Release of caspase activators (such as cytochrome c), changes in electron transport, loss of mitochondrial

transmembrane potential, altered cellular oxidation-reduction, and participation of pro- and anti-apoptotic Bcl-2 family proteins lead to apoptosis (Green and Reed, 1998).

It has been shown that various apoptosis-inducing stimuli can trigger both apoptotic and necrotic pathways (Coppola et al., 1995). Necrosis and apoptosis are generally recognized as two different mechanisms of cell death, regulated by intracellular ATP levels. The apoptotic pathway requires high levels of ATP, while low levels of ATP shift the cell death mode from apoptotic pathway to necrotic pathways (Eguchi et al., 1997; Leist et al., 1997). DNA fragmentation in ricin-treated cells was initiated after protein synthesis was reduced to a very low level and the inhibition of protein synthesis may trigger apoptotic pathway. This could explain why our GnRH-PAP conjugates induce apoptosis.

The Fas system recently has been implicated as a possible key regulator of cell apoptosis in the testis (Lee et al., 1997). Fas is a type I transmembrane receptor protein that belongs to the tumor necrosis factor (TNF) family (Watanabe-Fukunaga et al., 1992; Nagata and Golstein, 1995); Fas ligand is a type II transmembrane protein of the TNF family (Nagata and Golstein, 1995). Fas/Fas ligand interaction *in vitro* has been shown to trigger the death of cells expressing Fas (Nagata and Golstein, 1995). It has been also shown that tumor cells expressing GnRH receptors also expressed the Fas system (Imai et al., 1997). In this study, we have shown that GnRH-PAP conjugates induced apoptosis. In the future, it will be interesting to determine if GnRH-PAP has the ability to trigger expression of Fas/Fas ligand system.

In conclusion, the results suggest that GnRH-PAP conjugates induce cell death by blocking cell division and inhibiting protein synthesis, and also induce apoptosis, as evidenced by DNA fragmentation.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

Hormone-dependent cancers (such as prostate cancer in men, breast, endometrial, and ovarian cancers in women) are major cancers leading to death in America and many other parts of the world. The functions of the breast and prostate are regulated by steroid hormones such as estrogen and androgen, respectively. Moreover, these sex hormones are necessary for the growth and proliferation of hormone-dependent human breast and prostate tumors. Available options for the treatment of breast and prostate cancers are surgical castration, radiation therapy, hormone treatment and cytotoxic chemotherapy. Administration of GnRH analogs stimulates the secretion of FSH and LH by gonadotropes. When GnRH is administered chronically, desensitization of the GnRH receptors in the gonadotropes occurs and then secretion of FSH and LH is decreased, leading to a decrease of circulating concentrations of steroid hormones and thus inhibiting the growth and proliferation of breast and prostate cancers (Isaacs and Coffey, 1981). However, surgical and hormonal combination therapy to reduce the ovarian estrogen and testicular testosterone leaves an opportunity for the adrenal estrogen and androgen to stimulate the growth and proliferation of breast and prostate cancers. Moreover, hormone-independent breast cancers and prostate cancers can still grow and proliferate after these types of combinational treatments have been applied. In the case of cytotoxic chemotherapy, the standard cytotoxic agents primarily act against rapidly

growing and proliferating cancer cells, but prostate cancer is not a rapidly growing and proliferating cancer. Thus, it seems that it is time to try some new treatments. In view of non-specific toxicity of most chemotherapeutic agents against normal cells, the development of targeted chemotherapy is warranted. Efficient targeting of chemotherapeutic drugs to the cancerous area could be of great benefit for patients with advanced or metastatic tumors. Targeted cytotoxic conjugates (immunotoxins or hormonotoxins) are hybrid molecules composed of a cytotoxic moiety and a targeting moiety which binds to antigens or receptors on tumors. In this sense, the hormonotoxin approach is intriguing.

GnRH-toxin conjugates were constructed by chemical cross-linking to evaluate the potential of these conjugates to kill gonadotropin-secreting cells in the pituitary gland and thereby prevent reproduction (i.e. chemical castration) and destroy tumor cells that harbor GnRH receptors (i.e. treat breast, endometrial, ovarian, and prostate cancers).

Recently, we have developed a series of cytotoxic conjugates containing GnRH and PAP or RNase. In Chapter Two, The result showed that a sensitive, specific, and accurate sandwich ELISA has been established for measurement of GnRH-toxin conjugates. This new assay system will provide us a valuable research tool to understand the stability and half-life of GnRH-toxin conjugates in *in vitro* and *in vivo* systems.

In Chapter Two, the result also showed that biologically non-degradable linkers should be considered in conjugate design. To achieve the specific delivery of GnRH-toxin conjugates to targeted cells, the stability of linkers in systemic circulation should be maintained. This protection eliminates degradation of conjugates in circulation and improves therapeutic potential.

In Chapter Three, the results showed that the GnRH-PAP conjugates reduced growth and proliferation of cell lines expressing GnRH receptors. This information indicates that GnRH-PAP conjugates are good candidates to not only kill gonadotropin-secreting cells (gonadotropes) in the pituitary gland and thereby eliminate reproduction but also destroy cancer cells harboring GnRH receptors and thereby provide a direct treatment of these types of cancers.

In Chapter Four, the result showed that the GnRH-PAP conjugates-induced cytotoxicity via apoptosis concomitant with DNA fragmentation. This information gives us more understanding on the cytotoxic mechanisms of GnRH-PAP conjugates.

Overall, GnRH-PAP conjugate is a good candidate to selectively destroy cells expressing GnRH receptors on their surface to prevent reproduction and serve as a treatment for cancers harboring GnRH receptors.

CHAPTER SIX

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CHAPTER SEVEN

LIST OF ABBREVIATIONS

ACTH:	Adenocorticotropin
AN-152:	GnRH-doxorubicin conjugate
AN-207:	GnRH-2-pyrrolinodoxorubicin conjugate
CHO:	Chinese hamster ovary
cGnRH-PAP:	Cleavable GnRH-PAP conjugate
cGnRH-RNase:	Cleavable GnRH-RNase conjugate
DT:	Diphtheria toxin
ELISA:	Enzyme-linked immunoabsorbent assay
FSH:	Follicle-stimulating hormone
GH:	Growth hormone
GnRH:	Gonadotropin-releasing hormone
GnRHR:	Gonadotropin-releasing hormone receptor
HT:	Hormonotoxin
IT:	Immunotoxin
LH:	Luteinizing hormone
ncGnRH-PAP:	Non-cleavable GnRH-PAP conjugate
ncGnRH-RNase:	Non-cleavable GnRH-RNase conjugate
PAP:	Pokeweed antiviral protein

PE:	Pseudomonas exotoxin
PRL:	Prolactin
RIP:	Ribosome-inactivating protein
RNase:	Ribonuclease A
SAP:	Saporin
SDS:	Sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis
TSH:	Thyroid-stimulating hormone
TMB:	Tetramethylbezidine