Drug-Targeting Strategies for Prostate Cancer

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Abstract: Prostate cancer is the most frequently diagnosed cancer in North American men and accounts for 10% of cancer-related deaths in men. Despite advances in early detection and aggressive treatment of early disease, the overall mortality rate has not appear to have fallen, indicating that the current therapies are not beneficial for life expectancy and new strategies are required. Prostate cancer is a dynamic evolving process that develops in distinct steps, with each step liable to additional genetic hits that change the cancer cell phenotype and alter the patterns of gene expression. The molecular events in prostate cancer are beginning to be understood, including altered expression of tumor suppressor genes, pro- and anti-apoptotic genes, and oncogenes associated with the progression of the disease; and specific genes that are expressed predominantly or exclusively in prostate cells, prostate cancer cells, and prostate metastasis cells. These latter genes on the level of DNA, RNA and protein products are the targets of several new approaches to prostate cancer therapy and are the focus of this review.

Key Words: Prostate, Cancer, Peptidase, Prostate specific antigen (PSA), Prodrug, Gene Therapy, Androgen, antisense.

PROSTATE ANATOMY AND FUNCTION

The prostate is the gland of the male reproductive system responsible for producing the seminal fluid that accounts for the liquefication of the coagulated semen. It surrounds the neck of the urinary bladder and the posterior urethra in front of the rectum. The ejaculatory duct, a muscular tube carrying the sperm from the testes, enters the upper part of the prostate from behind, travels through the gland (about 2 cm), and deposits the sperm and seminal vesicle fluid into the urethra in the center of the prostate gland at the verumontanum. Some 15-30 excretory ducts enter the urethra as it passes through the prostate. These ducts are lined with secretory cells that respond to androgen stimulation by producing secretory proteins that are stored as viscous secretions in the ascinar spaces of the saccule ends or acini. Basal cells also line the prostate ducts and might be responsible for most types of prostatic hyperplasia that form due to uncontrolled prostatic tissue growth.

The prostate is divided into three distinct zones with different structural and functional characteristics: peripheral, central, and transition (Fig. **1**). The peripheral zone accounts for about 70% of prostate glandular tissue, and is the origin of most prostate tumors. The transition zone lies on either side of the proximal urethra, and its ducts empty into the verumontanum; it comprises some 5% of glandular tissue in younger men — a proportion that increases markedly with age as benign prostatic hyperplasia accumulates. The central zone, which constitutes about 25% of the gland volume, is conical shaped with its base constituting the greater part of the base of the gland. It follows the course of the ejaculatory ducts and branches near the base of the prostate. Prostate cancer can also form in this tissue. The prostate is surrounded by the prostate capsule, a tissue that separates it from the rest of the body. This is a critical area, since prostate cancer contained inside the prostate capsule is considered localized and treatable by surgery, while cancer that has punctured and spread outside the capsule has more limited treatment options (reviewed in [1-3]).

The prostate contributes the major part of normal human ejaculate, about 0.5 ml of the 3 ml of total ejaculate volume. During ejaculation, the prostatic fluid secretes citric acid, spermine and other basic molecules into the urethra to neutralize the seminal fluid, keep the sperm mobile, and protect it from the acid secretions of the female vagina. The proteins from the seminal vesicle cause the ejaculate to clot and form a coagulum within a few minutes after ejaculation, after which a serine protease secreted from the prostate, called prostatic specific antigen (PSA), lyses the clot. Semen has an immunosuppressive effect, which apparently explains why most women do not develop antibodies against sperm in the vagina. Other proteolytic enzymes in the secretions help sperm traverse cervical mucus, while the prostaglandins stimulate the female reproductive system to transport the sperm towards the ovum [3; 4].

The prostate gland typically enlarges as men grow older. It is about the size of a pea at birth, begins to enlarge rapidly at puberty until it reaches normal adult size and shape in the early twenties (25 gr), and starts to enlarge again in the mid-forties in most men through a process of cell multiplication of the transition zone called benign prostatic hyperplasia (BPH). BPH is seen in only 10% of men before the age of 40, and in some 80% by the age of 80. Prostate growth in

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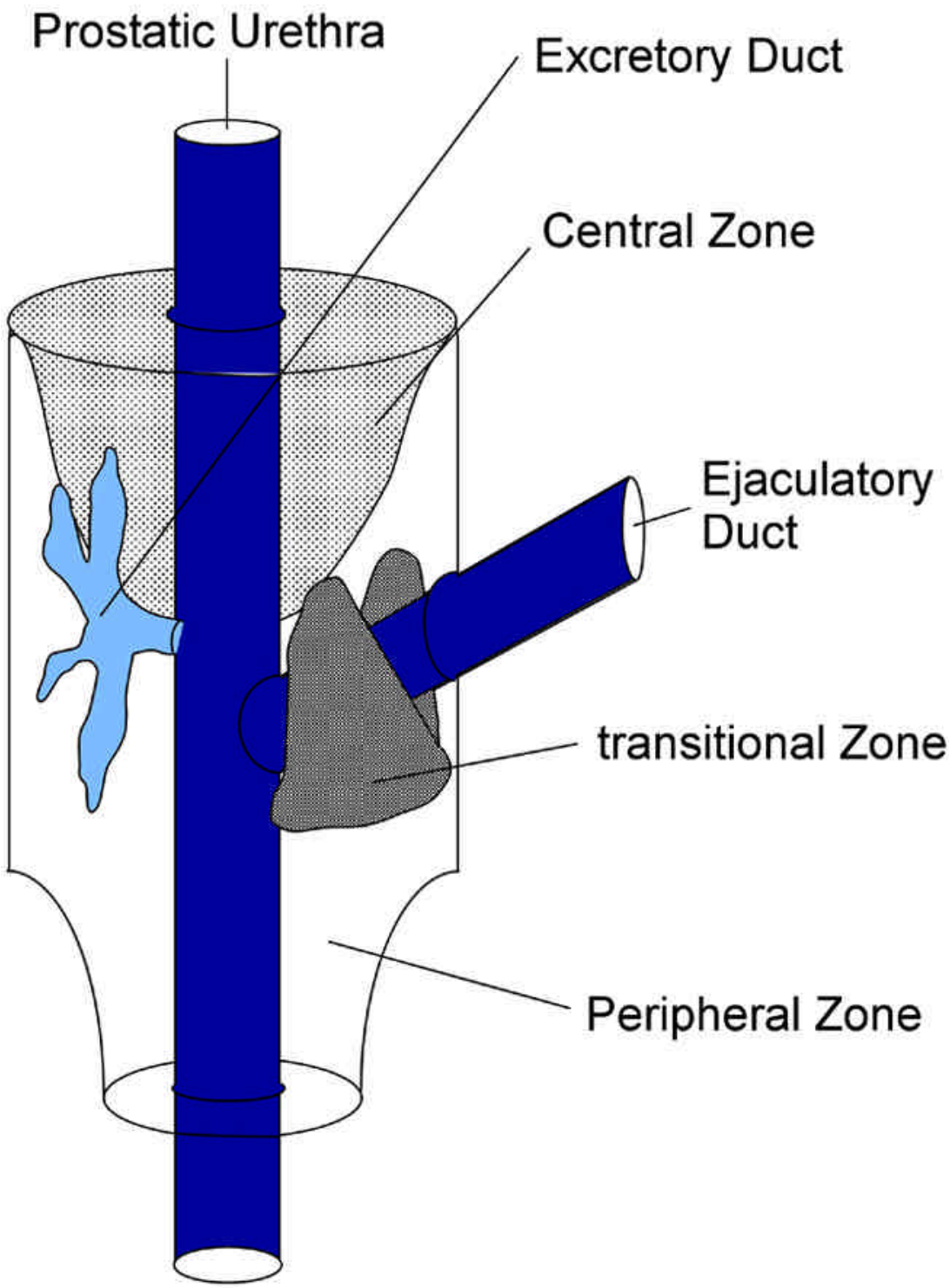

Fig. (1). Normal prostate anatomy. The three zonal anatomy of the prostate, the prostatic urethra, the ejaculatory duct and excretory duct are illustrated.

this area may block the bladder or urethra and prevent the flow of urine [5; 6].

Androgens, in particular a metabolite of testosterone, are essential for maintenance of the normal morphology and function of the prostate and seminal vesicles. Androgens control the growth of the prostate and formation of the prostatic secretions. Testosterone is synthesized in the testes under luteinizing hormone (LH) stimulation and is complexed in the serum with a steroid binding globulin. The

free form diffuses across the epithelial and stromal cell membranes and enters the prostatic cells where the testosterone is metabolized to a more androgenic substance called dihydrotestosterone (DHT) through reduction of a double bond at the 5-position of testosterone. The enzyme, termed 5-alpha reductase, forms the more potent DHT, which binds in a highly specific manner to the androgen receptor within the cell. The DHT-bound androgen receptor attaches to a promoter area on DNA at a sequence called the androgen responsive element (ARE). This binding participates in androgen-induced expression of genes such as PSA [7].

PROSTATE CANCER

The American Cancer Society estimated that in 2001 approximately 198,100 new cases of prostate cancer would be diagnosed in the United States and 31,500 men would die from prostate cancer. The lifetime risk for clinical prostate cancer is about 10% among U.S. men; approximately 3% die of this disease (SEER 1 Program Publication, National Cancer Institute; http://seer.cancer.gov/Publications/ProstMono/intro.pdf.;[8]).

Prostate cancers can be relatively harmless or extremely aggressive. Some are slow growing and cause few clinical symptoms; in these cases the patient will often die with prostate cancer rather than from prostate cancer. Aggressive cancers spread rapidly to the lymph nodes, and especially bone. Prostate cancer is graded and staged for aggressiveness and the extent to which it has spread. Stages A and B are confined to the prostate gland, stage C has spread outside the gland but only locally, and stage D has spread to lymph nodes or distant sites in the body.

Tumor stage determines the choice of treatment. Radical surgery (prostatectomy) or radiation is the major option for patients with prostate cancer confined within the prostate capsule without metastases. However, most prostate cancer patients are diagnosed at advanced stages of metastatic disease or are not cured by this therapy. Androgen withdrawal/ablation therapy is the common treatment for these patients, which ameliorates the symptoms and reduces tumor size. Since prostate cancer is extremely heterogeneous and spreads from several focal regions, the cancer inevitably progresses within 12-18 months to androgen resistance, which is incurable. The transition from the androgen-dependent to the androgen-independent stage results from genetic alteration undergone by the prostate cancer cells, or by androgen resistant cells that escape apoptosis and continue to proliferate and metastasize. Despite advances in early detection and aggressive treatment of early disease, the overall mortality rate has not appears to have fallen [9; 3], indicating that early detection is not beneficial for life expectancy and new therapies are required. The molecular events in prostate cancer are beginning to be understood, including altered expression of tumor suppressor genes, pro- and anti-apoptotic genes, and oncogenes associated with the progression of the disease; and specific genes that are expressed predominantly or exclusively in prostate cells, prostate cancer cells, and prostate metastasis cells. These latter genes on the level of DNA, RNA and protein products are the targets of several new approaches to prostate cancer therapy and are the focus of this review.

PRODRUGS

Chemotherapy remains the major systemic treatment of malignant diseases. However, it is not very effective against tumors, especially once they have metastasized, mainly because of insufficient drug concentrations in tumors, systemic toxicity, development of resistance, and lack of selectivity for tumor cells over normal cells. The cure rates achieved with chemotherapy are especially low in the treatment of solid tumors, where the majority of tumor cells are not dividing rapidly — only 3-5% of prostate cancer cell are in M phase [10-12]. The clinical efficacy of chemotherapy can be improved by selective delivery of the available drugs to malignant cells, which will reduce the toxicity of chemotherapy and permit much higher drug doses and more frequent treatments.

The development of relatively non-toxic prodrug forms of the anticancer agents that are activated only in the tumor tissue is one approach to this problem. The selective activation of the prodrug in the tumor tissue can be effected by metabolism or spontaneous chemical breakdown that results in an active anticancer agent. The ideal prodrug should be stable in the blood and body fluids, far less toxic in the prodrug form than in the activated form, and activated specifically in or within the microenvironment of the tumor cells.

PEPTIDASES

A number of strategies are being used to develop such prodrugs (reviewed in [13; 10; 11]). One approach is based on the activation of prodrugs by tumor-associated enzymes, particularly peptidases.

Prostate Specific Antigen (PSA)

PSA is a member of the human kallikrein family that exhibits serine protease activity. It is a 28,400 Da glycoprotein comprising 237 amino acid residues with approximately 8% carbohydrates (33-34 kDa on SDS gel). PSA has been shown to activate urokinase-type plasminogen activator, thought to be involved in tumor invasion and metastasis [14]. It was found to cleave insulin-like growth factor binding protein 3 (IGFBP-3), causing the release of active IGF-I, which could enhance tumor growth [15]. PSA may also inhibit tumor growth, evidenced by its ability to generate angiostatin from plasminogen [16; 17].

PSA is synthesized in the ductal and acinar epithelium of the prostate, where it is secreted at 0.5-2.0 g/L into the seminal plasma. Most PSA passes into the gland lumen, where it is mixed at the time of ejaculation with semen stored in the seminal vesicles to produce seminal liquefaction. A tiny proportion is absorbed into the bloodstream where levels should be less than 4 ng/ml. The prostate capsule creates a barrier that prevents the escape of

PSA into the peripheral circulation. The disruption of this capsule by disease allows PSA into the peripheral circulation and is used as diagnostic tool for prostate cancer.

The concentration of PSA in the prostate extracellular fluid is 1600-2100 nM in normal human prostate and primary human prostate cancer; 80-90% of this PSA is enzymatically active. In contrast, none of the PSA in the sera is enzymatically active [18]. The enzymatically active form of PSA (free PSA) forms complexes of 80-90 kDa with the serum prostate inhibitor α1-antichymotrypsin to create the predominant form of PSA in the serum. PSA also forms a complex with α2-macroglobulin and other serum enzyme inhibitors, but to a much lesser extent [19; 20].

Serum PSA levels correlate well with the number of malignant prostate cells, and higher levels of PSA are indicative of metastatic disease [21]. Approximately 75-85% of PSA exists as complexed PSA; the proportion increases to 90-100% in prostate cancer. Since the lower the level of free PSA in the serum, the higher the chances of malignancy [22], the distinction between complexed PSA and free PSA has become recognized as a clinically relevant feature of PSA tests. Tombal *et al*. and Graefen et al. [23; 24], on the other hand, demonstrated that the free/total PSA ratio is not indicative of organ-confined disease and PSA recurrence after radical prostatectomy.

The major proteolytic substrates of PSA are gel-forming proteins in the ejaculated semen, semenogelin I and II [25]. Cleavage maps following PSA treatment of human semenogelin I demonstrated that the most efficient cleavage occurred between Gln349 and Ser350 in semenogelin I [26; 27]. This led to the synthesis of short peptides that were efficiently hydrolyzed by PSA, and one of these peptides with the amino acid sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) was found to have a high degree of specificity for PSA [28]. This peptide was used to demonstrate that prostate cancer secretes enzymatically active PSA into the extracellular fluid, and that PSA becomes inactivated in the serum by the formation of a covalent complex with the plasma proteases inhibitors α1-antichymotrypsin and α2-macroglobulin. Thus, secreted PSA is only active in the microenvironment surrounding prostate cancer cells. This peptide was subsequently coupled through the C-terminal carboxyl group to the amino group of the drug doxorubicin (Dox) to yield a Dox-peptide conjugate that behaved *in vitro* as a targeted prodrug for PSA-secreting tumor cells. When it turned out that PSA was unable to hydrolyze the amino bond between the Dox amine and the C-terminal glutamine of the peptide, an additional amino acid L-leucine was linked to the primary amine of Dox (Leu-Dox). Leu-Dox was found to have activity against the human prostate cell line LNCaP with a median effective concentration (EC_{50}, the amount required to kill 50% of the tumor cells) of 50 nM; it was also shown to have less cardiac toxicity than Dox in animal models [29; 30]. Incubation of PSA collected from LNCaP cells grown in cell culture with the modified HSSKLQ-Leu-Dox prodrug resulted in more than 90% of the conjugate peptide hydrolyzed to Leu-Dox after 72 hours [31]. When nude mice bearing PSA-producing prostate cancer xenografts (PC-82) were given the Dox-peptide prodrug at four times the maximum tolerated dose (MTD) of Dox equivalent dose, there was a 57% decrease in tumor weight [32].

Based on this idea, systematic modification of the amino acid residues flanking the cleavage site was performed, leading to the synthesis of a slightly different Dox-peptide prodrug (N-glutaryl-(4-hydroxyprolyl)Ala-Ser-cyclohexa-glycyl-Gln-Ser-Leu-Dox) that can be cleaved by PSA releasing Leu-Dox as the active cytotoxic drug (Fig. **2**; [26]). This prodrug has the advantage of a minimum molecular weight that maintains high selective potency against PSA secreting cells: EC_{50} of 5 μM for PSA-secreting cells and more than 100 μM for non-PSA secreting cells – LNCaP and DuPRO, respectively. The prodrug has a rapid specific rate of hydrolysis by PSA: 30 minutes to hydrolyze 50% of the prodrug to Leu-Dox at a molar ratio of 1:100, respectively. *In vivo* experiments on human prostate cell xenografts (LNCaP) in nude mice revealed a 10-fold increase in MTD for the peptide-Dox prodrug compared to Dox *per se* (28.6 versus 2.8 μmole/Kg, respectively) [33]. This indicated a reduction in systemic toxicity of the prodrug prior to hydrolysis by PSA. In addition, tumor exposure to Leu-Dox was increased 2.5-fold compared to that achieved after an equimolar dose of Dox itself [34]. Notably, treatment of nude mice bearing LNCaP cells led to approximately 90% reduction in the level of serum PSA and the tumor weights with half the MTD of the Dox-peptide prodrug. The site-specific activation of this peptide prodrug was supported by additional experiments: nude mice carrying non-PSA-secreting cells or nude mice carrying non-PSA-secreting cells subjected to non-cleavable peptide-Dox exhibited no change in tumor weight. In addition, the distribution of the peptide-Dox was higher than that of Dox in tumor tissue, but lower in heart tissue [33]. Unfortunately, advancing this therapeutic approach to preclinical trials faces two major setbacks: 1) only one-third of the prodrug was metabolized to Leu-Dox in different laboratory animal models (mice, rats, dogs and monkeys); and 2) there was substantial non-PSA-specific formation of Dox, most likely the result of conjugate conversion to doxorubicin by both PSA-specific and non-PSA-specific proteolytic activities [34]. Until further modifications of the hydrolyzed peptide bring about greater specificity for PSA activity, the therapeutic usefulness of this prodrug is limited.

The site-specific release of a high concentration of Leu-Dox cytotoxic agent in the microenvironment surrounding prostate cancer cells by the proteolytic activity of PSA led to the search for other cytotoxic agents that might be conjugated to the hydrolyzed peptide. Isaacs *et al*. [35] and Christensen *et al*. [36] evaluated thapsigargin as the conjugated agent. Thapsigrgin induces apoptosis in human androgen-independent prostatic cancer cell lines (TSU-Pr1, PC-3, DU-145) with EC_{50} in the 10-100 nM range. It induces apoptosis of proliferatively quiescent G_0 cells by inhibiting the ubiquitous SERCA (sarco/endoplasmic reticulum calcium ATPase) pump. This leads to depletion of intracellular Ca2+ and the sustained elevation in cytosolic Ca2+ concentration that activates the apoptotic pathway. A series of thapsigargin analogues containing an amino acid applicable for conjugation to the peptide were tested for their ability to kill TSU-Pr1 human prostatic cancer cells. The conversion of thapsigargin into *O*-8-debutanoylthapsigargin

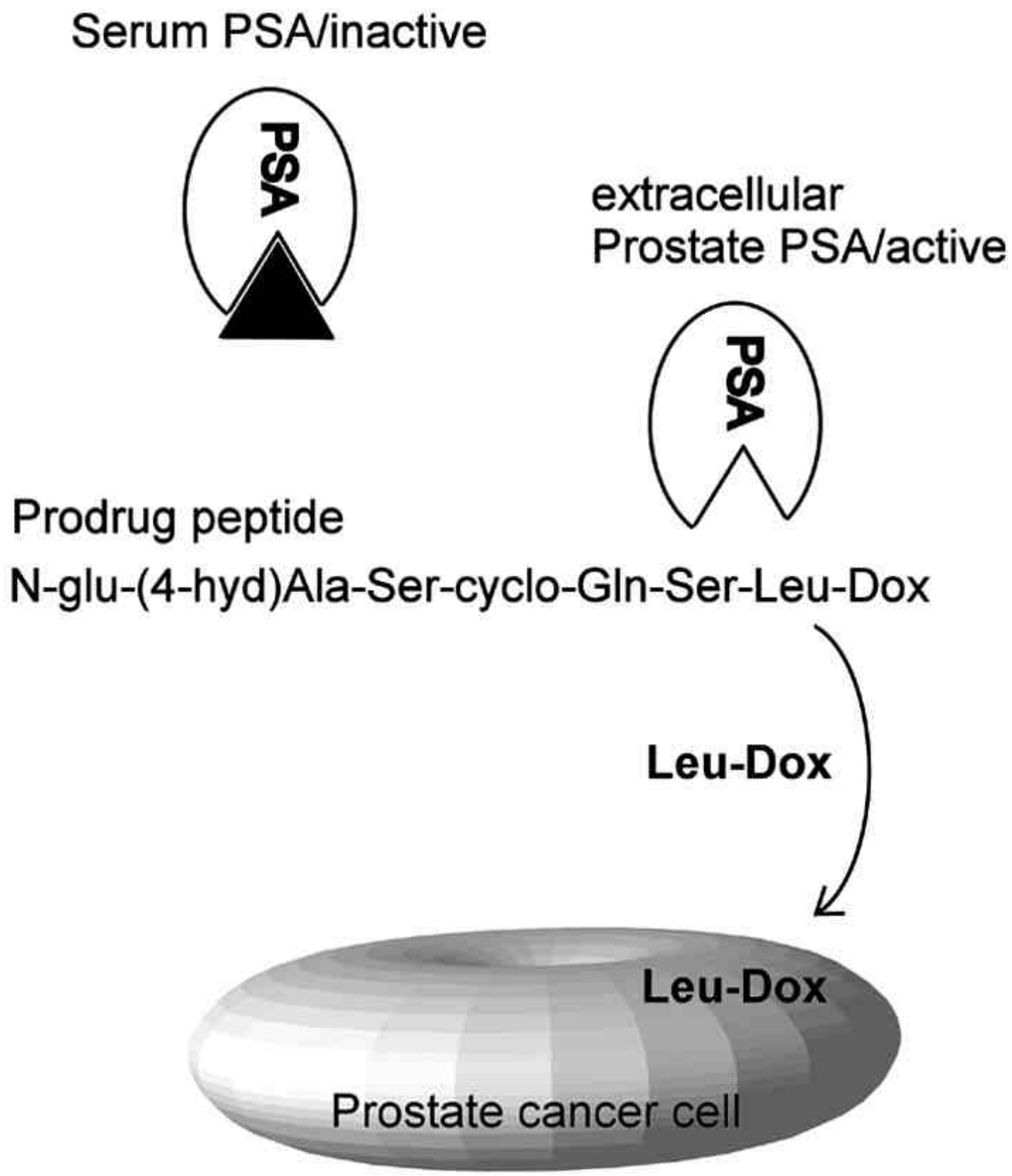

Fig. (2). General outline of the approach for site-specific activation of peptide-prodrug by PSA.

and esterifying the *O*-8 with amino acid linkers indicated that 12-(L-leuinoylamino) dodecanoyl gave an analogue equipotent with thapsigargin, making it a promising cytotoxic agent that can be conjugated to the hydrolyzed peptide [35; 36].

Prostate-Specific Membrane Antigen (PSMA)

PSMA is a glutamate carboxypeptidase, membrane-bound glycoprotein that is highly restricted to prostatic

epithelial cells and over-expressed in malignant human prostate tissues, especially in the hormone refractory disease (like the LNCaP cell line). The PSMA gene encodes PSMA and an alternatively spliced variant designated PSM [37]. PSMA is preferentially expressed in prostate cancer and all metastatic prostate carcinoma, while PSM is preferentially expressed in benign prostatic epithelium [38; 37]. The carboxypeptidase activity of PSMA cleaves terminal γ-linked glutamate residues from poly-γ-glutamated folates, which might be required to enable γ-glutamate transport into cells [39]. To target the prostate cell that over-expresses PSMA, a prodrug containing H-3 toxin was designed. H-3 is a toxin of 25 amino acids capable of inserting itself into lipid bilayers with a pore-forming activity. This pore formation leads to leakage of macromolecules or electrolyte imbalance of the cells, which results in cell death [40]. H-3 was modified by two glutamate residues linked to the C-terminal lysine of the toxin that inactivates the toxin. Selective activation of the prodrug by removal of the terminal glutamates by PSMA was demonstrated *in vitro* using two cell lines that overexpress or do not express PSMA: LNCaP versus PC-3 cell lines, respectively [41]. However, PSMA is also expressed in normal brain, and salivary gland tissues makes it unclear whether it can be used for targeted therapy *in vivo* [42-44].

Integrins

One of the essential steps in metastasis invasion is adherence of tumor cells to other cells or extracellular matrix proteins. Integrins are heterodimeric transmembrane receptors composed of 15 α and 8 β subunits that form 20 different αβ dimeric combinations on the cell surface. The extracellular regions of the α and β subunits are non-covalently binds to specific extracellular matrix proteins with ligand specificity determined by the particular combination of α and β subunits. Several integrins bind the canonical tripeptide sequence Arg-Gly-Asp (RGD), which prevents the cell from binding to other cells or the extracellular matrix. The interactions of prostate carcinoma with endothelium are mediated by α5β1, α3β1, and $\alpha_v\beta_3$ integrins. These interactions are sensitive to treatment with the RGD peptide [45]. The $\alpha_v\beta_3$ integrin is expressed in mature bone, where prostate cancer cells preferentially metastasize, and in highly invasive human prostate cancer PC-3 epithelial cells, but not in noninvasive LNCaP cells. Forced expression of $\alpha_v\beta_3$ in noninvasive LNCaP cells generates a cell migratory phenotype, suggesting that $\alpha_v\beta_3$ is involved in regulation of the migration of human prostate cancer cells and in the mechanisms that control metastatic spread of these cells [46]. Using a cyclic RGD peptide Chatterjee *et al.* [47] demonstrated a different pattern of expression of $\alpha_v\beta_3$, in LNCaP, and little expression in PC-3. Treatment of LNCaP but not PC-3 cells with the cyclic RGD peptide led to apoptosis due to cleavage of focal adhesion kinase which is involved in the integrin-mediated signal transduction pathway.

PHAGE DISPLAY

Prostate cell surface proteins are potential diagnostic markers and therapeutic targets. Identification of ligands for these proteins will enable investigation of receptor function in cancer progression and provide valuable diagnostic and therapeutic tools. Phage display is a powerful technique for searching for such ligands. Phage-displayed, random peptide libraries are generated by shotgun cloning of random oligonucleotide fragment into the phage genome and subsequent display of the encoded fusion protein on the surface of the phage [48]. Peptide phage display has been used to search for ligands that bind epitopes on the cell surface of LNCaP human prostate cell line [49]. A selected phage with biologically active ligand was able to alter the function of LNCaP and two more aggressive cell lines derived from LNCaP. Binding of the ligand to the putative receptor on the cell surface induced metastasis-associated function in the targeted cells growing in culture. Further identification of the receptor is required to elucidate the metastatic transformation of prostate cancer.

Phage display was also used to generate the optimal cleavage sites of PSA [50; 51], but it remains to be seen whether the technique can also be used to block the invasiveness and metastatic potential suggested for PSA.

GENE THERAPY

Gene therapy has been one of the most exciting and elusive areas of cancer therapy research. The concept of gene therapy is relatively simple – the efficient delivery of transgenes to correct a gene defect. Numerous techniques to enhance antitumor activity were developed over the last decade, including gene therapy targeted at tumor suppressor genes, anti-apoptotic genes, suicide genes, antiangiogenesis, and protocols aimed at strengthening the immune response. This relatively new and promising field experienced a major setback in 1999 with the death of an 18-year-old man who participated in a gene therapy trial after injection of an adenoviral vector to correct his ornithine transcarbamylase deficiency. This led to improvements in the delivery vectors, and enhanced safety and scrutiny of trial design involving regulating the timing and level of expression of transgenes. The ideal gene delivery vector is nontoxic to the patient, efficiently delivers the transgene into the cells, presents specifically to the cell of interest, and is nonimmunogenic and nonmutagenic. Unfortunately, a vector that meets all these criteria does not exist, leaving viral vectors the most common vehicle for gene transfer.

Several prototypes of gene therapy protocols have been investigated in clinical and preclinical studies for the treatment of cancer. These therapies include replacement of inactivated or defective tumor suppressor genes to restore normal growth control pathways, transfer or insertion of genes to stimulate the immune system, and delivery of genes that cause the activation of a prodrug that has selective cytotoxicity and destroys malignant cells. This review screens some recent developments in the field, which are detailed in a number of extensive reviews of gene therapy in prostate cancer [52-54].

Replication-deficient human adenovirus (Ad) serotypes 2 and 5 are the most common viral vectors used in prostate cancer gene therapy, with demonstrated efficient

transduction. These vectors offer several advantages: they can be produced in very high titers that allow efficient direct gene transfer, they have the capacity to infect both dividing and nondividing cells, and their DNA is not integrated into the host cells' chromosome, thereby eliminating long-term mutations effects. The major disadvantages of this vector are lack of target cell specificity, the transient expression of its DNA insert, and the induction of immune response to viral proteins and viral infected cells that may substantially inhibit the effect of repeated treatment with Adenovirus vectors [55].

Gene-directed enzyme prodrug therapy (GDEPT), also known as suicide gene therapy, is a two-step treatment: 1) a foreign enzyme is administered and directed to the tumor where it may be expressed by tissue-specific activation that depends on a tumor-restrictive promoter; 2) prodrugs are administered and activated by the foreign enzyme expressed specifically at the tumor. The common strategy of suicide gene therapy is to use a genetically engineered adenovirus for the transfer into the prostate of a recombinant thymidine kinase gene from the common herpes virus. This is followed by systemic administration of the prodrug ganciclovir, an antiviral drug. The recombinant thymidine kinase enzyme, which is not produced by normal human cells, phosphorylates ganciclovir during S phase, which is then incorporated into the DNA of dividing cells, causing cell death (Fig. **3**). This therapy was highly effective against mouse and human prostate cancer cells *in vitro*, as well as in a mouse model of metastatic prostate cancer [56; 57]. Scardino *et al*. was the first to demonstrate anticancer activity of this suicide gene therapy in patients with local recurrence of prostate cancer within the prostate. When they introduced the transgene vector into the prostate, 3 out of 18 patients treated in preclinical phase I had a 50% or more fall in serum PSA levels that was sustained for 6 weeks to 1 year [58]. Unfortunately, these suicide gene therapy systems are limited because the transgene expression is transient, and requires the S phase of the cell cycle for activity – a phase fraction that never exceeds 3-5% of cells in human prostate cancer. Nevertheless, repeated cycles of injection of this genetically engineered adenovirus into the prostate is safe [59], although a repeated cycle of gene therapy results in significant increases in serum PSA concentration [60].

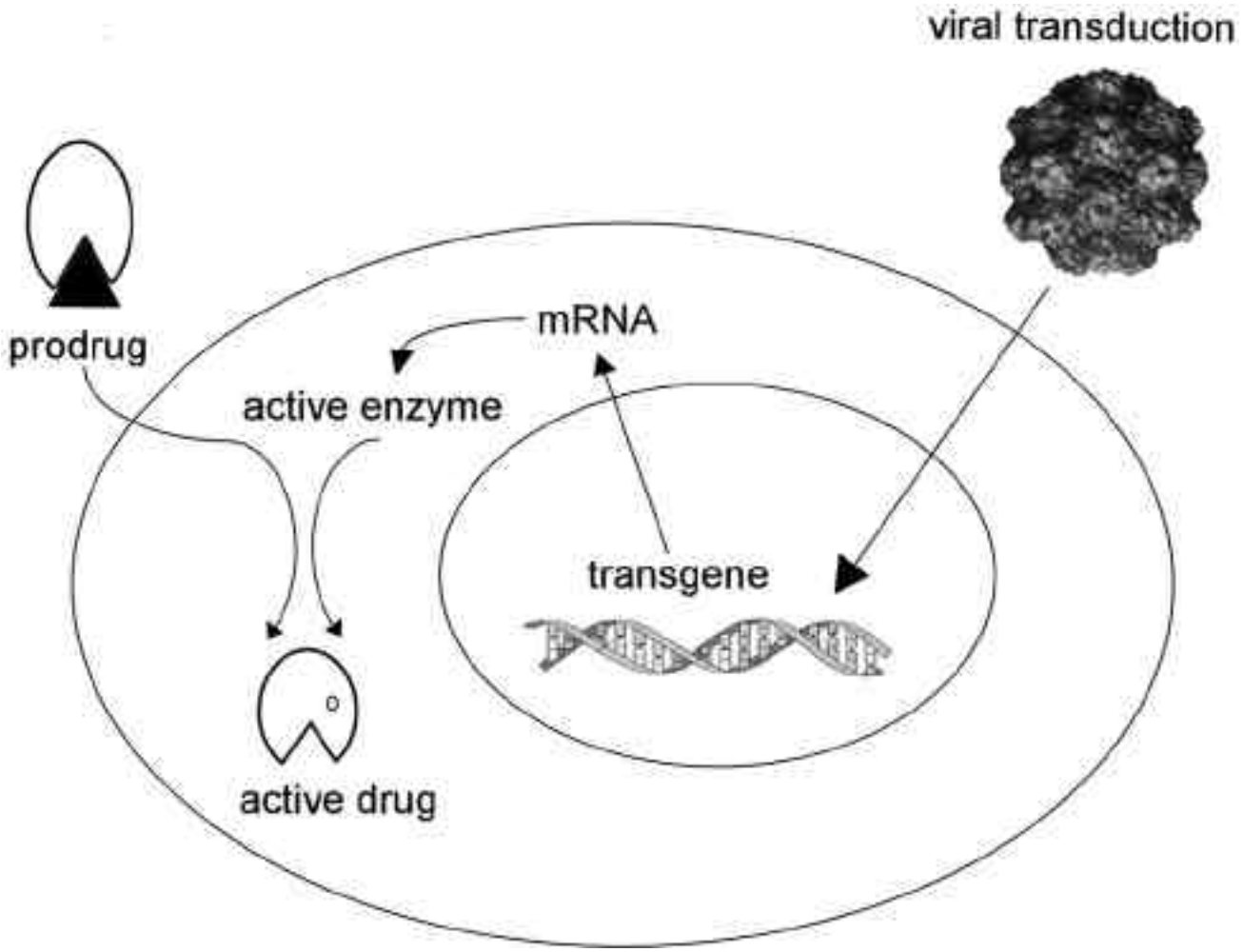

Fig. (3). General outline of the approach for GDEPT cancer therapy.

Most transgenes are driven by relatively strong viral promoters that achieve a constitutive, high level of transcription. But, tissue-specific promoters are used to limit expression of potential cytotoxic transgenes to the tissue of interest, for targeted approaches, and to enhance safety and specificity. In applications where the tissue-specific promoters are weak a chimeric promoters are constructed that retain a high transcription level of the viral promoters as well as tissue specificity of the tissue-specific promoters. Based on the molecular understanding of potential regulatory differences between normal and tumor cells, Henderson *et al*. demonstrated the efficacy of conditional replication-competent adenovirus with viral replication driven by a prostate tissue-specific promoter [61]. Subsequently, Chung *et al*. [62] developed a tissue-specific activation of a transgene that depends on tumor-restrictive promoter. The idea behind this system was the reciprocal cellular interaction between prostate metastasis cancer and bone stromal cells that leads to permanent phenotypic and genotypic alterations of the cells. Osteocalcin (OC), a noncollagenous Gla protein, was found to be produced exclusively by differentiated osteoblasts and deposited onto bone matrices at the time of bone mineralization [63; 64]. Investigators used a tissue-specific and tumor-restrictive OC promoter to drive the replication of adenovirus for the treatment of prostate cancer metastasis in an experimental, human prostate cancer, skeletal xenograft model (reviewed in [65]). Using mouse OC promoter to drive viral replication through the regulation of E1a, an adenoviral early gene required for viral replication, the researchers showed inhibition of human prostate tumor previously established in the skeleton; the inhibition was irrespective of the tumor PSA and androgen receptor status. Thus, placing these transgenes under the transcriptional control of tissue-specific promoters enhances safety, avoids the immune response, and reduces toxicity to normal tissues.

Several additional chimeric vectors using regulatory elements of prostate-specific expression genes were generated to retain a high degree of tissue discriminatory gene therapy. *In vivo* apoptosis of LNCaP in a xenograft tumor was demonstrated by activation of the artificial death switch of inducible caspase-9. The prostate-specific targeting of this system was generated by composite chimeric promoters/enhancers containing the androgen receptor-bonding site and rat probasin promoter element on adenovirus vector (ARR2PB; [66]). A similar chimeric ARR2PB vector was constructed to direct expression of Bax gene [67]. Bax is a pro-apoptotic protein that forms a heterodimer with the anti-apoptotic Bcl-2. In prostate cancer, the cells are protected from death by over-expression of Bcl-2, where much of the Bax is presenting Bcl-2/Bax heterodimer. Apoptosis was induced in LNCaP cells *in vitro* when infected with the Bax expression vector [67]. Wu *et al*. [68] generated chimeric vectors containing promoter elements of PSA gene that retained tissue specificity to target the expression of these vectors in xenograft models. Uchida *et al*. [69] used suicide gene therapy in an *in vitro* model to demonstrate the utility of the PSMA promoter/enhancer in prostate gene therapy. This might be the target of choice for patients who undergo androgen ablation, but the low level of expression of PSMA in normal brain and salivary gland tissues make it unclear whether it can be used for targeted gene therapy [42; 43; 70]. Molecular and biochemical studies of prostate cancer have identified restrictive expression of several genes in prostate tissues. Elements of the promoter/enhancers of these genes may prove useful in selective target prostate cancer for gene therapy.

ANTISENSE TECHNIQUES

The use of a short stretch of nucleic acid - DNA or RNA - to disrupt the expression of disease-related genetic code has potential applications in a vast number of illnesses, including prostate cancer. Antisense technology uses various methods to interrupt the process by which disease-causing proteins are produced. The first is antisense oligonucleotides, short synthetic pieces of antisense DNA or RNA (usually modified nucleotides or backbones) that can bind to the mRNA of a specific protein and stop its translation. Another antisense approach is the use of ribozymes that catalyze RNA cleavage and inhibit the translation of RNA into protein. These and other technologies, including peptide nucleic acids (PNAs) DNA-like molecules that are potential antisense and antigene agents, are described below. Antisense therapy is considered to be a form of gene therapy because it is a modulation of gene function for therapeutic purposes, although oligonucleotides differ somewhat from standard gene therapies in that they do not give rise to proteins but only block the expression of existing genes [71; 72].

Antisense Oligonucleotides

The apoptotic pathway is highly regulated by anti-apoptotic molecules, such as members of the Bcl-2 family. Bcl-2 is a mitochondrial membrane protein that acts at various levels of the apoptotic cascade. Indeed, it appears to play a critical role in the delay or prevention of apoptosis by a variety of death-promoting signals, suggesting that it interacts with multiple components of the death-signaling pathway [73]. Bc1-2 is over-expressed during the transition from androgen-dependent to androgen-independent prostate cancer. This over-expression of Bcl-2 in prostate cancer decreases the pro-apoptotic response to irradiation, chemotherapy, and androgen withdrawal. Thus, Bcl-2 is an attractive target for prostate cancer therapy. Stable transfection of LNCaP cells with antisense Bcl-2 decreased the intracellular Bcl-2 protein by 50%, resulting in 50% growth arrest [74-76]. The therapeutic benefit of Bcl-2 down-regulation was examined by synthesizing a phosphorothioate antisense oligonucleotide complementary to the first six codons of the initiating sequence of the human Bcl-2 mRNA (Genasense or G3139). The phosphorothioate oligonucleotides contain a sulfur atom substituted for an oxygen atom at a nonbridging site at each phosphorus atom in the oligonucleotide chain, which renders the oligonucleotide nuclease resistant. But, the phosphorothioate oligonucleotides are toxic to the cell, and the binding affinities of phosphorothioates are lower than for the parent phosphodiester oligonucleotides [77]. On the other hand, the phosphorothioate substitution is fully soluble in aqueous solutions. Phosphorothioate oligonucleotides were shown to bind to a wide variety of proteins, mostly proteins that bind heparin [78-80]. This non-sequence-specific binding may

produce numerous biological effects in addition to any observed sequence-specific, antisense effects [81].

The formation of RNA-DNA duplex between the antisense oligonucleotide and the Bcl-2 mRNA resulted in RNase H-mediated cleavage of Bcl-2 mRNA, reducing Bcl-2 translation in the cell [82]. In animal models, the Bcl-2 antisense oligonucleotide partially inhibited Bcl-2 expression, delayed the transition time from the androgen-dependent to androgen-independent stage, and enhanced the effects of chemotherapy by increasing apoptosis [74; 75; 83-85]. Reduction of Bcl-2 expression in hormone-refractory prostate cancer markedly increased the antitumor efficacy of docetaxel, a semisynthetic taxane, with response rates in the range of 30% for hormone-refractory prostate cancer and 50% for PSA response in phase II preclinical trials [86]. However, a similar trial that used a combination of Genasense and mitoxantrone, a standard chemotherapy for patients with hormone-refractory prostate cancer, had little effect on refractory prostate cancer: only 2/26 had more than 50% reduction in PSA level, suggesting that the combination of Genasense with docetaxel may be a more promising regimen for synergistic activity [87]. Docetaxel is believed to have a two-fold mechanism of antineoplastic activity: inhibition of microtubular depolymerization, and attenuation of the effects of Bcl-2 and Bcl-xL gene expression [88]. However, the pathways for docetaxel-induced apoptosis appear to differ in androgen-dependent and androgen-independent prostate cancer cells [89].

The nonspecific effects of the phosphorothioate backbone, and the possibility that this antisense induces non-specific degradation of PKC-α mRNA, raise the possibility that the "chemosensitive" phenotype occurs as a direct result of PKC-α or Bcl-2 down-regulation, and that the chemosensitive phenotype is due to sequence-specific and non-specific effects [90]. However, in the advanced melanoma trial, Genasense in combination with the anti-neoplastic agent dacarbazine induced objective responses in 6 of 14 heavily pretreated patients [91], and the compound is currently in phase III clinical trials with advanced melanoma.

Progression to the androgen-independent stage results, in part, from the up-regulation of anti-apoptotic genes following androgen withdrawal. Testosterone-repressed prostate message-2 encodes the anti-apoptotic protein clusterin, which is enhanced in prostate cancer cells following androgen withdrawal therapy [92]. Antisense oligonucleotide against clusterin reduced clusterin level by 50% in prostate cancer xenograft models. It also significantly delayed androgen-independent prostate cancer, and increased the cytotoxic effects of the drug paclitaxel in the Shionogi tumor mouse model. The Shionogi tumor model regresses after castration and later recurs as androgen-resistant tumor. This is similar to the progress of the disease in humans, which progresses from androgen dependence in the first phase treated by androgen withdrawal, to androgen independence and death in most cases within a few years. The Human LNCaP cell line progresses from androgen dependence to androgen independence, and thus is an excellent *in vitro* model to follow disease progress.

Antisense cDNA

A different approach to antisense therapy uses stable transfection of antisense PAR cDNA in DU145 cells [93]. The PAR gene function is unknown but has a higher expression in tumor cells. In cell culture, stable transfection of this antisense cDNA in DU145 cells resulted in decreased cell proliferation in tissue culture, arrest of these cells in the G2/M phase, and a marked decrease in cell density. This suggests cellular function of PAR in malignant transformation.

Antisense therapy is easy to perform and relatively specific for down-regulating basal gene expression. It permits the straightforward examination of protein degradative pathways unencumbered by continuous protein replenishment. However, other modified nucleotides or backbones, except phosphorothioate, might increase specificity and reduce toxicity, especially the morpolino oligonucleotides.

Ribozyme

Ribozymes are catalytic RNA molecules capable of cleaving phosphodiester linkages without the aid of protein-based enzymes, enabling specific inhibition of gene expression by targeting mRNA for catalytic cleavage. Ribozymes bind to substrate RNA through Watson-Crick base pairing, which offers sequence-specific cleavage of transcripts. Their activity can be targeted against specific mRNAs by selection of unique sequences flanking a conserved catalytic motif. In synthetic ribozymes, specificity, stability, and cell permeability can be dramatically improved by the incorporation of chemically modified ribonucleotides. Ribozymes are unique in that they can inactivate specific gene expression, making them helpful in identifying the function of a protein or the role of a gene in a functional cascade. Moreover, ribozymes are able to discriminate between closely related, or even mutated, sequences within gene families [94].

Metallothioneins are a class of low-molecular-weight (6-7 kDa), cysteine-rich proteins that are known to modulate three fundamental processes: 1) the release of gaseous mediators such as hydroxyl radical or nitric oxide; 2) apoptosis; and 3) the binding and exchange of heavy metals such as zinc, cadmium or copper [95]. In the prostate, metallothioneins expression is highest in the peripheral zone, moderate in the transition zone, and weak in the central zone [96]. Over-expression of metallothioneins was found in a great variety of human cancers, raising the possibility that enhanced expression of metallothioneins predisposes the peripheral zone to cancer development. Expression of metallothioneins was found to correlate positively with tumor histologic grade and negatively with patient survival, suggesting that metallothioneins play a role in the oncogenesis of prostate cancer. Moreover, higher levels of metallothioneins expression in cancer cells confer radiation and chemotherapy resistance (reviewed in [97]), and antisense-targeted or metallothionein expression in various cancer cell lines leads to cell cycle arrest or cell death [98]. Ribozyme-targeting of metallothioneins-II_a expression in the prostate cancer cell line PC-3 lowered cellular metallothioneins-II_a mRNA levels, induced massive cell

death via apoptosis, and down-regulated Bcl-2 and c-*myc* expression. These findings suggest that metallothioneins are required for cell survival probably as anti-apoptotic factors [99].

The RAD51 protein is a major component of homologous recombinational repair at the S/G_2 phase of the cell cycle; levels of RAD51 protein are elevated during cell cycle progression to a maximum at G_2 (reviewed in [100; 101]). Transfection of LNCaP cell line with ribozyme directed against RAD51 mRNA resulted in significant down-regulation of RAD51 to 20–50% of original levels. The survival of these cells, i.e. their sensitivity at low doses of radiation, was shown to be correlated with the amount of RAD51 within the cells [102]. This observation targeted RAD51 for ribozyme therapy in tumor radiosensitisation.

Peptide Nucleic Acids

Peptide nucleic acids, or PNAs, are oligonucleotide analogs whose phosphodiester backbone is replaced with a polyamide structure. PNAs represent nucleic acid analogues with unique biochemical properties that make them of great interest to the developers of therapeutic agents. PNA analogues have been synthesized in an attempt to improve biological activities, stability, and efficiency of delivery to target cells. PNAs hybridize to DNA and RNA with high efficiency, forming highly stable duplexes; those containing a high pyrimidine:purine ratio are able to form triple helices (reviewed in [103]). However, PNAs are restricted in their ability to penetrate the nucleus. To overcome this drawback, Boffa *et al*. [104] conjugated the biologically active form of testosterone to a PNA vector to target a unique sequence in the second exon of c-*myc* gene in prostatic carcinoma cells. The presence or absence of the androgen receptor in the LNCaP and DU145 cell lines was used to demonstrate the specific uptake of the conjugate into the nucleus only in the androgen receptor express LNCaP cell line. This finding indicates that testosterone can be a cell-specific target in anti-gene therapy: it can facilitate the uptake of PNA into the nucleus of prostate cancer cells that express androgen receptor, thereby regulating their growth and proliferation.

CONCLUSIONS

We have reviewed the recent advanced drug-targeted strategies for prostate cancer: peptidase, phage display, gene therapy, and antisense techniques. The key genes and pathways involved in the molecular and biochemical mechanisms contributing to prostate cancer growth, resistance, and metastatic spread are just beginning to be identified. Further studies will reveal additional pathways for targeted therapy for prostate cancer, pointing the way to new treatment modalities with the potential to increase therapeutic efficacy.

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