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<http://dx.doi.org/10.1089/hum.2009.210>

Gene Transfer Vectors Targeted to Human Prostate Cancer: Do We Need Better Preclinical Testing Systems?

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Abstract

Destruction of cancer cells by genetically modified viral and nonviral vectors has been the aim of many research programs. The ability to target cytotoxic gene therapies to the cells of interest is an essential prerequisite, and the treatment has always had the potential to provide better and more long-lasting therapy than existing chemotherapies. However, the potency of these infectious agents requires effective testing systems, in which hypotheses can be explored both *in vitro* and *in vivo* before the establishment of clinical trials in humans. The real prospect of off-target effects should be eliminated in the preclinical stage, if current prejudices against such therapies are to be overcome. In this review we have set out, using adenoviral vectors as a commonly used example, to discuss some of the key parameters required to develop more effective testing, and to critically assess the current cellular models for the development and testing of prostate cancer biotherapy. Only by developing models that more closely mirror human tissues will we be able to translate literature publications into clinical trials and hence into acceptable alternative treatments for the most commonly diagnosed cancer in humans.

Introduction

THE LIMITED LONG-TERM *in vivo* efficacy of biological therapies for human prostate cancer (Maitland *et al.*, 2004) can be ascribed to a number of different causes: First, the entire concept could be wrong, that is, the gene or pathway to be exploited, perhaps discovered in cell line experiments, is simply not differentially expressed to the same extent *in vivo*; second, the agent itself could be wrong or inactive, perhaps unable to survive in the *in vivo* environment, or unable to penetrate cells while retaining its activity against the primary target; last, and often neglected, is that although the concept might be strong, and the agent active, the testing system used to define efficacy (and safety) could be defective.

Validity of Cell Lines That Represent Prostatic Disease

Prostate cancer has been poorly served by the establishment of cell lines. In general, new agents for prostate cancer are tested mainly in three cell types; PC3, LNCaP, and DU145. A review of the gene therapy literature suggests there are 2650 PubMed references that include the terms

prostate cancer and *gene therapy*. Of those, 562 employ only LNCaP cells; PC3 cells are used in 153 and DU145 cells in 179. The remainder employ a mixture of all three. In reality, only relatively few published studies make it through to the clinic. The *Journal of Gene Medicine* database (March 2009; <http://www.wiley.co.uk/genmed/clinical/>) lists the actual number of prostate cancer clinical trials as considerably lower (105): a conversion rate of 4%. In fact, many of the reported trials are derivative, so the actual rate is closer to 1%. Later stage clinical trials have been dominated by vaccine approaches to augment antitumor responses without any need to target the tumor itself (Small *et al.*, 2004).

The LNCaP, PC3, and DU145 cell lines were derived almost 30 years ago from therapy-resistant metastatic cancers (Table 1). The TSU-Pr1 cell line was also considered to be from prostate, but was subsequently shown to be derived from the human bladder cancer cell line T24 (van Bokhoven *et al.*, 2001). What is frequently lacking from these models is any representation of untreated low Gleason grade tumors within the prostate (Gleason, 1966), which have a high degree of normal prostate differentiation, or less structured,

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TABLE 1. CELL LINES FROM HUMAN PROSTATE FOR VECTOR TESTING

Cell line	Androgen sensitivity	Derivation	Ref.
Normal/nonmalignant			
PNT2	No	Derived from normal prostate epithelium by transfection of an <i>ori</i> mutant of SV40	Cussenot <i>et al.</i> (1991)
PNT1	No	Derived from normal prostate epithelium by transfection of an <i>ori</i> SV40 plasmid	Cussenot <i>et al.</i> (1991)
BPH1	No ^a	Benign prostatic tissue after SV40 large-T antigen immortalization of primary cells	Hayward <i>et al.</i> (1995)
PZ-HPV-7	No	Derived from epithelial cells cultured from normal tissue from the peripheral zone of the prostate by transfection with HPV18 DNA	Weijerman <i>et al.</i> (1994)
RWPE-1	No ^a	Peripheral zone of a histologically normal adult human prostate transfected with a single copy of HPV18	Bello <i>et al.</i> (1997)
PWR-1E	No ^a	Human prostatic epithelial cells, derived from a normal prostate with mild hyperplasia, were immortalized with an adenovirus 12--SV40 hybrid virus (Ad12-SV40)	Webber <i>et al.</i> (1996)
WPMY-1	ND	A myofibroblast stromal cell line derived from stromal cells from the peripheral zone of the histologically normal adult prostate, immortalized with SV40 large-T antigen gene	Webber <i>et al.</i> (1999)
RC156N/hTERT	No	hTERT-immortalized nonmalignant human prostate epithelial cell	Miki <i>et al.</i> (2007)
Premalignant			
P4E6	No	Derived from Gleason 2 + 2 (low-grade cancer) by retrovirus-mediated introduction of an HPV16 E6 gene	Maitland <i>et al.</i> (2001)
RWPE-2	No ^a	Derived from RWPE-1 cells by transformation with <i>Ki-ras</i> , using the Kirsten murine sarcoma virus (Ki-MuSV): RWPE2-W99 was further selected by cloning in soft agar to select cells that show high expression of <i>Ki-ras</i>	Bello <i>et al.</i> (1997)
WPE1-NA22 WPE1-NB14 WPE1-NB11 WPE1-NB26	No ^a	WPE1-Nxx cells were derived from RWPE-1 cells after exposure to <i>N</i> -methyl- <i>N</i> -nitrosourea	Webber <i>et al.</i> (2001)
WPE1-NB26-64 WPE1-NB26-65	ND	WPE1-NB26-64 and WPE1-NB26-65 (CRL-2890; ATCC) were derived from a subcutaneous tumor in a nude mouse injected with WPE1-NB26	Rivette <i>et al.</i> (2005)
WPE-stem WPE-int	ND	WPE-stem and WPE-int cells were derived from the RWPE-1 cell line after two consecutive cycles of single-cell cloning	Tokar <i>et al.</i> (2005)
Carcinoma			
MDA PCa 2b	Yes	MDA PCa 2b was established from a bone metastasis of androgen-independent adenocarcinoma of the prostate	Navone <i>et al.</i> (1997)
NCI-H660	ND	Has the appearance and many (but not all) of the properties of small-cell carcinoma of the prostate	Gazdar and Minna (1996)
LAPC-4	Yes	Grown from androgen-sensitive lymph node metastasis of prostate cancer	Klein <i>et al.</i> (1997)
CA-HPV-10	Yes	CA-HPV-10 was derived from cells from a prostatic adenocarcinoma of Gleason grade 4/4. The cells were transformed by transfection with HPV18 DNA	Weijerman <i>et al.</i> (1994)
DU145 22Rv1	No Yes	Cultured from a brain metastasis of prostate cancer 22Rv1 is a human prostate carcinoma epithelial cell line derived from a xenograft of a primary prostate cancer that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft	Stone <i>et al.</i> (1978) Sramkoski <i>et al.</i> (1999)

(continued)

TABLE 1. CONTINUED

Cell line	Androgen sensitivity	Derivation	Ref.
RC-92a/hTERT	No	hTERT-immortalized intraprostatic tumor epithelial cell	Miki <i>et al.</i> (2007)
DuCaP	Yes	Established from a metastatic lesion to the dura mater of a patient with hormone-refractory prostate cancer	Lee <i>et al.</i> (2001)
VCaP	Yes	This line was established in 1997 from a vertebral bone metastasis from a patient with hormone-refractory prostate cancer. It was passaged as xenografts in mice and then cultured <i>in vitro</i>	Korenchuk <i>et al.</i> (2001)
LNCaP	Yes	Derived from a lymph node metastasis of prostate cancer after estramustine treatment	Horoszewicz <i>et al.</i> (1980)
C4-2	No	Androgen-independent subclone of LNCaP parent	Wu <i>et al.</i> (1994)
PC-3	No	Derived from an androgen-independent bone metastasis of a treatment-resistant prostate cancer	Kaighn <i>et al.</i> (1979)
PC3M		The M variant was selected for enhanced metastatic spread	

Abbreviations: ATCC, American Type Culture Collection; HPV, human papilloma virus; hTERT, human telomerase reverse transcriptase; ND, not determined; SV40, simian virus 40.

^aCan be induced to express androgen receptor and luminal markers by culture in three dimensions and/or androgens.

untreated but “undifferentiated” higher Gleason grade tumors. As screening and diagnostic techniques improve for prostate cancer (Schröder *et al.*, 2009), “clinical” prostate cancers will be downgraded to lower Gleason grades, which will comprise the primary target for new therapies. At present, however, most biotherapies are used as a “last resort” after failure of hormone therapy, radiotherapy, and chemotherapy.

It is common in studies of new agents for prostate cancer to demonstrate their efficacy on one or more cancer cell lines, but only rarely do the experiments offer any estimation of damage to the normal prostate, as the prostate is considered a nonessential organ. Nonmalignant cell lines (Maitland *et al.*, 2001; Peehl, 2005) are listed in Table 1 along with many of the existing cancer cell lines. Primary cell cultures (see later), grown in a serum-free or low-serum medium, are frequently used as a comparator with the established cancer cell lines. These primary cultures are of a basal cell type, unless induced to differentiate by growth in a medium supplemented with serum, calcium, and androgens (Collins *et al.*, 2005). Similarly, most nonmalignant epithelial cell lines from prostate are also basal in phenotype, and express cytokeratin-5/14, but not the androgen receptor.

Thus, when studying vector-mediated gene transfer efficiencies in prostate cell cultures, one must be careful which parameter is under study, for example, between androgen-sensitive and androgen-insensitive cancers. In this case the comparison is frequently between LNCaP and PC3/DU145. This is more precisely a comparison of luminal cells and basal cells, and a better comparison would be LNCaP with one of the many derived androgen receptor-negative subclones such as C4-2 (Wu *et al.*, 1994). The comparison of the more recently established wild-type androgen receptor-expressing PC346C with one of its androgen receptor-negative subclones provides an even more clinically relevant alternative (Marques *et al.*, 2005, 2006).

It was the aim of the GIANT (Gene Therapy: An Integrated Approach to Neoplastic Treatment) program

(www.giant.eu.com) to assess the validity of the preclinical testing procedures for near-to-patient testing. As mentioned previously, cell culture models of prostate epithelium often lack the luminal differentiation present in tumors, unless derived from a later stage metastatic tumor. As illustrated in Fig. 1A, a normal human prostate acinus consists of an upper luminal layer (60% of epithelial cells), in close contact with a basal cell layer (40% of epithelial cells). These proportions are grossly disrupted in higher Gleason grade prostate cancers. Most untreated hormone-naïve prostate cancers have a luminal phenotype (express androgen receptor and luminal cytokeratin-18) and lack or contain <1% basal cells (no p63/cytokeratin-5/14 expression). The LNCaP cell line (Horoszewicz *et al.*, 1980) is frequently used to study hormone-sensitive (luminal) cancer, but LNCaP was isolated from a patient who had failed a primitive form of anti-hormone therapy (estramustine chemotherapy) and the cells express a mutant androgen receptor, which responds equally well to other steroid hormones (Veldscholte *et al.*, 1990).

Toward Better Preclinical Systems: Vector Adhesion in Two-Dimensional Cell Cultures

Initial testing of infectious agents in cell cultures is often optimized for viral replication or infectivity, for example, attachment of viruses to the cell surface. Many viruses use adhesion molecules as receptors and adenovirus is no exception (reviewed in Greber and Gastaldelli, 2007). For example, the main adenovirus receptor, the coxsackievirus-adenovirus receptor (CAR), forms homodimers between contacting cells and serves as a cell adhesion molecule (van Raaij *et al.*, 2000), but adenoviruses also require a secondary receptor in the form of integrins for high-efficiency transduction. Of these, $\alpha_v\beta_3$ is probably the key integrin involved in virus uptake (Wickham *et al.*, 1993; Nemerow, 2000). CAR protein is also expressed in mobile membrane lipid rafts (Ashbourne Excoffon *et al.*, 2003) and coimmunoprecipitates with ZO-1, a tight junction protein (Cohen *et al.*, 2001).

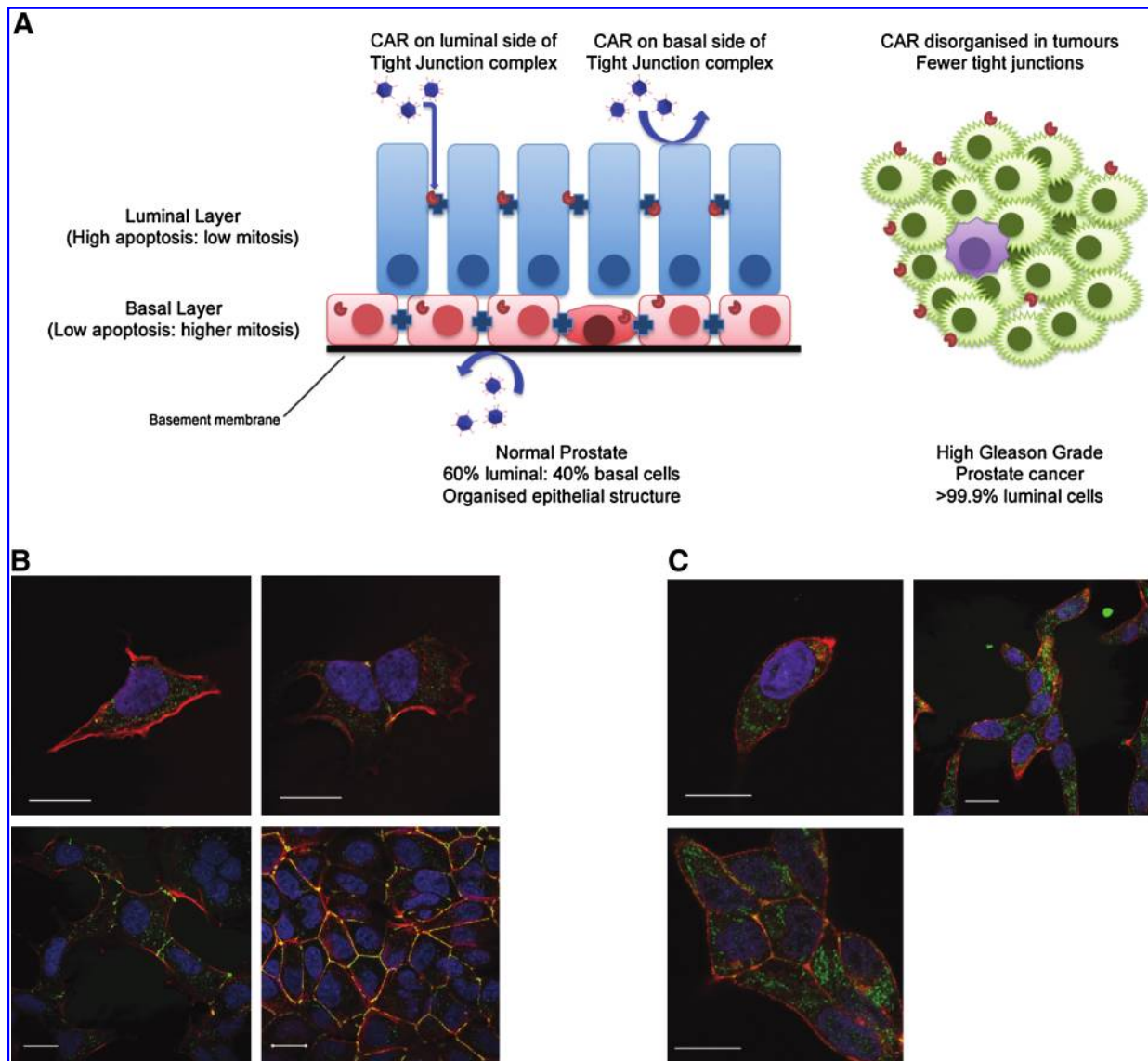


FIG. 1. A model for virus binding and penetration in prostate epithelium. **(A)** Differences between (*left*) an organized bilayered normal prostate epithelium and (*right*) the mainly luminal, less organized cancer epithelium (in a higher grade tumor). The principal adenovirus attachment protein, CAR (red dots), may be associated with the apical or basal side of the tight junction. If associated with the apical side then it is available for primary binding to the fiber knob of adenovirus. If below the tight junction then it is not available for binding. It is known that disruption of the tight junctions increases infection with adenovirus, probably because of increased accessibility of CAR on the basal side of the tight junction. In prostate cancers the structural disruption due to aberrant differentiation could prevent viral spread and penetration, in a therapeutic setting. **(B and C)** Immunocytochemical detection of coxsackievirus–adenovirus receptor protein in cultured prostate cancer cells. CAR (green) is expressed at lateral cell junctions in **(B)** HEK-293 cells and **(C)** LNCaP cells and colocalizes (yellow) with F-actin (red). DAPI (blue) was used to visualize nuclei. Note the various CAR densities, the localization of CAR at intracellular junctions, and the striking changes in CAR expression, according to cell density, in the two cell cultures. Scale bars: 50 μm .

In normal prostate tissues, CAR is located at the lateral junctions between luminal cells, and at the apical border, whereas in basal cells mostly cytoplasmic expression is observed (Rauen *et al.*, 2002). Prostate cancer is mainly luminal in phenotype, which suggests it should express more CAR, as application of hormones to rat seminal vesicle and prostate epithelium results in a reorganization and differentiation-regulated increase in junction formation (Ortiz and Cavicchia, 1990; Mitra *et al.*, 2006). However, in sections of prostate cancer, the expression of CAR is lost at the cell

junctions and also decreases at the protein level (Li *et al.*, 1999; Okegawa, 2000). Therefore the viral vectors may find alternative means of entry, for example, adenovirus may enter through direct contact of the penton base with integrins or other receptors. The loss of CAR was confirmed in our laboratory, where we could not detect expression at the apical border of primary prostate cancer samples (K. Chambers, unpublished data). Although the common productive cell line for adenoviral culture, HEK-293, has extensive intracellular CAR (Fig. 1B), in prostate cancer cell lines, such as

LNCaP, CAR is present at lateral junctions but is expressed mainly in the cytoplasm of single cells without adhesions (Fig. 1C; and K. Chambers, unpublished data). CAR is, however, still detectable in subconfluent prostate cancer cell cultures even after disruption of the junctions for flow cytometric analysis (Pandha *et al.*, 2003).

Because of the expression of CAR at the tight junctions, the availability of the receptor from the luminal surface is blocked by their proximity to or incorporation into tight intracellular junctions (Balda and Matter, 1998; Cohen *et al.*, 2001). Access and spread can be governed by restricted access to receptor complexes, as the mean distance between cells in tight and gap junctions is 1.5 and 4 nm, respectively, whereas the full diameter of many nonviral particles is at least 50 nm and that of the rigid adenoviral particle is 100 nm.

With prostate cancers, there is also the question of the cell surface available to be infected in a structured and polarized epithelium (present in most lower Gleason grade tumors). *In vivo*, it is likely that infection will occur through the luminal surface (see Fig. 1A) rather than through basal contact via the basement membrane, which adenoviruses can fail to penetrate, although genetic modification of the virus by replacement of the E3 gene to encode the relaxin-degradative enzyme can improve penetration (Kim *et al.*, 2006). Other strategies to improve infectivity via CAR include internalization of tight junction proteins such as occludin to expose the tight junction receptor CAR, without affecting the distribution of other tight junctional proteins (Coyné *et al.*, 2007), and increased exposure of CAR through actin remodeling (Coyné and Bergelson, 2005).

In tissues, the damage induced by direct injection results in effective infection restricted to a few millimeters around needle tracks (Patel *et al.*, 2009). Therefore it will probably never be possible *in vivo* to saturate a tumor with prodrug-activating cells after gene transduction by intratumoral injection of even high titers of the most efficient vectors, without facilitating intratumoral spread. It was for this reason that an oncolytic approach was taken for the initial trials in GIANT (Cheng *et al.*, 2006), where intracellular spread occurs after infected cell lysis.

However, in immunocompetent patients such spread is a matter of timing between the triggering of a potent immune response against adenoviruses and the ability of the virus to reinfect multiple times. Although the immune response may be restrictive in the spread of the virus (Parato *et al.*, 2005), there is evidence to suggest that patients who respond immunologically to a therapeutic viral infection also mount a powerful immune response against tumor cell antigens. The strong adjuvant effect of high levels of viral antigens is thought to be responsible, resulting in regression of not only the primary tumor, but also of distant metastases (Freytag *et al.*, 2002). This type of outcome emphasizes the need to use virotherapies in immunocompetent patients to achieve maximal therapeutic efficacy. Predictably, in most phase 1 (in which safety assessment is the object) but more surprisingly in phase 2 (in which efficacy is sought) clinical trials of biotherapies, this is seldom the case.

Primary Tissue Infections

An excellent model, which takes into account differential patient susceptibility to infection, is direct infection of needle

biopsies from fresh prostate cancer tissues, in which tissue architecture and viability can be preserved in culture for a period of up to 2 weeks in a basal medium. Such tests provide, on a patient-to-patient basis, a measurement of susceptibility not only to gene therapy agents but also to many common chemotherapies. However, ethical restrictions in obtaining fresh tissues, or the risk of compromising pathological diagnosis, can compromise the quantities of tissue available to do such studies in a rational and quantitative way.

A good alternative would be to establish and test the transduction of fragments of tissue as primary xenografts in immunocompromised mice. Short-term "culture" of human tissues is possible in xenografts, with maintenance of both tissue architecture and hormone responsiveness at subcutaneous, subrenal capsule, and orthotopic mouse prostate sites. Although the production of such xenografts can be labor intensive, and they are limited in size and scope for the testing of agents, they do offer the best measurement of therapeutic effectiveness as a patient-specific medicine, the "gold standard" for sophisticated and targeted biotherapies. Within the GIANT program we have used xenograft models, established for many years (Table 2).

Primary Epithelial Cell Cultures

As an interim solution, *in vitro* cultures of primary prostate cancers can be employed. Primary cells have a limited life span in culture, especially in medium containing high fetal calf serum concentrations, which is used to promote rapid growth of not only the primary epithelial cultures, but also the fibroblasts. These culture conditions also stimulate the terminal differentiation of basal epithelial cells. Normal prostate luminal epithelium has a limited ability to persist *in vitro* and *in vivo*, whereas it is the basal epithelium that is responsible for driving organ regeneration after castration (Isaacs and Kyprianou, 1987) and has a much higher frequency of mitosis compared with the luminal epithelium. Apoptotic rates are higher (De Marzo *et al.*, 1998) and mitoses are barely detectable in normal luminal cells (Fig. 1), which can be considered the end-stage of a differentiation process, in which cells are shed into the lumen and often appear in the bloodstream as apoptotic or preapoptotic bodies. This is also true in tumors, where circulating tumor cells are mostly of a differentiated phenotype (PSA⁺, CK18⁺), many of which are not viable (Riethdorf and Pantel, 2008). It is perhaps due to the high apoptotic rate in the luminal fraction that successful primary cultures of normal human prostate are of a basal cell type. We have not yet solved the problem of both differentiation and apoptosis within these cultures, which is frequently linked to the presence of high amounts of prostate stroma copurified from the dissociated tissues (Cunha, 1984; Hall *et al.*, 2002). To eliminate or reduce the stromal content, cholera toxin can be used along with other epithelial growth-promoting factors within the cell culture medium (Chung *et al.*, 1988; Marques *et al.*, 2005). Tissue dissociation and cell fractionation, for example, by means of immunomagnetic beads, effectively removes all prostate stroma and additional purification by promoting adherence to basement membrane or collagen provides further reduction of both luminal and stromal cells (Richardson *et al.*, 2004).

TABLE 2. HUMAN PROSTATE CANCER XENOGRFT MODELS^a

Name	Derived from:	Androgen responsive	PSA	AR	Year derived
PC-82	Prostate	Yes	Yes	Yes	1977
PC-133	Bone	No	No	No	1981
PC-135	Prostate	No	No	No	1982
PC-EW	Prostate	Yes	Yes	Yes	1981
PC-295	LN	Yes	Yes	Yes	1991
PC-310	Prostate	Yes	Yes	Yes	1991
PC-324	TURP	No	No	No	1991
PC-329	Prostate	Yes	Yes	Yes	1991
PC-339	TURP	No	No	No	1991
PC-346	TURP	Yes	Yes	Yes	1991
PC-346I	PC-346	No	Yes	Mutant	1992
PC-346B	TURP	Yes	Yes	Yes	1991
PC-346BI	PC-346B	No	Yes	Yes	1992
PC-374	Skin	Yes	Yes	Yes	1992
TEN12	Prostate	Yes	Yes	Yes	1985
LuCaP 23.1	Lymph node	Yes	Yes		1996
LuCaP 23.8	Lymph node	Yes	Yes		1996
LuCaP 23.12	Liver	Yes	Yes		1996
LuCaP 35	Lymph node	Yes	Yes	Yes	2003
LuCaP 35V	LuCaP 35	No			2003
LuCaP 49	Metastasis	No	No	No	2002
LAPC-3	AI TURP	No	Yes/no	Yes	1999
LAPC-4	AI LN	Yes	Yes	Yes	1997
LAPC-9	AI Bone Met	Yes	Yes	Yes	2001
CWR22	AD Met	Yes	Yes	Mutant	1993
CWR21	AD Met	Yes	Yes	Yes	1993
CWR31	AD Met	Yes	Yes	Yes	1993
CWR91	AD Met	Yes	Yes	Yes	1993
MDA Pca-31	Liver	N/A			1998
MDA Pca-40	Liver	N/A			1998
MDA Pca-43	Adrenal	N/A			1998
MDA Pca-44	Skin	N/A			1998

Abbreviations: AD, androgen dependent; AI, androgen independent; AR, androgen receptor; LN, lymph node metastasis; Met, metastasis; N/A, not applicable; PC, prostate cancer; PSA, prostate-specific antigen; TURP, transurethral resection of the prostate.

^aReproduced with permission from van Weerden *et al.* (2009).

To at least partly restore luminal differentiation, reconstructions of the multilayered and multicellular prostatic epithelium can be achieved in two dimensions by allowing the epithelial cells to grow out of monolayer and form a bilayer. Induction of luminal differentiation is further enhanced by addition of prostate androgen-responsive stroma and medium supplements such as dihydrotestosterone and calcium, resulting in a polarization of the epithelial layers. At this point the characteristic patterns of expression of cell surface receptors are found, as gene expression switches from basal to luminal cytokeratin expression in the upper (luminal) layer (Swift *et al.*, 2010).

Three Dimensions: Better Than Two?

Whereas two-dimensional cell culture presents a single surface for virus infection, tissues exist *in vivo* as multiple layers bordered by tight junctions and desmosomes, and

containing a multitude of different structurally differentiated cell types, which will inhibit vector penetration. One way to model this is to generate three-dimensional structures in cell culture. To assess the effects of differentiation, three-dimensional cultures can be induced to both polarize and differentiate by suspension in an appropriate matrix, the stimulus to polarize being provided by signals from prostate stromal cells (Lang *et al.*, 2001).

Adenoviruses have a limited ability to extravasate from blood vessels, unless the capillaries are severely damaged or are irregular in development, such as is the case with intratumoral capillaries (Wang and Yuan, 2006). When adenovirus was administered via the intravenous route in mice xenografted with a human prostate tumor, infection was observed mainly in endothelial cells of healthy vessels in the tumor. Few infected tumor cells were detectable after intravenous administration. However, when virus was administered intratumorally in the same model, many infected tumor cells were detected, although the distribution pattern remained concentrated around the needle track (R. Kraaij, unpublished). Complex three-dimensional structures *in vitro* can also address the issue of penetration.

In another tissue, that is, infections of the eye, adenoviruses were also unable to penetrate the outer layers of epithelium to infect the basal epithelium, whereas enveloped viruses of less rigid structure, such as baculovirus (mean diameter, 60 nm), could do so (Kinnunen *et al.*, 2009). This suggests that particle diameter is not the only parameter to take into account in determining tissue penetration.

To model tissue penetration in the GIANT program, three-dimensional spheroids grown from a number of different prostate cell lines were employed. Different morphologies of spheroid were derived from the different cell types (Fig. 2). Some were rigid and heavily structured, whereas others were much more loosely associated, in which the requirement to form structures and to communicate with other cells had been gradually lost either as a result of oncogenic changes, or through long-term culture. In such full three-dimensional models (Lang *et al.*, 2001) it became apparent that most vectors, including adenoviruses, had difficulties in penetrating basement membranes such as Matrigel, which reflects restrictions on their spread in tissues *in vivo* as well.

However, even more striking was the inability of adenoviruses to pass through and penetrate organized spheroids as shown in Fig. 2A and B. Furthermore, P4E6 cells (derived from a well-differentiated prostate cancer) form a highly organized spheroid (Fig. 2C), which also contains a stretched layer of thin epithelium to provide structural integrity. Both by immunofluorescence (Fig. 2A and B) and by electron microscopy (Fig. 2D), virus replication could be seen within the outer layer of spheroid epithelium, but spread within the spheroid was almost totally absent. When yields of oncolytic virus in this system were compared with the yields from a replicating monolayer culture and subsequently with xenografts (see later), the three-dimensional culture more closely matched the situation in xenograft models (L. Georgopoulos, H. Evans, and R. Nugent, unpublished data).

Cellular Heterogeneity in Prostate Cancer

For most cancer medicines, there has long been the assumption that all the cells in a tumor grow more quickly than

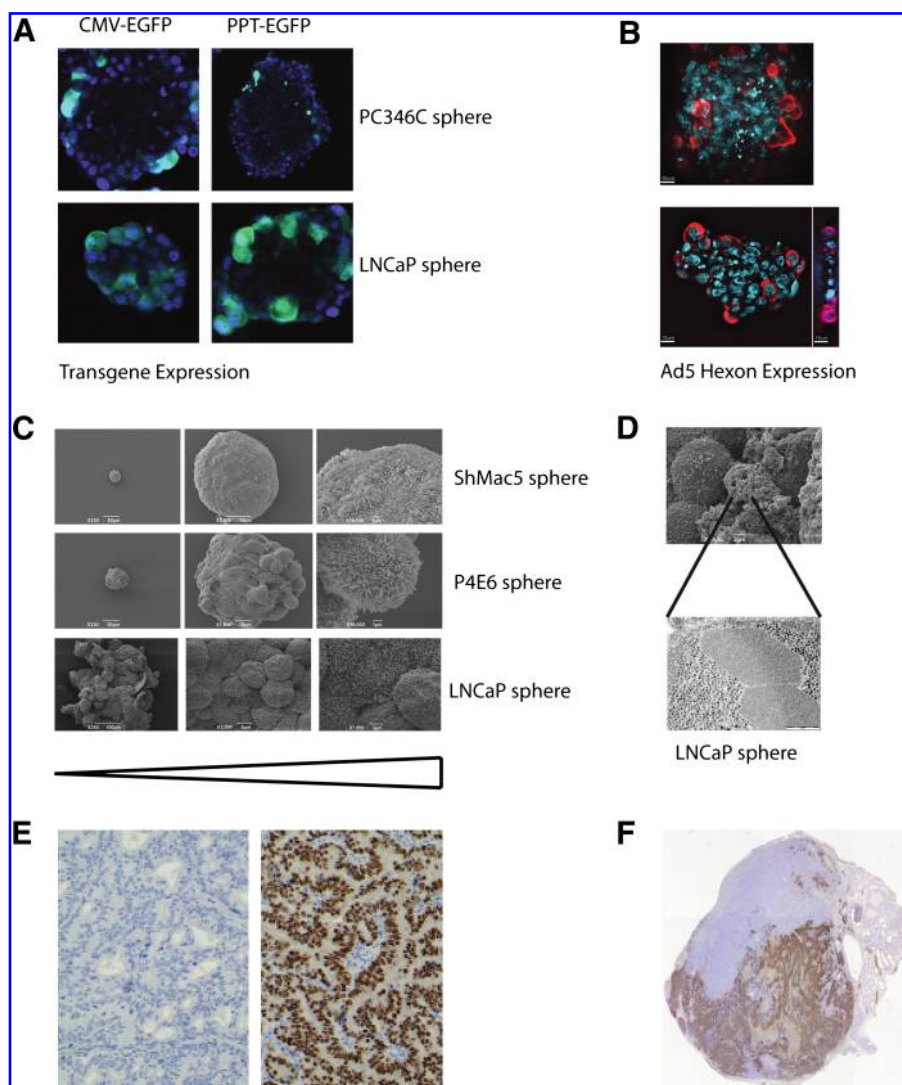


FIG. 2. Advanced models of prostate cancer. (A) Infection of PC346C and LNCaP spheroids with adenoviruses expressing GFP from constitutive cytomegalovirus (CMV) and prostate-targeted (PPT) promoters. Note the lack of GFP signal in the center of the spheroids, indicating lack of viral penetration. (B) Three-dimensional confocal rendering of staining for Ad5 hexon protein (red) in the peripheral layers of a PC346C spheroid infected with prostate-specific PPT oncolytic adenovirus. Note the restriction of viral replication to the perimeter of the spheroid. (C) Scanning electron micrographs (at increasing magnification) of three different human prostate cancer cells generating spheroid structures. (D) *Top*: High-power scanning electron micrograph of LNCaP spheroid infected with PPT-oncolytic adenovirus (72 hr postinfection). *Bottom*: A segment of a transmission electron micrograph of an infected cell from an infected spheroid, showing multiple viral particles and adenoviral proteins: lack of viral spread is not due to poor viral replication or assembly. (E) Prostate cancer xenografts in immunocompetent mice: Histologies of androgen-responsive PC82 tumor (*left*, hematoxylin staining) and PC310 tumor (*right*, androgen receptor staining). (F) Xenograft infection with oncolytic targeted adenovirus: Histology of PC346C tumor 3 days after infection with the replication-competent Ad-ZH/3 vector (brown, Ad5 hexon staining; purple, hematoxylin staining).

their normal counterparts, and that they homogeneously express the tissue-specific mRNA patterns of a prostate tumor. The rapid emergence of therapy-resistant tumors, even for biotherapies, has been cited as proof of the existence of a preexisting therapy-resistant fraction, sometimes termed *cancer stem cells* or *tumor-initiating cells* (Diehn *et al.*, 2009). In human prostate cancer, this fraction seems not to respond to androgens, which form the basis of much of the tissue-specific targeting strategies employed in virus targeting (Maitland and Collins, 2008). As suggested in a review (Short and Curiel, 2009), the development of new genera-

tions of vectors with the ability to eliminate cancer stem cells, as part of a combined gene therapy approach to shrink tumor bulk, should extend the effectiveness of such therapies.

Contamination of Cell Models

High-throughput testing of infectious agents, relying on both primary cells and cell lines, also presents a number of dangers with respect to the validity of the cells to be employed. It is now mandatory for publication in some journals that all cell cultures be regularly genotyped. The standard

applied by the American Tissue Culture Collection (ATCC, Manassas, VA; <http://www.atcc.org/>) is the PowerPlex system (a microsatellite genotyping kit; Promega, Madison, WI). All cells to be employed in therapy testing should be genotyped initially as primary cells, in comparison with the original tissue DNA (or lymphocytes). If an established cell line is used the reference PowerPlex fingerprint is available from the ATCC. Even primary cultures, from normal or tumor tissues, have a limited life span as a true reflection of the original tumor, for example, glioblastoma cell cultures (Lee *et al.*, 2006). This life span can be extended by purifying different cell populations, including stem cells, as described previously (Collins *et al.*, 2005).

Second, cells derived from xenografts or primary cultures passaged on inactivated mouse stromal feeder layers require routine assessment of human cell content and the presence of murine cells and DNA, derived either from murine infiltrates in the xenografts or "breakthrough" of feeder cells resulting from inadequate inactivation, by polymerase chain reaction (PCR) using repetitive species-specific centromeric sequences (Becker *et al.*, 2002).

The last restriction when working with primary tissues, xenografts (in particular), and established cell lines, is the necessity to eliminate mycoplasma contamination, which frequently prevents vector attachment and penetration. A number of excellent commercial PCR-based assays are available for mycoplasma detection.

Ex Vivo Clinically Relevant Testing for Antivector Immunity

One aspect of gene therapy, which cannot be determined in a standard cell culture environment, concerns exposure of the infectious agent to the bloodstream and immune system of the host. The current standard *in vivo* system, a xenograft of the original cell line used in cell culture in an immunocompromised mouse, suffers 2-fold in this respect.

First, there is no strong humoral or T cell-mediated immune response against the infectious particles, as the commonly used nude mouse is athymic. The nonobese diabetic-severe combined immunodeficient (NOD-SCID) mouse lacks more of the T cell immunity in addition to B cells, but is now being replaced in practical use by even more immunocompromised hosts such as the Rag2/ γ c double-negative hosts, which most notably also lack most natural killer (NK) cell functions (Le Dévédec *et al.*, 2009). These hosts still retain complement responses, but show different abilities to engraft with human tumor cells. However, all hosts share the inability to mount any immune response to tumor cell lysis products, acknowledged as one of the major influences on tumor destruction (see previously).

Second, infectious particles, in particular adenoviruses, interact in a different way with blood components in murine and human hosts. It has been shown that more than 90% of adenovirus administered to human blood *ex vivo* binds to human erythrocytes whereas only a negligible fraction (<0.1%) of adenovirus binds to blood cells in freshly isolated murine whole blood (Lyons *et al.*, 2006). Human erythrocytes bind and inactivate adenovirus serotype 5 (Ad5) by expressing both the coxsackievirus-adenovirus receptor (CAR) and the complement receptor-1 (CR1) (Carlisle *et al.*, 2009). Mouse erythrocytes do not express either CAR or CR1. Be-

cause complement receptors are important for induction of neutralizing antibodies (Seregin *et al.*, 2009) mouse blood does not mimic the situation in human blood, that is, the effective dose of virus in humans is now calculated to be much lower than that originally predicted from the critical preclinical studies in mice.

As part of the GIANT project, we sought to overcome this by testing vectors in a human *ex vivo* blood loop system, which had previously been developed for the testing of other biomaterials (Hong *et al.*, 2001). This system is a powerful preclinical model for studies of viral and nonviral vector interactions with all components of whole blood, omitting the need for high-level anticoagulants that can alter the results by chelating the system of positive ions such as Ca²⁺ and Zn²⁺. Furthermore, in contrast to the use of sealed small-volume tubes, the loop system also benefits from reproducing shear forces, which are important for platelet function and the continuous flow of the bloodstream.

We have previously shown (Georgopoulos *et al.*, 2009) that a major complement response in blood loops was mounted against a nonhuman virus, baculovirus. Significant binding of IgM and complement components was observed and strong immunoreactivity was characterized by blood clot formation. The importance of the complement responses to baculovirus, against which humans should have no immunological memory, was indicated by clot prevention by use of complement inhibitors such as compstatin (Nilsson *et al.*, 1998). The life span of the vector particles could therefore be extended in serum by coinoculation with specific complement inhibitors.

Within the GIANT network, we have used the blood loop system to assess the immune response in whole human blood to an Ad5 vector and immunologically "stealthed" PEGylated Ad5. Such PEGylation of Ad5-based vectors has proven efficient in reducing the immune response and increasing the circulation times in mouse models (O'Riordan *et al.*, 1999; Gao *et al.*, 2007) and reducing virus uptake by mouse Kupffer cells (Mok *et al.*, 2005). PEGylation reduced Ad5 adhesion to blood cells, and both complement activation and cytokine release to a certain degree, especially when the neutralizing anti-Ad5 antibody titer was low (Danielsson *et al.*, 2010). However, the reductions were lower than would have been predicted from mouse models (O'Riordan *et al.*, 1999; Mok *et al.*, 2005; Gao *et al.*, 2007; Wortmann *et al.*, 2008). The reason may well be the difference in cell adhesion of Ad5 to human and mouse blood cells and the fact that PEGylation has only a limited protective effect when the neutralizing anti-Ad5 antibody titers are high. This finding simply emphasizes the importance of using a relevant model for evaluating viral vectors.

Toward More Clinically Relevant *In Vivo* Testing

Current modeling of human prostate cancer in animal models still does not accurately represent the clinical nature of the human disease. A consensus meeting in the United States (Pienta *et al.*, 2008) concluded that newer, more biologically relevant models were still required. Although pure transgenic models have permitted the dissection of basic biological processes underlying prostate cancer, such as the importance of PTEN (phosphatase and tensin homolog) haploinsufficiency (i.e., Di Cristofano *et al.*, 1998), as murine

cells are not particularly susceptible to vectors targeting human cancers, and oncolytic adenoviruses do not grow in murine cells, testing systems are usually restricted to human xenografts.

Just as with cell culture lines, until the early 1990s, the limited number of xenograft models for prostate cancer represented predominantly late-stage disease, whereas models of untreated tumors were underrepresented (i.e., radical prostatectomy samples). The more recent establishment of "early-stage" xenograft models and cell lines has largely overcome this limitation (Tables 1 and 2). The latter xenograft models (van Weerden *et al.*, 1996, 2009; Marques, 2006) have become even more relevant as prostate cancer is being diagnosed at an earlier stage. Until more recently, for the testing of gene therapy vectors, the field still remained heavily dependent on the LNCaP, PC3, and DU145 cell lines.

PC3/DU145 Xenografts

PC3 cells form reproducible tumors at most murine sites of inoculation, as subcutaneous, subrenal capsule, and intraprostatic orthotopic grafts (see review by Hoffman, 2007). The tumors are capable of killing the host with an inoculation of about half a million cells within 28 days. This extremely rapid growth from an undifferentiated epithelial cell mass has been used extensively as the target for many prostate-specific therapies. DU145 cells are similarly tumorigenic in immunocompromised animals, although they have been used less frequently (Nemeth *et al.*, 1999).

New variants expressing indicator genes such as luciferase and green fluorescent protein (GFP) have been developed, and more metastatic variants of PC3 have been selected by prolonged passage in nude mice. It could be argued that the lack of structure in the tumors and the rapid growth are not comparable with human prostate cancers, which are much slower growing and display elements of organization and structure even in their least differentiated forms, as described originally by Gleason (1966). One complication with PC3 is that bone tumors, which can be obtained by direct inoculation into bone (Nemeth *et al.*, 1999), are oncolytic rather than osteoblastic, as is commonly found in advanced human cancers (see review by Dotan, 2008).

LNCaP Xenografts

In contrast, the LNCaP cell line expresses androgen receptor (albeit a mutant androgen receptor), which typifies luminal differentiation in well-differentiated common prostate cancers. However, xenografting with LNCaP can be technically difficult, in particular to subcutaneous sites. It is also capable of throwing off many substrains and the C4-2 series originally described by Thalmann and colleagues (1994) clearly has the capacity not only to engraft but also to induce metastasis.

The success of LNCaP xenografts can, however, be substantially improved by coinoculation with prostate stromal cells (Tuxhorn *et al.*, 2002). This again mimics much more closely the situation in humans.

Other Prostate Epithelial Cell Line Xenografts

The BPH1 cell line, which derived originally from benign prostatic hyperplasia epithelium by introduction of SV40T

antigen (Hayward *et al.*, 1995), can be induced to form aggressive tumors by grafting subrenally in the presence of carcinoma-associated fibroblasts (CAFs) from prostate (Hayward *et al.*, 2001). This again emphasizes the importance of the epithelial-stromal interaction in the development of prostate cancers both *in vitro* and *in vivo*. The mechanism for this oncogenic transformation of a previously benign cell type simply by the presence of fibroblasts is thought to occur through a complex series of interactions, at least some of which involve transforming growth factor (TGF)- β signaling (Ao *et al.*, 2007). To authentically model lower grades of prostate cancer in the mouse environment will probably require such multicellular grafts.

PC346C Xenografts

The more recently established PC346C cells reliably form undifferentiated epithelial tumors after subcutaneous or intraprostatic orthotopic inoculation of 1 million cells within 20 days. Importantly, PC346C is androgen sensitive, expresses a wild-type androgen receptor, and secretes large amounts of prostate-specific antigen, which typifies well-differentiated common prostate cancers. Prolonged culture of PC346C cells in the absence of androgen and/or in the presence of the antiandrogen hydroxyflutamide, as well as *in vivo* passage in castrate recipient mice, has resulted in several castration-resistant variants (Marques *et al.*, 2005, 2006). Furthermore, orthotopic injection of PC346C has provided variant sublines derived from metastatic lesions (lymph node and lung) that are equally tumorigenic (W.M. van Weerden, unpublished data). At present, PC346C variants expressing indicator genes such as luciferase and GFP or RFP fluorescence are being developed, which will permit real-time monitoring of tumor cell fate.

Within the GIANT program we chose to exploit the latter xenografts (for a review see Van Weerden *et al.*, 2009), which have been selected to span a range of pathologies, from androgen dependency through to androgen independency and a number of different morphologies and histological grades (see Table 2).

Tumors of early prostate cancer, as represented by the androgen-responsive PC82, PC295, and PC310 xenografts, are well-differentiated tumors with a cribriform growth pattern that continue to express the prostate antigens and more critically a wild-type androgen receptor (Fig. 2E). In contrast, xenografts that represent later stage disease, such as PC133, PC135, and PC339, are relatively undifferentiated.

Xenografts are propagated by tumor fragment transplantation without additional human stromal cells. Instead, tumors are supported by infiltrating murine prostate stroma, which provides the required vascularization and putative stroma-derived factors that may be required for xenograft growth. These xenografts have growth rates that are variable between the various models, with relatively slow growth rates for the androgen-responsive tumors (tumors reaching a volume of 500 mm³ by 60–70 days after transplantation) and faster growth rates for the less differentiated xenografts (tumors reaching a volume of 500 mm³ by 30–40 days after transplantation). Also, as the tumors continue to secrete prostate-specific antigen (PSA), the principal biomarker for clinical studies of prostate cancer (van Weerden and Schröder, 2008), tumor growth can be readily monitored.

The limitation of the xenograft panel largely lies in the fact that these tumors, except for PC346, can be propagated only subcutaneously, as we have been unable so far to establish permanent cell lines from these xenograft models. Because subcutaneously grown tumors generally do not metastasize, these tumors cannot be used to study metastasis from the primary site. However, PC346C cells that are inoculated into the prostate have been shown to spread to other organs (metastases in the lymph nodes and lung). For preclinical testing, the GIANT program has employed the PC346C tumor, which has the great advantage of being able to be grown *in vitro* and as a serially transplanted xenograft. The ability of PC346C cells to sustain virus growth has been previously demonstrated (Kraaij *et al.*, 2005), and several prostate-specific oncolytic adenoviral vectors, such as Ad[I/PPT-E1A] (Cheng *et al.*, 2006) and Ad-ZH/3 (Magnusson *et al.*, 2007), have now been preclinically validated using the orthotopic PC346C xenograft model. (Fig. 2F; and R. Kraaij, unpublished).

Future Considerations/Outlook

The potential of gene therapy to provide an alternative treatment for prostate cancer has been discussed since the early 1990s (Sanda *et al.*, 1994). The early promise has not yet been fulfilled, although we now know a great deal more about how to kill the correct cells, and perhaps more importantly how to target potent therapies to the correct cell types. Sadly, the testing strategies have failed to maintain the rapid pace of advance in agent development, which could account in part for the failure to convert our basic knowledge into clinical trials.

The translation from cell lines in culture, and of rapidly growing xenografts of the same cell lines, to a heterogeneous and relatively slowly growing tumor in an immunocompetent patient is rarely considered in vector development strategies. We have attempted to highlight the difficulties in achieving total tumor infection as a result of restrictions in vector spread by using adenoviral vectors as a common example. The restriction of spread from a needle injection in tissues remains limited to at best a few millimeters, requiring precise location of the tumor to be infected (Patel *et al.*, 2009). By treating the vector inoculum as a medicine, requiring formulation just like a small molecule, we can perhaps reduce the *in vivo* restrictions. For example, blockade of the innate immune response, and incorporation of tissue disruptors such as relaxins (or equivalent small molecules) to open tight junctions and reveal receptors, would aid such spread.

However, the most important consideration must remain patient selection: the stage of prostate cancer to be targeted, and the critical realization that responses in each individual human will vary and also differ from those in mouse models. Increasingly, these responses can now be modeled in the laboratory, restricting unnecessary time and expenditure on premature clinical trials.

Acknowledgments

The authors thank all the collaborators on the EU-funded FP6 Project GIANT for discussions and contributions. The authors particularly thank Michelle Scaife for assistance in manuscript preparation. All original research reported was funded by the GIANT Integrated Project (contract number

LSHB-CT-2004-512087). N.J.M. also received invaluable core support from Yorkshire Cancer Research.

Author Disclosure Statement

No competing financial interests exist connected with the information conveyed in this review.

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Received for publication November 16, 2009;
 accepted after revision December 23, 2009.

Published online: April 12, 2010.

