Gene Ther Mol Biol Vol 2, 41-58. August 7, 1998.

Exploiting stromal-epithelial interaction for model development and new strategies of gene therapy for prostate cancer and osteosarcoma metastases (review)

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Received 15 May 1998; accepted 20 May 1998.

Summary

Results of toxic gene therapy for the treatment of localized and disseminated prostate cancers showed that: (i) Ad-OC-TK expressed high levels of TK in both androgen-dependent and androgen-independent human prostate cancer cell lines; (ii) in parallel with the expression of Ad-OC-TK in tumor cell lines, the efficacy of Ad-OC-TK toxic gene therapy in target cells is directly correlated with the levels of TK expression in vitro; (iii) in two experimental models of human prostate cancer, C4-2 and PC-3, we demonstrated that Ad-OC-TK, when applied together with ACV, induced tumoricidal effects in vivo. Significant histomorphologic improvement of human prostate cancer growth in the bone was supported by bone scans in vivo. In the C4-2 model, we obtained evidence that Ad-OC-TK plus ACV diminished serum PSA, which is confirmed by the improvement of histomorphologic appearance of this tumor in the skeleton. Finally, we have focused our effort in the development of combined adenovirus and chemotherapy (i.e. chemogene therapy), the development of a concept of loco-regional delivery of therapeutic genes and drugs, and the exploration of using the homing mechanism to treat prostate cancer skeletal metastasis in vivo. Taking advantage of the reciprocal cellular interaction between prostate cancer and bone stroma, we have developed two novel gene therapy approaches to target prostate cancer growth in the bone. We have achieved for the first time the use of Ad-OC-TK/ACV as a novel therapeutic agent that can selectively target and induce the killing of both prostate and osteoblast lineage cells.

I. Introduction

Molecular therapeutic strategies such as gene therapy are being used with increasing frequency. The exponential expansion of knowledge in the field of molecular medicine has led to therapy that is based on understanding the molecular events underlying a disease process. Currently, molecular based gene therapy protocols are used predominately for life-threatening diseases like cystic fibrosis (Boucher et al., 1994; Crystal R.G., 1994), ADA (Blaese R.M., 1995), and cancer (Sanda et al., 1994). Such approaches will rapidly expand into other areas of medicine in the near future. To understand the uses of gene therapy for the treatment of both localized and metastatic prostate cancer and osteosarcoma, our laboratory focused on the development

of animal models that mimic human prostate cancer progression and osteosarcoma dissemination and explored new therapeutic approaches, particularly gene therapy, for the treatment of both localized and disseminated diseases. In the development of animal models for prostate cancer metastasis, we observed intense reciprocal cellular interaction between prostate cancer cells and bone stroma. We demonstrated that in an in vivo castrated condition, bone stroma cells "select" or "induce" the androgen-dependent human prostate cancer cell line LNCaP to acquire androgen-independent phenotypes and exhibit metastatic potential. These metastatic human prostate cancer models were used to evaluate the therapeutic actions of gene therapy under various growth conditions.

In this review, we will discuss the concepts and the models developed in our laboratory to study the molecular mechanism underlying human prostate cancer progression and metastasis. To understand the molecular basis of bone stromal cell targeting, we also established an osteosarcoma metastatic model in a rodent inoculated with either human or rat osteosarcoma cells. The models will be used as targets for in vivo gene therapy by delivering therapeutic toxic genes using a tissue-specific promoter (Ko et al., 1996). The ability to combine adenoviral gene therapy and chemotherapy, such as the development of chemogene therapy strategy (Cheon et al., 1997) and the systemic delivery of adenoviruses for the treatment of prostate cancer skeletal metastases and osteosarcoma pulmonary metastases will be discussed (Shirakawa et al., 1998). Finally, we will discuss the use and development of an ex-vivo gene therapy that utilizes stably transduced bone stromal cells to deliver the toxic genes and their by-products to the site of prostate cancer skeletal metastasis (Gardner et al., 1998).

II. Prostate cancer growth and metastasis: model development

A. Introduction

According to 1997 Cancer Statistics, it is predicted that prostate cancer diagnosis will alter the daily life of a man every 3 minutes, and end the life of another man every twelve minutes (Parker et al., 1997). The majority of the morbidity and mortality from this disease is caused by androgen-independent progression, in which tumors develop a metastatic phenotype after an unpredictable period of androgen ablation. The most common anatomical site for these metastases is bone (Franks, 1956). Numerous therapeutic options are under investigation and progress has been made, but none have demonstrated significant advantages in improving patient survival. New models to study novel therapeutic approaches toward the treatment of metastatic disease are needed.

Cancer progression is a multi-step process involving initiation, promotion, and progression, which often are difficult to study directly in human patients and their tissues. For this reason, most of the literature describing human cancer development often cite examples from epidemiological surveys or accidental exposure of human populations to drugs (e.g. diethylstilbestrol and thalidomide), radiation (e.g. the Hiroshima radiation exposure), and carcinogens (e.g. aryl hydrocarbons). Fortunately, significant conservation of genes and responses to drugs, chemicals, and hormones allows a close monitoring of cancer progression in animal models which facilitated research and achieved significant levels in understanding the molecular basis of these processes. The benefit of understanding the multi-steps of carcinogenesis is apparent. For example, in prostate cancer, an individual's prostate may undergo initiation of carcinogenesis in his 40's, but the actual progression and manifestation of clinical diseases will only become apparent in his mid-70's. This significant lag time could be a great opportunity for therapeutic intervention, which could significantly reduce the mortality and morbidity of patients who are predisposed to prostate cancer development. Moreover, understanding the molecular steps of cancer progression could also allow us to assess more accurately the natural history of the disease and hence improve strategies for treating and preventing the disease processes.

To accomplish this goal, we have established several cellular models of human prostate cancer and osteosarcoma in order to understand the molecular and cellular events associated with disease progression and to evaluate the efficacies of drug and gene therapy for the treatment of prostate cancer metastasis. Below is a description of three separate models: (**i**) The growth of osteosarcoma, a bone tumor that shares many molecular similarities with prostate osseous metastasis. Reciprocal interactions between prostate cancer and bone stroma and the critical supporting role of the bone stroma cells for prostate cancer growth demand a better understanding of the biology of bone tumors. (**ii**) The LNCaP progression model, which demonstrates that bone stromal cells support and facilitate androgen-independent progression of human prostate cancer cells in castrated hosts. (**iii**) A subcutaneous prostate cancer osteoblastic growth model in which a pluripotent and cloned bone stromal cell line, when co-inoculated with an androgen-independent human prostate cancer cell line, C4-2, formed osteoblastic prostate tumors subcutaneously.

B. Osteosarcoma model simulates aberrant osteoblastic growth.

The terminal form of prostate cancer involves the development of an androgen-independent metastatic disease. The morbidity and mortality are derived mostly from the osseous metastases that occur at the end stage of prostate cancer (Franks, 1956). Unlike the majority of bone metastases from other predominantly osteolytic tumors, prostate cancer bone metastases are osteoblastic (Scher and Chung, 1994). Prostate cancer promotes bone deposition and growth where many other cancers promote bone resorption and destruction. Interaction between prostate cancer cells and bone stroma appears to be reciprocal (Chung and Cunha, 1983; Cunha and Chung, 1981; Djakiew et al., 1966), maintaining a symbiotic relationship. To destroy this reciprocal interaction, we focused on the possibility of establishing a condition where individual components of prostate cancer metastasis, i.e. the osteoblastic lesion and prostate epithelium, can be studied separately. With respect to the osteosarcoma model, we used a rat osteosarcoma 17-2.8 (ROS) cell line and a human osteosarcoma (MG-63) cell line as the starting material for subcutaneous inoculation, which consistently formed osteosarcoma in vivo. These cell lines can also be readily grown in vitro to study the molecular interaction between the transduced therapeutic genes and osteosarcoma growth. ROS cells, when injected intravenously in syngeneic animals, formed pulmonary metastases. These characteristics of the ROS cells have been used as a model to study the effect of gene therapy alone and a combination of gene and chemotherapy for the treatment of osteosarcoma pulmonary metastasis (Cheon et al., 1997).

C. LNCaP progression model mimics human androgen-independent prostate cancer progression

Dr. Huggins (Huggins and Hodges, 1941) first demonstrated therapeutic intervention in localized and metastatic prostate cancer by manipulating the host hormonal status through surgical castration and/or administration of an androgen antagonist, diethylstilbestrol. The original discovery that androgen deprivation could lead to symptomatic responses in patients with prostate cancer led to a Nobel Prize for Dr. Huggins. One of the difficulties in understanding the tumor biology of prostate cancer is the insidious nature of this cancer, which grows virtually unnoticed and only shows itself symptomatically in the very late stages of the disease. To understand the molecular and cellular basis of androgen-independent progression, our laboratory has developed a mouse model of human prostate cancer progression. In this model, we observed that a marginally tumorigenic LNCaP cell line, when co-inoculated with a human non-tumorigenic osteosarcoma cell line, MS, consistently formed PSA-producing tumors in vivo (Thalmann et al., 1994). Upon castration of the athymic hosts, the tumors undergo androgen-independent progression by enhanced proliferation and increased PSA production in the absence of testicular androgen (Thalmann et al., 1994). By employing bone stroma cells as inductors in “selecting” or “inducing” the parental LNCaP cells in castrated animals to acquire androgen independence and metastatic potential, we developed a LNCaP subline, C4-2, which grew in castrated hosts and metastasized to the bone in castrated male hosts. C4-2 cells are an attractive model to study prostate cancer progression for the following reasons: (**i**) C4-2 cells are capable of growing and metastasizing in castrated athymic mice. (**ii**) C4-2 cells share a cell lineage relationship with parental LNCaP cells. This cell-lineage relationship allows the detailed biochemical and molecular analysis of genotypic and phenotypic changes of cells during disease progression. (**iii**) C4-2 cells produce PSA and contain androgen receptor (AR), which allows the study of ligand-dependent and -independent regulation of gene expression in human prostate cancer cell lines both in vivo and in vitro. (**iv**) The C4-2 cell line exhibits many biochemical and molecular characteristics resembling human prostate cancer. For example, C4-2 cells, when metastasized to bone or when injected intraosseously, produce an osteoblastic response. C4-2 cells produce a protein factor, prostate-specific antigen (PSA)-stimulating autocrine factor (PSAF), which induced human prostate cancer cells to synthesize and secrete PSA (Hsieh et al., 1993). The biological activity of this factor was found to be present in human bone marrow aspirate obtained from men with androgen-independent disease. This attractive model allows us to examine the molecular events that regulate prostate growth and gene expression and to design and test various forms of therapeutic modalities in a pre-clinical model of human prostate cancer metastasis.

One drawback of this in vivo model of prostate carcinogenesis is that the latent period between tumor cell inoculation and the actual development of solid skeletal tumor nodules (e.g. orthotopic or subcutaneous administration of C4-2 cells in athymic mice will take a mean of 6.8 months prior to the observation of tumor metastasis to the skeleton (Thalmann et al., 1994). Recently, we demonstrated that intraosseous administration of tumor cells (C4-2) to athymic mice form reproducibly PSA-secreting tumors in vivo. The parental LNCaP cells injected similarly failed to form tumors in vivo (Wu, 1998). Histomorphologic observation reveals that the tumors formed in the bone appear to be osteoblastic and stain positively by PSA antibody. Using this intraosseous injection of tumor cells in vivo as a model, we found no correlation between

serum PSA and circulating prostate cancer cells, as detected by RT-PCR of PSA mRNA (Wu, 1998). This model established for the first time rapid prostate cancer growth in the bone with a confirmed osteoblastic reaction, which can be used to study the pharmacokinetic relationship with circulating cells in the blood, as well as to study prostate cancer gene therapy.

D. Subcutaneous osseous prostate cancer growth model

Stromal epithelial interactions are vital to the development of the prostate gland and the maintenance of homeostasis in the growth and gene expression of the prostate gland (Chung et al., 1993). To study this interaction, we developed a cellular model of human prostate cancer growth and its androgen-independent progression (see above). While this model provides an opportunity to study prostate cancer-bone stromal interaction, the elicited osteoblastic responses in bone sometimes are difficult to discern. Recently, we (Gardner et al., 1998) showed that a mouse pluripotent bone stromal cell line, D1 (Cui Q., 1997; Diduch D.R., 1993), when co-inoculated with an androgen-independent human prostate epithelial cell line C4-2 formed a radio-opaque osteoblastic tumor subcutaneously in athymic mice **(Figure 1)**. This exciting observation established for the first time an osteoblastic growth of human prostate cancer as subcutaneous deposits. This model provides a mechanism to study molecular events governing the development and maintenance of prostate cancer osseous metastasis. This model was used to study “bystander” cell-kill using drug and/or gene therapy (see below). This model is the first instance of establishing an osteoblastic human prostate metastasis in the subcutaneous space of an animal. The benefit of this new model is that the chimeric tumor can be easily x-rayed, and the response of the tumor to various therapies can be monitored accurately and conveniently. The chimeric nature of the mouse bone stromal cell and the human prostate cancer cell allows us to evaluate the types of autocrine/paracrine growth factors and extracellular matrices derived from each cellular compartment and their actions in conferring growth and differentiation signals to the prostate gland.

Figure 1. Radiographic and histological appearance of subcutaneous osseous metastasis human prostate cancer model. Co-inoculation of a mouse bone stromal cell (D1) and androgen-independent human prostate cancer (C4-2) revealed osteoblastic lesion demonstrated by x-ray and histology.

E. Summary

The development of in vitro and in vivo models for studying human disease is vital to understanding the molecular mechanisms leading to disease. These model systems can allow the testing of hypotheses, but clinical trials remain the gold standard for the efficacy of a therapy. The elucidation of the molecular mechanism underlying each of the disease states (e.g. prostate cancer, osteosarcoma) will allow the expedient development of novel molecularly-based therapies which can be tested and modified subsequently at the stages of pre-clinical trials. Model systems will facilitate the more rapid development of experimental therapeutics which ultimately will be applied clinically with the potential of curing prostate cancer and its distant spread.

III. Gene therapy approaches to cancer

A. Introduction

The term 'Gene Therapy' in its simplest definition refers to the therapeutic application of genetic materials. Two prototype gene therapy protocols have been chosen for clinical or pre-clinical evaluation for the treatment of cancer. The first strategy is corrective gene therapy. This involves either replacement of defective genes or inactivation of activated genes in neoplastic cells to restore normal growth control pathways (Boulikas, 1997; Gotoh et al., 1997; Ko et al., 1996). The second strategy is ablative or cytoreductive gene therapy, which is based on the targeted destruction of malignant cells (Bonnekoh et al., 1995; Cheon et al., 1997; Ko et al., 1996; Shirakawa et al., 1998; Tanaka T., 1996; Trinch et al., 1995). Ablative or cytoreductive gene therapy involves the delivery of gene(s) to target cells that catalyses cell-kill or arrest of cell cycle progression through metabolic activation of prodrugs or direct interference with cell survival (Cheon et al., 1997; Gotoh et al., 1997; Ko et al., 1996).

The key components of a gene therapy approach include, but are not limited to: (**i**) the selection of genetic materials which are comprised of therapeutic genes; (**ii**) the appropriate tissue-specific or universal promoters, where in some instances the promoters may be inducible by a heavy metal, a hormone, or an antibiotic; (**iii**) the appropriately selected vectors, such as retrovirus (e.g. Moloney leukemia virus and lente virus), adenovirus, adeno-associated virus, liposomes and/or naked DNA; and (**iv**) the appropriate route of delivery, such as aerosol, intralesional injection, loco-regional perfusion, or systemic administration. Our laboratory has focused on an adenoviral system to deliver therapeutic genes for the treatment of prostate cancer in pre-clinical models. The model systems described above have allowed us to develop and evaluate new therapeutic approaches for the treatment in particular of androgen-independent prostate cancer. Below is a summary of our use of a tissue-specific promoter-directed expression of therapeutic gene, applied alone or in combination with chemotherapy, for the treatment of both localized and disseminated prostate cancer and osteosarcoma in experimental models.

B. Rationale of adenoviral approach for cancer gene therapy

The adenovirus has many attractive features for the treatment of cancer, such as its aptitude for infecting a wide range of cell types irrespective of their status of cell cycle progression. Its high infectivity of epithelial cells made this form of virus particularly attractive for the treatment of cancer. The adenovirus is well suited for ablative gene therapy because of the following: (**i**) the adenoviral genome is well known and is capable of incorporating large foreign genes into the vector; (**ii**) adenovirus is not incorporated into the host genome, and thus functions as an episome with much reduced host genome toxicity; and (**iii**) adenovirus is highly infectious to both dividing and non-dividing cells. Currently, the DNA size limitation of the E1-deleted adenovirus is approximately 7.5 kb (Graham and Prevec, 1991), but as more complete deletion vectors are constructed (e.g. "gutless" version of adenovirus), the DNA size that could be accommodated into adenovirus could be enhanced up to 38 kb.

The adenoviral vector also has limitations. The adenoviral proteins can cause a host immune response.

This can be beneficial to cancer gene therapy, causing a vaccine-like immune response to the tumor, but this immunity also limits the ability for the adenovirus to exert itself over a long period because of the mounting host immune rejection of these foreign adenoviral proteins. The development of neutralizing antibodies can block an initial host response and improve the therapeutic efficacy of this treatment. Current attempts to delete most of the adenoviral genome from the adenovirus will overcome this problem in part. Due to its transient nature of expression in cells as an episome, adenovirus is not the appropriate choice for long-term applications, such as the use of gene therapy for corrective purposes.

C. Vector designs and modes of action of toxic genes

The adenovirus subtype 5 has been modified with an E1 deletion (Graham and Prevec, 1991) to allow the insertion of the desired expression cassette. The expression cassette contains a fixed region that allows a homologous recombination event to occur in 293 cells and a variable region used to insert a desired DNA sequence. Because this recombination event has deleted the E1a region of the adenoviral genome, the recombinant adenovirus becomes replication-defective. There are two parts of the expression cassette virus that can be engineered: First, the *promoters* that regulate the transcription of downstream genes. Second, the *therapeutic gene(s)* of interest which are regulated by the promoters. In the first category we have employed prostatic-specific antigen (PSA) and osteocalcin (OC) promoters as tissue-restrictive promoters for the delivery and expression of therapeutic genes in target prostatic cancer cells. Because of the highly specific expression of PSA and OC proteins by prostate cancer cells, the promoters of these genes are suitable candidates for the delivery and expression of therapeutic genes in prostate cancer cells. Our studies showed that: (**i**) PSA (Gotoh et al., 1998) or OC (Cheon et al., 1997; Ko et al., 1996; Shirakawa et al., 1998) promoter-mediated expression of therapeutic genes are equivalent to those mediated by universal promoters, such as CMV and RSV, except that the expression of genes are highly regulated in a tissue and tumor-restricted manner. (**ii**) Delivery of toxic genes such as thymidine kinase (TK) and cytosine deaminase (CD) genes that are capable of exerting "bystander" effects against the tumor cells have achieved significant direct as well as "bystander" cell-kill.

The molecular mechanisms of TK and CD are as follows. Upon TK expression, this form of enzyme will be able to convert a prodrug, ganciclovir (GCV) or acyclovir (ACV), into a biologically active drug, phosphorylated GCV, which is incorporated into an elongated DNA strand, and interrupts DNA synthesis and causes early chain termination, initiating an apoptotic process. Similarly, CD gene can convert 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), which can be incorporated into cellular RNA, and interrupts RNA synthesis in both dividing and non-dividing cells. 5-FU is also considered an inhibitor for TK, thus also interrupting cellular DNA synthesis. Both TK and CD genes are known to exert bystander effects on their neighboring cells. It is proposed that the phosphorylated form of GCV or acyclovir (ACV) can be transported to neighboring cells through gap junctions and interrupt DNA synthesis in neighboring cells. Unlike TK, the CD gene converts 5-FC into 5-FU, which is readily diffusible and can serve as a direct toxin to neighboring cells without the necessity of gap junctional transfer. Both of these strategies have been demonstrated as efficacious in causing regression of a number of solid tumors, including metastatic colon carcinoma to the liver, gastric carcinoma, and malignant mesothelioma.

D. Viral production and delivery.

Recombinant adenovirus containing the selected expression cassette (PSA-TK, OC-TK, CMV-TK, etc.) is produced by co-transfecting a shuttle vector containing the expression cassette (e.g. pΔE1sp1B-PSA-P-TK) and recombinant adenoviral vector (pBHG-11) plasmids in a human fetal kidney 293 cell line, as described (Graham and Prevec, 1995). Recombinant adenovirus was cloned from individual plaques, amplified, and purified by the CsCl centrifugation method. The virus stock was then dialyzed, concentrated, and titered. The plaque-forming unit (PFU) of the viruses was measured by a standard biologic plaque forming assay (Graham and Prevec, 1991).

A number of methods of viral delivery can be implemented. For example, adenovirus can be injected intralesionally (Cheon et al., 1997; Ko et al., 1996), loco-regionally by perfusion (Kao et al., 1998), and intravenously (Shirakawa et al., 1998). In our laboratory, we have successfully delivered and expressed adenovirus by all of the above routes in pre-clinical models of cancer growth and metastasis. Some of these results will be illustrated below.

IV. Utilizing a tissue specific promoter to target the growth of prostate cancer and osteosarcoma

A. Introduction

Several groups, including ours, have constructed vectors containing tissue-specific promoters to restrict the expression of transduced cytotoxic genes to the

tissue of interest (Gotoh et al., 1998; Ko et al., 1996; Macri and Gordon, 1994; Shimizu, 1994; Shirakawa et al., 1998; Vile and Hart, 1993). Several studies have described the use of retroviral vectors mediated by tyrosinase promoter for the treatment of melanoma (Vile and Hart, 1993) , albumin promoter for the treatment of hepatoma (Kuriyama et al., 1991; Macri and Gordon, 1994) , myelin basic protein promoter for the treatment of brain tumors (Shimizu, 1994), short PSA promoter for the treatment of prostate cancers (Ko et al., 1996; Pang et al., 1995), and carcinoembryonic antigen (CEA) promoter for gastric carcinoma cells (Tanaka T., 1996) . Our laboratory has developed several adenoviral gene therapy protocols for the treatment of prostate and bone tumors in vivo. Recently, we observed that both prostate cancer cells and their supporting bone stroma expressed a non-collagenase bone matrix protein, osteocalcin (OC) (Ou et al., 1998). Osteocalcin is commonly associated with the turnover of bone cells (McKee et al., 1993; Price, 1985), and is a marker for ossification, which is commonly associated with prostate cancer and its metastases (Arai et al., 1992; Beresford et al., 1984; Shih et al., 1990; Tarle et al., 1989). For these reasons, an ablative gene therapy using the OC promoter to deliver herpes simplex virus-thymidine kinase (TK) (Ishii-Moirta H., 1997) was developed for the treatment of prostate cancer osseous metastasis. In the presence of TK, acyclovir (ACV) or ganciclovir (GCV) will be converted to a toxic guanine analogue capable of disrupting DNA synthesis as described in the preceding section.

There are compelling reasons to believe that prostate cancer-bone stromal interaction occurs in vivo, and such communication may contribute to local prostate cancer growth and its distant metastasis (Arai et al., 1992; Curatolo et al., 1992; Ekman and Lewenhaupt, 1991; Shih et al., 1990; Tarle et al., 1989) and associated osteoblastic reactions. Thus, the rationale of this approach is to devise a promoter that will be expressed by both prostate cancer and bone stroma, and use a therapeutic gene that has well-documented bystander effects (Gagandeep S., 1996; Ishii-Moirta H., 1997). By using Ad-OC-TK construct a highly infectious adenovirus, we hope to achieve maximal cell-kill by interrupting cellular communication between the prostate cancer and the bone stroma, and by the direct cytotoxicity exhibited by this version of gene therapy.

Both osteosarcoma and androgen-independent prostate cancers remain major challenges for the orthopedists, urologists and medical oncologists involved in the care of these patients. These seemingly unrelated diseases, however, came together through a molecular analysis of the gene(s) that may be over-expressed in these two forms of cancer during disease progression. Osteocalcin, a noncollagenous Gla protein, was thought to be produced specifically in osteoblasts. OC is synthesized, secreted and deposited at the time of bone mineralization (Price, 1985).

Interestingly, OC expression was upregulated in several forms of solid tumors, including osteosarcoma and both androgen-dependent and androgen-independent human prostate cancer cell lines. Furthermore, OC expression and secretion were higher in men with metastatic prostate cancer (Coleman et al., 1988; Curatolo et al., 1992), and serum levels of OC were even higher in prostate cancer patients subjected to hormonal deprivation, suggesting that OC may be negatively regulated by testicular androgen (Tarle et al., 1989).

B. Osteocalcin promoter-based tissue-specific gene therapy (Ad-OC-TK) for osteosarcoma

1. Molecular rationale

Osteocalcin is a molecular marker present in the serum of patients suffering from either osteosarcoma or prostate cancer. A recent study showed that immunohistochemical staining of osteocalcin was positive in primary osteoblastic osteosarcoma and chondroblastic osteosarcoma specimens, as well as in five of seven fibroblastic osteosarcomas. We have shown that strong osteocalcin staining was associated with both primary and metastatic prostate cancer (Ou et al., 1998). Since osteocalcin is the protein most commonly secreted by osteosarcoma cells of the osteoblastic lineage and also a marker of osteoblastic differentiation, we have chosen to use the osteocalcin promoter to achieve tissue-specific expression of the toxic TK gene in rat and human osteogenic sarcoma

Figure 2. Tumor-specific targeting of osteosarcoma by the osteocalcin promoter (OC). The normal cell does not have the transcriptional factors that are required to activate the osteocalcin promoter to drive thymidine kinase (TK) gene expression. Without the thymidine kinase enzyme, acyclovir (ACV) has no effect on the cell. In contrast, the osteosarcoma cell can activate the osteocalcin promoter and drive transcriptional gene expression of TK. With the thymidine kinase enzyme present, ACV leads to cell death when the cell attempts to divide.

cell lines (Cheon et al., 1997; Ko et al., 1996; Shirakawa et al., 1998) **(Figure 2).**

2. Results

Significant growth inhibition of rat osteoblastic osteosarcoma (ROS) and a human osteoblastic osteosarcoma (MG-63) occurred when infected with 20 MOIs of Ad-OC-TK and ACV (10 μg/ml). Cells either infected with Ad-OC-TK (20 MOIs) or treated with ACV (10 μg/ml) alone did not exhibit altered growth or morphologic changes during an 8-day observation period. Consistent with their low levels of TK activity, the growth of WH (human bladder cancer) and NIH-3T3 (fibroblast) cells were not affected by Ad-OC-TK infection, despite the addition of pro-drug ACV in the tissue culture medium. Intralesional injection of Ad-OC-TK followed by ACV administration led to the marked growth inhibition of both ROS and MG-63 subcutaneous xenografts in syngeneic mice. These findings are further supported by the growth attenuation of ROS pulmonary lesions by systemic Ad-OC-TK as described below.

C. Chemogene therapy for osteosarcoma: combining methotrexate with osteocalcin promoter based tissue-specific gene therapy

1. Molecular rationale

To improve the efficacy of the above gene therapy in preclinical osteosarcoma models, we explored the use of methotrexate (MTX) chemotherapy combined with gene therapy. MTX was selected for several reasons. MTX is a proven first line chemotherapeutic agent for treating osteosarcoma patients (Damron and Pritchard, 1995). MTX, as a competitive inhibitor of dihydrofolate reductase, leads to a decreased accumulation of tetrahydrofolate which then interferes with both purine and pyrimidine biosynthesis (Gorlick et al., 1996), thus diminishing the available nucleotide pool necessary for DNA synthesis in dividing cells. This, combined with the mechanism of TK and ACV, which produces a poisonous phosphorylated ACV (a purine analog known chemically as ACV-triphosphate) through enzymatic catalysis by TK causing DNA chain termination and inhibition of cell division, would allow for enhanced cell-kill. The aim of this study was to investigate the

Figure 3. Proposed mechanism of chemogene therapy. First, MTX decreases the availability of both purines and pyrimidines in all cells. MTX as a competitive inhibitor of dihydrofolate reductase leads to decreased tetrahydrofolate, which then interferes with both purine and pyrimidine biosynthesis, required for DNA synthesis in dividing cells. Second, MTX-induced diminished nucleotide pools are further contaminated by a poisonous phosphorylated ACV (a purine analog known chemically as ACV-triphosphate), which is produced through enzymatic catalysis by TK, and causes DNA chain termination and inhibition of cell division. Thus, the additive effects of chemogene therapy can be attributed to both the efficacy of MTX in decreasing the nucleotide pool size in cells and Ad-OC-TK plus ACV which produce phosphorylated ACV and causes cessation of tumor cell DNA synthesis. The lack of systemic toxicity can be explained by the TK gene expression being controlled by tissue-specific promoter, thus limiting TK expression only to tumor cells and a bystander tumor cell-kill in localized areas.

possible utility of chemogene therapy in order to combine treatment modalities by maximizing fractions of tumor cell-kill with minimized toxicities. Thus, the additive effects of chemogene therapy can be attributed to both the efficacy of MTX in decreasing the nucleotide pool size in cells and Ad-OC-TK plus ACV which produce phosphorylated ACV and causes more severe cessation of tumor cell DNA synthesis **(Figure 3).** The lack of systemic toxicity can be explained by the TK gene expression being controlled by tissue-specific promoter, thus limiting TK expression only to tumor cells and a bystander tumor cell-kill in localized areas.

2. Results

After determining the IC_{10} for MTX in both the ROS and MG-63 cell lines, we demonstrated in vitro that low dose MTX (IC_{10}) and Ad-OC-TK plus ACV have additive therapeutic effects as compared to MTX(IC_{10}) or Ad-OC-TK plus ACV treatment alone. In vivo, using a subcutaneous models of murine osteosarcoma, we demonstrated that treatment of Ad-OC-TK plus ACV in combination with low dose MTX chemotherapy inhibited osteosarcoma tumor growth more efficiently than either Ad-OC-TK plus ACV or MTX alone. These data suggest that Ad-OC-TK-induced tumor regression was more efficient and significant when

Figure 4. Treatment of human osteosarcoma (MG-63) with Ad-OC-TK/AVC and MTX chemogene therapy at 180 Days. The untreated mouse has a large flank tumor (left panel) and the treated group demonstrated a marked growth inhibition (right panel).

combined with low dose and non-toxic MTX in tumor-bearing animals during a 35 to 45-day study period. At 45 days, 100% and 80% of the animals bearing ROS subcutaneous tumors were alive after gene therapy or chemogene therapy, respectively. Conversely, no animals bearing ROS tumors after PBS or MTX therapy alone were alive at 35 days **(Figure 4).** The growth inhibition can be demonstrated six months after therapy. In another study, we demonstrated that intravenous Ad-OC-TK plus intraperitoneal ACV significantly improved pulmonary metastases of osteosarcoma (see below).

D. Systemic delivery of tissue specific gene therapy

1. Introduction

Ablative gene therapy for the treatment of cancer continues to gain prominence in preclinical research, but remains limited in clinical application because of an inability to deliver the toxic gene to the tumor cells with specificity. Many vectors (e.g. retroviruses, retroviral producing cells, adenoviruses, liposomes, and others) can deliver genes (therapeutic or ablative) to target cells. Localized delivery and restricted gene expression to the primary tumor have been accomplished via direct injection of therapeutic viruses in animal models

(Bonnekoh et al., 1995; Cheon et al., 1997; Eastham et al., 1995; Ko et al., 1996) and clinical trials (Eck et al., 1996; Treat et al., 1996) . This approach is not feasible for the treatment of metastatic disease because of the presence of multiple lesions that would each require separate injection and manipulation. Therefore, alternative approaches to the treatment of metastatic disease with gene therapy must be developed.

To study the potential therapeutic efficacy of systemic cancer gene therapy for the treatment of pulmonary metastases, osteosarcoma is an attractive model because a significant number of these patients eventually develop lung metastasis. Initially, surgical resection of the primary lesion and adjunctive chemotherapy are the mainstay of today's therapy. For the 20% that present with metastatic disease, 80 % will require additional therapy for relapse; while of the 80% that present with local disease, 35% will require additional therapy for relapse after surgery and adjunctive chemotherapy (O'Reilly, 1996) . Therefore, 44% of patients diagnosed with osteosarcoma will fail conventional first line therapy. Patients developing recurrent disease usually have a poor prognosis, dying within one year of the development of metastatic disease (Malawer et al., 1993; Naka et al., 1995; Saeter et al., 1995; Ward et al., 1994). New therapeutic approaches that can be applied either separately or in conjunction with current modalities in treating osteosarcoma pulmonary metastases are needed.

2. Mechanistic rationale

Systemic delivery of therapeutic genes is attractive for targeting metastatic disease, particularly pulmonary metastases. Because the pulmonary vascular system would be the first encountered, the adenovirus would be trapped in the lung parenchyma, allowing for higher infectivity. Experimental models using the systemic delivery of liposomal p53 (Lesoon-Wood et al., 1995) and retroviral (Vile et al., 1994) tumor specific TK have been promising. Compared to liposome or retrovirus, adenovirus has several advantages in a systemic delivery strategy, such as its high infectivity *in vivo*, further aided by the ability to achieve high viral titers through in vitro production. However, a recent report (Brand et al., 1997) demonstrated that systemic administration of adenovirus containing TK under the control of a universal promoter (CMV) supplemented with GCV treatment induced severe hepatotoxic effects.

Osteocalcin promoter (OC) has been shown above to be highly effective in directing the transcription of reporter genes in both rat and human osteosarcoma cell lines (Ducy and Karsenty, 1995; Ko et al., 1996) . Since lung epithelium contains the first capillary bed encountered by therapeutic agents given systemically, several investigators have explored the use of a venous system to deliver therapeutic genes to the lung by cationic liposomes (Lesoon-Wood et al., 1995; Philip et al., 1993; Thierry et al., 1995; Zhu et al., 1993) or retroviral vectors (Vile et al., 1994) . Since osteosarcoma metastasizes primarily to the lung, and lung vasculature is considered as the first major capillary bed that a systemically-given therapeutic agent encounters, we designed a strategy to target osteosarcoma pulmonary metastasis by the administration of Ad-OC-TK/ACV in an animal model.

3. Results

To prove the principle that a tissue specific promoter regulated gene expression could be achieved with a systemic adenoviral approach. ß-galactosidase reporter gene expression under the transcriptional control of the osteocalcin promoter is specifically expressed in osteosarcoma cells rather than the normal lung parenchyma of syngeneic animals bearing pulmonary ROS lesions. In comparison to control animals, systemically delivered Ad-OC-TK plus ACV (via an intravenous route) significantly retarded the growth of osteosarcoma pulmonary metastases and improved the survival of treated animals. While a limited number of tumor cells in the lung may be infected by Ad-OC-TK, as judged by the immunostaining of a comparable virus that mediates the expression of a reporter gene, ß-galactosidase (ß-gal) Ad-OC-ß gal, a surprisingly potent growth-inhibiting effect by Ad-OC-TK/ACV was noted in osteosarcoma lung metastases. The treated animals bearing ROS pulmonary lesions had markedly less nodules of smaller size and a statistically improved survival. This biologic effect is most likely derived from the existence of close gap junctions between osteosarcoma cells (Donohue and Miller, 1991) which allows the phosphorylated form of ACV to exert its full bystander effect.

E. Osteocalcin promoter-based tissue-specific gene therapy (Ad-OC-TK) for prostate cancer

1. Molecular rationale

Prostate cancer's propensity for the bone environment and the phenotype of osteoblastic growth suggest that it would be susceptible to an osteocalcin promoter based gene therapy. Our laboratory has confirmed the presence of osteocalcin protein by immunohistochemical staining of primary and metastatic human prostate cancer and human prostate cancer cell lines (unpublished data). This finding combined with the clinical findings of serum osteocalcin elevations in men with metastatic disease would also suggest that the

osteocalcin promoter would be active in prostate cancer **(Figure 5)**. Since prostate cancer cells have been shown to interact with surrounding stromal cells, it is reasonable to hypothesize that an osteocalcin promoter based toxic gene would exert an effect on the both the prostate cells and the osteoblastic stromal cells, thus potentiating the bystander effect.

2. Results

To assess whether Ad-OC-TK may drive the expression of the TK gene in cells of human prostate cancer, we compared the expressions of TK activities in prostate and

Figure 5. Tumor-specific targeting of prostate cancer by the osteocalcin promoter (OC). The normal cell does not have the transcriptional factors that are required to activate the osteocalcin promoter to drive thymidine kinase (TK) gene expression. Without the thymidine kinase enzyme present, acyclovir (ACV) has no effect on the cell. In contrast, the prostate cancer can activate the osteocalcin promoter and drive transcriptional gene expression of TK. With the thymidine kinase enzyme present, ACV leads to cell death when the cell attempts to divide.

non-osteoblastic, non-prostatic cell lines after exposure to 20 MOIs of Ad-OC-TK per target cell. The mean TK-mediated [^{3}H]-GCV phosphorylation per 10^6 cells was determined and was designated as the TK activity unit. LNCaP and its androgen-independent lineage-derived sublines, C4, C4-2, C4-2B, and an androgen-independent PC-3 expressed high levels of TK activity in the cell lysates. The TK activity was minimal for several other human prostate cancer cell lines, DU-145 (derived from a brain metastasis), ARCaP (derived from ascites fluid), and those of non-osteoblastic and non-prostatic origin. This directly correlated with the amount of in vitro growth inhibition demonstrated by each of these cell lines. Significant growth inhibition of androgen-independent human prostate cancer cell lines, PC-3 and C4-2, occurred when infected with 20 MOIs of Ad-OC-TK and ACV (10 µg/ml). Cells either infected with Ad-OC-TK or treated with ACV (10 mg/ml) alone did not exhibit altered growth or morphologic changes during an 8-day observation period. Consistent with their low levels of TK activity, the growth of DU-145, WH and NIH-3T3 cells

were not affected by low levels of Ad-OC-TK infection, despite the addition of pro-drug ACV in the tissue medium.

PC-3 xenografts were induced by the subcutaneous injection of athymic mice with PC-3 cells. After tumors were palpable (>4 mm^3), animals were treated with either PBS alone (n=6), ACV alone (n=6), Ad-OC-TK alone (n=8), or Ad-OC-TK plus daily ACV intraperitoneal injection (n=8). ACV markedly suppressed the growth of PC-3 tumors during a 45-day observation period following Ad-OC-TK infection; Ad-OC-TK infected PC-3 tumors or ACV treatment of tumor xenografts alone did not significantly affect the rate of tumor growth. Gross and histological findings of representative tumors for each group demonstrated a marked treatment effect. Photomicrographs at low magnification illustrate an increased degree of tumor necrosis and migration of lymphocytic cells into the tumor in the Ad-OC-TK plus ACV treatment group versus controls. One week after PC-3 cells were inoculated intraosseously into the marrow space of femurs of nude mice, Ad-OC-TK was injected directly into the femur bone marrow space and followed by 2 weeks of ACV treatment. In the group that received no treatment, X-rays showed that most of the femurs exhibited an intense osteolytic response, while Ad-OC-TK plus ACV treatment clearly inhibited PC-3-induced osteolytic responses with much improved the structure of the femurs.

Similarly, we established an intraosseous model for the growth of C4-2 cells, an androgen-independent PSA-producing human prostate cancer cell line. Intraosseous administration of C4-2 but not parental LNCaP cells to the femurs of athymic mice formed reproducible PSA-secreting prostate tumors (Wu, 1998). After intraosseous C4-2 cell inoculation, animals (5 mice/group) were randomized to control (PBS-treated), ACV treatment, Ad-OC-TK treatment, and combined Ad-OC-TK and ACV treatment. Serum PSA in mice was followed weekly. Once serum PSA was clearly measurable (> 1 ng/dl), mice were treated with intraosseous injections of adenovirus and intraperitoneal injections of ACV. Serum PSA of both control and treated animals was followed at 1, 3, and 5 weeks. The absolute values of serum PSA (mean + SEM) in animals treated with either ACV, Ad-OC-TK alone, or combined Ad-OC-TK and ACV demonstrated a rise in all groups except for the treatment group. Because of the PSA decline between week 3 and week 5, we calculated the net percentage of PSA elevation [defined as PSA(Wk 5) - PSA(Wk3) / PSA(Wk 3)] was greater in control or ACV-treated mice than Ad-OC-TK alone or the combined Ad-OC-TK and ACV. In fact, the treatment group had negative PSA elevation (-40%) and was lower than that of the Ad-OC-TK treatment alone (+25%). These results were corroborated by the histomorphologic data of the skeletal tumor specimens obtained from control and treated hosts. A characteristic C4-2 intraosseous lesion with viable C4-2 cells confirmed by PSA staining (data not shown) was demonstrated in the control group, while the histomorphologic characteristics of the treatment group demonstrated marked irregularity of the cellular morphology and tumor structure with evidence of many dying tumor cells, not seen in the control group.

F. Potential clinical applications of tissue-specific promoter-mediated gene therapy

Current Phase I trials targeting prostate cancer patients utilize a toxic gene therapy strategy that involves the universal promoter (RSV) or the prostate specific antigen promoter and TK. Both of these promoters may not be suitable for targeting androgen-independent and osseous metastatic patients because: (**i**) Direct injection of Ad-RSV-TK virus can leak out and infect neighboring normal cells and can damage these cells. Intravenous delivery of Ad-RSV-TK has caused significant mortality in mice (Brand et al., 1997). In sharp contrast, we demonstrated that systemic delivery of Ad-OC-TK plus intraperitoneal ACV led to improved survival in athymic mice with osteosarcoma pulmonary metastases (Shirakawa et al., 1998). (**ii**) PSA promoter-mediated toxic genes (e.g. Ad-PSA-TK) may be only killing prostate specific antigen secreting cancer cells but not their supporting osteoblastic cells. There are indications that PSA expression may be greatly reduced in poorly differentiated prostate tumors, a result that seems to be in sharp contrast with OC, whose expression is augmented in metastatic AI prostate cancers (unpublished observation). Therefore, Ad-OC-TK may be the superior agent for targeting end-stage of prostate cancer patients.

Rodriguez et al. (1997) reported another strategy to make adenovirus become replication competent under regulation by the short version of the PSA promoter which drives the expression of E1a protein of adenovirus. Since E1a is an essential protein for adenovirus replication, PSA promoter in theory should limit adenoviral replication only in PSA-positive cells and hence induce cytolytic activity in prostate cells only. However, Hitt and Graham reported that "wide variation in E1a expression levels has little effect on virus replication" raising the question of the applicability of this concept (Hitt and Graham, 1990). Further study in suitable animal models capable of adenoviral replication should proceed the initiation of any clinical trials using this approach. Based on the pre-clinical results above, we have proposed a phase I trial for the intralesional treatment of recurrent and metastatic prostate cancer

that is currently under FDA review. This study will assess the safety and efficacy of this approach. At the completion of our trial, we hope to have the safety information to propose future trials evaluating the efficacy of this strategy. Subsequently, once safety and efficacy have been established, we will propose a form of regional systemic administration of Ad-OC-TK targeting all forms of prostate cancer.

G. Summary

Ad-OC-TK is a tissue specific recombinant adenovirus developed to target osteoblastic cell (i.e.. osteosarcoma) and prostate cancer based on the molecular similarities of these two cancers, that is, their common expression and secretion of OC proteins. In pre-clinical model systems, Ad-OC-TK/ACV demonstrates both in vitro and in vivo tumoricidal activity. We have achieved for the first time the use of Ad-OC-TK as a novel therapeutic agent that can selectively target and induce the killing of both prostate cancer and cells of an osteoblast lineage. Ad-OC-TK/ACV may be used as the potential gene therapy agent for prostate cancer and osteosarcoma patients who have very debilitating osseous lesions associated commonly with bone pain. By the combination of low-dose MTX plus an osteocalcin promoter-based toxic gene therapy we have developed a novel therapeutic strategy for the treatment of osteosarcoma. We demonstrated that combination treatment is superior to a single drug modality in inducing cell-kill of both rat and human osteosarcoma models (ROS and MG-63). Potentially, chemogene therapy could be a better therapeutic strategy for patients with osteosarcoma as a neoadjuvant, adjuvant, or possibly primary combination therapy. Chemogene therapy could potentially be used to reduce the tumor burden and pain associated with primary metastasis and to eradicate osteosarcoma pulmonary metastasis. Chemogene therapy could ultimately improve both overall survival and the quality of life of patients suffering from osteosarcoma.

In summary, we have shown for the first time that recombinant adenovirus can be given systemically without systemic toxicity to achieve a therapeutic effect on osteosarcoma lung metastasis. Ad-OC-TK/ACV dramatically inhibited the growth of lung nodules and significantly increased the survival of animals bearing osteosarcoma pulmonary metastases. This approach will open new avenues for targeting pulmonary metastasis using tissue-specific or tumor-specific promoters to guide the expression of therapeutic genes.

For the treatment of prostate cancer, Ad-OC-TK plus ACV could have two significant effects to cause the shrinkage of prostate cancer growth in the skeleton. First, Ad-OC-TK plus ACV significantly inhibited the growth of prostate tumor cells in vitro and tumor growth subcutaneously and intraosseously in animal models. Second, Ad-OC-TK plus ACV could significantly inhibit the growth of tumor supporting bone stroma because of its osteoblastic lineage. Bone stromal cells have been proposed to be important for the adhesion of tumor cells and to maintain their survival, either through cell attachment or by providing tumor cells with soluble growth factors. In addition, bone stromal compartment potentially could secrete important humoral factors (e.g. TGF-b), which could protect the host immune response at the site of tumor growth. Ad-OC-TK plus ACV thus could exert not only a direct tumoricidal effect on prostate cancer in the osseous environment, but also block its association with tumor stroma, which presumably plays a vital role in the survival of prostate cancer in the skeleton.

V. Ex-vivo gene therapy using bone homing

A. Introduction

The importance of stromal-epithelial interaction in prostate cancer has been well established (Chung and Cunha, 1983; Cunha et al., 1987). The reciprocal molecular interactions between prostate cancer (epithelial component) and bone (stromal component) have been well documented (Chung et al., 1991, 1984). These investigations have led to the development of animal models of prostate cancer metastasis and novel therapeutic approaches that have been applied in several preclinical studies. Efforts have also been made to identify putative autocrine and paracrine factors that may be responsible for prostate cancer-bone stroma interaction, and these pathways could have important clinical implications. Based on the theory that prostate cancer cells need the nurturing environment of the bone to survive, we have designed two therapeutic strategies directly targeting the growth of both prostate cancer cells and bone stroma with a therapeutic gene that is also known to exert a bystander effect. Secondly, we have genetically engineered bone stromal cells and have observed the ability of these cells to home to the bone and to exert bystander cell-kill of tumor epithelium in vivo.

Our novel therapeutic approach relies on the natural bone homing mechanism of a genetically modified bone cell to deliver therapeutic genes to a target lesion. For example, a bone cell transduced with a toxic gene capable of elaborating a bystander effect to the neighboring cells (i.e. prostate cancer cells) can be an effective therapy for the treatment of prostate cancer bone metastasis. A pluripotent bone stromal cell, D1, was derived from the bone marrow of a mouse and

maintains its natural ability to home to the bone after both intravenous and intraosseous injections (Cui Q., 1997; Diduch D.R., 1993). This cell line has been genetically modified to express TK and ß-galactosidase. TK, upon the administration of the prodrug ACV, can convert the prodrug into its active form, killing the D1 cells and resulting in a bystander cell-kill of prostate cancer cells. Bone stromal cells tagged with the ß-galactosidase gene have been shown to home to the bone marrow space, and are widely distributed throughout the bone stroma upon direct intraosseous injection. The strategy of employing genetically engineered bone stromal cells with the potential to home back to the bone, where prostate cancer micrometastasis and lesions may occur, may present an attractive new opportunity for treating patients with bony metastases on an individualized basis.

B. In vitro demonstration of bystander cell kill

Osteoblastic cells, D1 and ROS, infected with 20 MOI Ad-CMV-TK and Ad-OC-TK were subsequently co-cultured with LNCaP and its sublines C4-2 and C4-2B. Upon acyclovir administration, D1 cells exhibited a greater bystander growth inhibition on LNCaP than its lineage-related androgen independent sublines. D1 cells transduced with TK but not antisense TK, when co-cultured with LNCaP or its sublines exhibited variable cytotoxicity of the co-cultured tumor cells on plastic dishes. D1-TK, co-cultured with LNCaP or its sublines in the three-dimensional microgravity chamber, demonstrated organoid formation which was altered in size, consistency, and morphology upon administration of ACV. In contrast, the resulting tissue from a 1:1 co-culture of D1-TK and C4-2 in an untreated chamber or in a chamber that was supplemented with ACV for 8 days revealed a larger amount of tissue mass measured by wet weight and a more compact morphology and tissue architecture as demonstrated by gross tissue/organoids isolated from the untreated chamber. The PSA production measured every 48 hours was minimal in the treated group, while observed to steadily increase in the untreated group.

C. In vivo demonstration of bystander cell kill

Using the subcutaneous co-culture of D1-TK and C4-2 cells, a series of animals bearing chimeric tumors were established as described above. The administration of ACV in the treatment group led to a decreased soft tissue component in D1-TK plus C4-2 chimera and decreased serum PSA production. A significant effect on histology was also demonstrated. The genetically engineered D1 cells maintained their ability to preferentially migrate to the bone after several retroviral transfections. At a time point beyond two weeks, after tail vein injection, there was preferential accumulation of D1-TK as demonstrated by X-Gal staining. The same accumulation was demonstrated after tail vein injection or intraosseous injection of D1-TK cells in SCID or athymic nu/nu mice bearing C4-2 intraosseous lesions.

D. Summary

This approach is also targeted at disrupting the homeostasis of an osseous prostate cancer metastasis by infiltrating its supportive stroma with cells that can be killed by administration of acyclovir. The D1 cell's ability to home to and populate an osseous metastasis in an animal model suggests that this approach has potential as an ex vivo form of gene therapy. By combining this bone homing D1 cell with C4-2 in the subcutaneous tissue of an athymic mouse, we were able to (**i**) generate a novel model to study the bone stroma-prostate cancer cellular interaction and (**ii**) demonstrate a significant bystander effect on the growth of prostate cancer cells mediated by the genetically-engineered bone stomal cells.

The implications of a bone homing approach are two-fold. (**i**) The ability of D1-TK cells to exhibit bystander cell-kill in vitro and in vivo in a subcutaneous model mimicking prostate cancer osseous metastasis suggests that the homeostasis of an osseous metastasis may require bone stromal cells and can be disturbed by removing a bone stromal component. (**ii**) The ability of the D1 cell to maintain its bone homing ability after several ex vivo manipulations suggests that there is a possibility that human bone stromal cells may maintain their skeletal-homing potential. Genetically manipulated bone marrow stem cells have been applied for the treatment of malignancies utilizing autologous bone marrow transplantation.

Increased knowledge of the stromal-epithelial interactions of osseous metastasis will allow us to dissect this process and uncover potential new targets for therapy. The bystander effect has not been explained completely. The transfer of toxic metabolites through gap junctions or via the incorporation of apoptotic bodies are the two leading theories (Ishii-Moirta H., 1997; Richards C.A., 1995). Recently, it has been proposed that an immune mediated event may be responsible for the observed cytotoxicity (Gagandeep S., 1996). The pre-clinical models developed in our laboratory may help discern the molecular mechanisms of the bystander effects on prostate cancer growth. A better understanding of the bystander effect will allow us to design and implement more effective therapies.

Among many subtypes of prostate cancers, androgen-independence and osseous metastasis have caused significant mortality and morbidity in patients because there is no available curative therapy. Even after hormonal therapy with the most active agent currently available, a significant number of prostate cancer patients ultimately develop androgen-independent osseous metastases. To develop new therapeutic modalities for treating end-stage prostate cancer patients, we have explored the possibility of targeting prostate cancer osseous metastasis with toxic gene therapy mediated by an osteoblastic tissue-specific promoter (osteocalcin) and a bone homing mechanism to deliver ablative gene therapy to osseous metastases. The first approach utilizes an osteoblastic tissue-specific promoter that will restrict the transcription of toxic genes to prostate cancer cells and bone stromal cells. Ablative gene therapy has been demonstrated to exert a bystander effect in achieving maximal cell-kill. In theory, Ad-OC-TK/ACV can exhibit both the expected TK-associated bystander effect on the growth of prostate cancer cells and their supporting bone stromal cells, while also exhibiting an indirect bystander effect by killing the nurturing bone stromal cells and interrupting intracellular communication between prostate cancer cells and bone stroma. The second approach utilizes a natural bone homing mechanism to deliver genetically engineered bone stromal cells to prostate cancer skeletal metastasis, also yielding promising results. This technology needs to be further developed to yield maximal cell-kill at multiple sites of prostate cancer metastases.

VI. Prospects

Molecular therapeutics such as gene therapy are being used with increasing frequency. The exponential expansion of knowledge in the field of molecular medicine has led to therapy based on understanding the molecular pathways of the underlying disease processes. Currently, molecular based gene therapy protocols have been applied predominately for the treatment of life-threatening diseases (i.e. cystic fibrosis, ADA, and cancer). With such great potential, molecular approaches will be expanded rapidly into other areas of medicine in the near future.

In this review, we have focused our discussion on the concepts and models that we have developed in our laboratory to study the molecular mechanisms underlying human prostate cancer progression and metastasis. These models have been selected and utilized to test the efficacy of various gene therapies using delivery systems containing therapeutic toxic genes, including tumor suppressors and cytotoxic genes driven by tissue-specific promoters. To understand the use of gene therapy for the treatment of both localized and metastatic prostate cancer, our laboratory focused on the development of animal models that mimic human prostate cancer progression for the exploration of new therapeutic approaches. In the development of animal models, we observed intense reciprocal cellular interaction between prostate cancer cells and bone stroma. We demonstrated that bone stromal cells "select" or "induce" an androgen-dependent human prostate cancer cell line, LNCaP, to acquire androgen-independent phenotypes particularly in castrated hosts, with resulting LNCaP sublines that exhibit metastatic potential.

Results of toxic gene therapy for the treatment of localized and disseminated prostate cancers showed that: (**i**) Ad-OC-TK expressed high levels in both androgen-dependent and androgen-independent human prostate cancer cell lines; (**ii**) in parallel with the expression of Ad-OC-TK in tumor cell lines, the efficacy of Ad-OC-TK toxic gene therapy in target cells is directly correlated with the level of TK expression in vitro; (**iii**) in two experimental models of human prostate cancer, C4-2 and PC-3, we demonstrated that Ad-OC-TK, when applied together with ACV, induced tumoricidal effects in vivo. Significant histomorphologic improvement of human prostate cancer growth in the bone was supported by bone scans in vivo. In the C4-2 model, we obtained evidence that Ad-OC-TK plus ACV diminished serum PSA, which is confirmed by the improvement of the histomorphologic appearance of this tumor in the skeleton. Finally, we have focused our efforts on the development of combined adenovirus and chemotherapy [i.e. chemogene therapy (Cheon et al., 1997)], the development of a concept of loco-regional delivery of therapeutic genes and drugs, and the exploration of the homing mechanism to treat prostate cancer skeletal metastasis in vivo (Gardner et al., 1998). Taking advantage of the reciprocal cellular interaction between prostate cancer and bone stroma, we have developed two novel gene therapy approaches to target prostate cancer growth in the bone. We have achieved for the first time the use of Ad-OC-TK/ACV as a novel therapeutic agent that can selectively target and induce the killing of both prostate and osteoblast lineage cells.

Our ex vivo approach generated a unique prostate cancer bone growth model, and an osteoblastic reaction was observed when prostate cancer cells were co-inoculated with appropriate bone stromal cells subcutaneously. By introducing genetically engineered bone stromal cells, we observed that the bone stromal cells can confer cytotoxicity to their neighboring prostate cancer cells via a bystander effect. These observations will be developed to improve the delivery of therapeutic genes to the sites of prostate cancer metastases. We anticipate a new array of novel therapeutic approaches

that can be applied in the near future to treat prostate cancer in general and its skeletal metastasis in particular.

Acknowledgments

Supported by the CaP CURE Foundation (TAG, LWKC), IMClone Scholar for the American Foundation of Urologic Diseases (TAG), NIH Grant #1R29CA74042-01(CK), and NIH Training Grant #5-T32-DK07642 (TAG). We also thank our families for supporting our efforts.

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Exploiting Stromal-Epithelial Interaction for Model Development and New Strategies of Gene Therapy for Localized and Metastatic Prostate Cancer.

Abstract

To understand and appreciate the potential uses of gene therapy for the treatment of both localized and metastatic prostate cancer, our laboratory focused on the development of animal models that mimic human prostate cancer progression which allow for the exploration of new therapeutic approaches, particularly the use of gene therapy for the treatment of both localized and disseminated cancers. Reciprocal stromal-epithelial interaction occurs when prostate cancer cells metastasize to the bone; both laboratory and clinical evidence suggest that bone microenvironment is vital for the development and survival of prostate cancer cells. This review highlights our efforts to develop novel gene therapy protocols targeting the growth of both prostate cancer and its surrounding bone stroma. A bone homing mechanism was exploited to deliver therapeutic genes to prostate cancer osseous metastases. These models will then be used as targets for gene therapy by delivering therapeutic toxic genes. Bystander cell-kill using adenoviral-mediated expression of thymidine kinase (TK), either regulated constitutively or by an osteoblastic tissue-specific promoter, osteocalcin (OC), was developed.

The adenovirus containing TK under the transcriptional control of the OC promoter (Ad-OC-TK) was constructed and tested in several in vitro and in vivo models of human prostate cancer and osteosarcoma. Ad-OC-TK combined with acyclovir (ACV) significantly inhibited the growth of several osteoblastic cell lines (ROS, MG-63) and prostate cancer cell lines (PC-3, LNCaP, C4-2) in vitro and intraosseous and subcutaneous prostate tumors in vivo. Additionally, we have combined adenovirus and chemotherapy (i.e. chemogene therapy) and the development of systemic and a loco-regional delivery of therapeutic genes for the treatment of cancers in vivo.

A bone stromal cell line, D1, was stably transfected with both b-galactosidase and TK genes to allow for in vitro and in vivo localization and TK expression. The D1 cell line was selected because of its unique ability to localize to the bone upon intravenous injection. D1 cells expressed TK constitutively (D1-TK) and were able to exert strong bystander cell-kill upon the administration of ACV by inhibiting the growth of human prostate cancer cells when grown in vitro in tissue culture, in microgravity chambers, and in vivo as chimeric tumors. In vivo, the potent bystander effect exerted by D1-TK on C4-2 tumor growth was demonstrated radiographically, histologically, and was accompanied by a sharp decrease of serum PSA to a non-detectable level upon ACV administration.

We have demonstrated that stromal-epithelial interaction, which is vital to prostate cancer survival, can be interfered with by two novel gene therapy approaches in preclinical models of human prostate cancer. Both adenoviral delivery of TK under transcriptional control by OC and a constitutive expression of TK by bone stromal cells elicit significant prostate cancer cell-kill, and warrant further development.

Biosketch

Thomas A. Gardner completed both his undergraduate and medical school training at the George Washington University in Washington, DC. He completed a two years of a General Surgery residency followed by 4 years of a Urologic residency at the New York Hospital-Cornell Medical Center. To study the molecular mechanisms of prostate cancer and explore novel therapies for patients with prostate cancer, he pursued a urologic oncology fellowship with Leland W. K. Chung at the University of Virginia and supported by the American Foundation of Urologic Diseases. He currently is an Assistant Professor of Molecular Urology and Therapeutics within the Department of Urology at the University of Virginia Health Sciences Center. His present interests include the understanding of molecular events of urologic malignancies and designing molecular therapies that are based on tthose events.

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