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STATE-OF THE-ART IN THE MANAGEMENT OF CANCER

PROSTATE CANCER — PART II

DIAGNOSIS, STAGING, PROGNOSIS, SCREENING, AND NOVEL MOLECULAR MARKERS

MEETING COVERAGE

REPORT FROM THE SIXTH INTERNATIONAL CONFERENCE ON GENE THERAPY OF CANCER

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This article updates material that appeared in FUTURE ONCOLOGY, pp 287-291 and 401.

DIAGNOSTIC AND SCREENING TESTS

Tests used in the screening, diagnosis, staging and prognosis of prostate cancer, and those in development for such applications, are detailed in FO, pp 287-291. In actuality, measurement of prostate-specific antigen (PSA) levels in the circulation is the only *in vitro* test approved for the diagnosis, monitoring and screening of prostate cancer in the USA. Another test, prostatic acid phosphatase (PAP), originally the only available *in vitro* diagnostic test for prostate cancer, has been virtually supplanted in most cases by PSA.

Prostate-specific Antigen (PSA)

PSA is the most common marker associated with prostate cancer used for diagnosis, screening and monitoring of the disease (also see FO, pp 287-288). Although often referred to as one of the most important tumor markers in clinical oncology, PSA possesses neither the specificity nor sensitivity of an ideal diagnostic or screening test. PSA is prostate-specific rather than prostate cancer-specific, and is elevated in other non-malignant conditions such as benign prostatic hyperplasia (BPH), a very common disorder of aging males.

PSA is a glycoprotein that forms complexes with protease inhibitors. Generally, presence of elevated PSA in the systemic circulation indicates some kind of disruption of barriers that normally contain PSA within the gland; levels of PSA in the seminal fluid are a million-fold greater than those present in the systemic circulation. It has been shown that disruption of these barriers is associated with prostatic intraepithelial neoplasia (PIN) and prostate cancer. Circulating PSA is found in two forms (see Exhibit 1), free (fPSA) and complexed (cPSA). Excess PSA, usually the hallmark of prostate cancer, is bound by glycoprotein antiproteases released by the liver to prevent PSA's enzymatic activity in semen liquefaction. In serum, most of immunoreactive PSA is complexed with α_1-antichymotrypsin (ACT). A small fraction is also complexed with or α_2-microglobulin (AMG), but this PSA is not detectable by immunoassay because its epitopes are masked. Total PSA (tPSA), therefore, is the sum of fPSA and PSA-ACT.

Because of the financial burden, as well as patient anxiety, associated with false-positive results, several approaches are being attempted to improve the specificity of PSA testing, including obtaining values of PSA velocity (PSAV), PSA density (PSAD), age-related PSA, as well as the different forms of circulating PSA (Exhibit 1). PSAV appears to be a specific marker for the presence of prostate cancer. When changes that occur in PSA over an elapsed time of 1.5 to 2 years are adjusted, fewer than 5% of men without prostate cancer, but approximately 70% of those with prostate cancer will have a PSAV ≤0.75 ng/ml per year (Carter HB and Pearson JD, Urol Clin North Am, May 1997, 24(2):333-8).

Another means of improving PSA specificity is direct measurement of either fPSA and/or cPSA. Using the ratio of fPSA/tPSA has made it possible to distinguish BPH from prostate cancer because BPH is associated with a higher proportion of fPSA in serum; the higher the fPSA/tPSA the more the likelihood of benign disease. In those with biopsy-confirmed prostate cancer, fPSA is usually under 25% of tPSA. However, using cut-off levels of this ratio that may correctly identify most BPH cases, thus preventing many unnecessary biopsies, may also fail to detect a number of prostate cancer cases. Researchers believe that cancers missed by this test are very few and, generally, less aggressive.

In March 1998, the FDA approved Hybritech's fPSA test that is targeting men whose PSA value falls between the gray area of 4 ng/ml and 10 ng/ml and who would normally be referred for a biopsy. In a multicenter clinical trial involving 773 men, aged between 50 and 75 years, with PSA values within this gray area, fPSA detected 95% of prostate cancers. Abbott Laboratories, in collaboration with EG&G Wallac, holds the USA and European patents for fPSA and is expected to obtain FDA approval for its fPSA test by the end of 1998. The fPSA patents were issued to Dr. Ulf-Hakan Stemna of the Helsinki University Central Hospital in Finland and Dr. Hans Lilja of Lund University in Sweden, and assigned to EG&G Wallac. Abbott is currently suing DPC Bierman, the German subsidiary of Diagnostic Products Corporation, for patent infringement. Taking a different route, Bayer Diagnostics (Tarrytown,

Exhibit 1
Types of PSA Tests

Test	Description
Total PSA (tPSA)	tPSA is the sum of free PSA (fPSA) and PSA complexed with α_1-antichymotrypsin (PSA-ACT); cutoff is 4 ng/ml.
Free PSA (fPSA)	Free PSA is the measure of PSA circulating unbound in the blood stream. It has five epitopes available but three of them are masked by PSA-ACT. It is the least represented form of PSA found in the circulation but is the major component of seminal plasma. Free PSA makes up a higher proportion of total PSA in BPH. Hybritech obtained FDA approval to market its fPSA test in March 1998.
fPSA/tPSA ratio	Free PSA results are used to calculate the fPSA/tPSA ratio in the case of patients who have PSA values between 2.5 and 10 ng/ml and who may be at risk for prostate cancer but would like to determine the degree of the risk before opting for a biopsy.
PSA-ACT or complexed PSA (cPSA)	PSA complexed with α_1-antichymotrypsin (ACT), or PSA-ACT, accounts for approximately 90% of the identifiable PSA in the blood. This form of PSA has three epitopes available. A cPSA test is under development by Bayer Diagnostics.
PSA-AMG	PSA complexed with or α_2-microglobulin (AMG), or PSA-AMG, has no epitopes detectable by currently available assays.
PSA velocity (PSAV)	PSAV can best be defined as the speed at which a series of PSA values increases or decreases. It is believed that PSAV is an indicator of the way prostate cancer may be developing in patients; cutoff is 0.75 ng/ml/year.
PSA density (PSAD)	PSAD, also known as PSA index, is a measure of the concentration of PSA in the prostate and is calculated by dividing the amount of serum PSA by the ultrasonographic volume of the prostate gland; it is expressed in ng/ml/ml. Studies have shown that carcinomas demonstrate a 10-fold increase in PSAD compared to BPH (NEJM. 317:1990). A cut-off value of 0.30 ng/ml/ml for PSAD is predictive of capsular or lymph node invasion with a diagnostic accuracy of 94% (Doublet JD, etal, Prog Urol, Apr 1995, 5(2):211-9).
Urine PSA (uPSA)	Measurement of uPSA in the follow-up of radical prostatectomy patients may identify localized relapse earlier than PSA, and indicate localized response to radiotherapy or hormonal therapy; the cut-off is 0.1 ng/ml (Marzano D, etal, Arch Ital Urol Androl, Feb 1997, 69(Suppl 10):105-8).

NY) is developing a PSA test to directly measure PSA-ACT. The test will be filed for FDA approval for monitoring prostate cancer. With the exception of tPSA, all these tests are for diagnostic/monitoring applications only.

Researchers have also discovered that when cells in the circulation of prostate cancer patients stain positively for both PSA and the monocyte marker CD14, there is a low risk for metastasis. Therefore, CD14 may be helpful in assessing prognosis of organ-confined prostate cancer, and may be used to select patients at low-risk of developing bone metastasis despite presence of circulating prostate cancer cells. Actually, circulating PSA-positive cancer cells do not provide a reliable means of assessing risk of metastasis. In a comparison between 17 controls (8 with bladder cancer and 9 with BPH) and 27 patients with prostate cancer (16 with Stage N0M0 disease and 11 with documented Stage M1 prostate cancer that had metastasized to the bone), the number of PSA/CD14 doubly-positive cells were almost equal in all instances except in Stage M1 disease where there were virtually non-existent (Burkhard B, etal, JNCI, 2 Jan 97, 89 (2):174).

One of the most important contributions of PSA has been its role in prostate cancer screening which is now accomplished either by use of PSA testing or digital rectal examination (DRE), or both. PSA-based screening in the USA increased dramatically in the past decade but appears to have reached a plateau (see Exhibit 2). A similar trend is seen abroad, but PSA-screening levels outside the USA are nowhere near domestic levels. Manufacturers estimate the 1997 USA volume of diagnostic PSA tests at approximately 7.5 million and the overall USA PSA testing volume, including screening tests, at between 22 million to 30 million. Testing volume in Europe and Japan, and other foreign markets are estimated at approximately 21 million.

Despite aggressive screening programs in the USA, there is little evidence to support screening of prostate cancer using PSA and/or DRE in asymptomatic patients. PSA-based screening as practiced today, although a remarkable tool, is not foolproof and remains controversial. Although high total PSA values (>4.0 ng/ml) are more likely to indicate malignancy, low values do not always preclude it. For instance, among 760 men tested, one cancer (<0.2%) was detected among 559 men with total PSA values <2.0 ng/ml and 8 (3.9%) were detected among 201 men with PSA values between 2.1 and 4.0 ng/ml. Also, low PSA values remained unchanged over a 3-year period indicating that screening men with PSA <2.0 ng/ml may be unnecessary (Harris CH, etal, J Urol, May 1997, 157:1740-3).

PSA has been recommended for use in screening of asymptomatic men, in combination with a DRE, by the American Cancer Society, American College of Radiology and American Urologic Association (AUA). However, in 1997, the American College of Physicians concluded that there was no evidence of benefit from routine screening using PSA and recommended against regular screening using this test. The AUA, that is backing legislation for Medicare coverage of screening tests for eligible men over 50 years of age, claims that PSA and DRE screening increases

the rate of early cancer diagnosis from 30% to 40% for those not screened to 70% to 85% for those screened with PSA. The problem is high false positives leading to unnecessary biopsies. However, newer PSA tests that may offer higher specificity in differentiating between BPH and prostate cancer, may prove more useful as screening tools. For instance, Hybritech believes that its newly approved fPSA test that is to be performed when the initial PSA screening test is elevated, will reduce biopsies by 20%.

In a questionnaire survey of a random sample of 462 general practitioners in New Zealand, to ascertain their opinion as to the value of screening in asymptomatic patients, approximately 50% of the 317 physicians who completed the questionnaire believed DRE and PSA were effective and that asymptomatic men should be screened, while 40% believed all men aged 50 years or older should be screened using either DRE or PSA. Regardless of beliefs about the efficacy of the tests, the majority of the general practitioners screened at least some of the men aged 50 years or more on their lists using DRE or PSA. Interestingly, significantly more general practitioners in the age group >50 years, believed asymptomatic patients should be screened with DRE or PSA (Morris J and McNoe B, New Zealand Medical J, 23 May 1997, 110:1044 178-82).

PSA has also been recommended as a monitoring test. The American Society for Therapeutic Radiology and Oncology, in a consensus statement, is recommending that PSA measurements should be obtained every 3 to 4 months during the first 2 years post radiotherapy and/or surgery, and if no untoward PSA rise is detected, every 6 months thereafter (Int J Radiat Oncol Biol Phys, 1997, 37:1035-41). However, increases in PSA post-chemotherapy treatment may be attributable to the treatment itself. For instance, researchers discovered that treatment with TNP-470, an angiogenesis inhibitor under development by Takeda (Osaka, Japan), in collaboration with TAP Pharmaceuticals (Deerfield, IL), causes PSA to rise *in vitro*. In a human prostate cancer line, introduction of TNP-470 reduced the number of cancer cells by 40% but increased PSA levels by 52% (Dixon SC, etal, AACR98, Abs. 298:44).

Numerous PSA tests are on the market or in development, worldwide (see Exhibit 3). Estimates of the worldwide market for PSA tests at the manufacturers' level vary based on the price of the test that has been estimated at between $4.50 and $9.00 for the diagnostic version to $2.50-$5.00 for the screening version. Prices and end-user costs depend on testing volume. The USA and global markets for PSA tests are estimated at $121.3 million and $245.8 million, respectively (see Exhibit 4). Other industry sources place the USA market at $150 million.

In the USA, it is estimated that Abbott Laboratories controls over 50% of unit test sales, with Hybritech, a distant second, with 15% of unit sales. Bayer Diagnostics and Chiron Diagnostics are also significant market participants, with under 10% and 5% of the USA market, respectively. Abbott's lead is mostly attributed to its automated assays. Efforts are also underway at Beckman Coulter (Fullerton, CA) to automate the Hybritech Tandem PSA using the Access Immunoassay technology acquired from Sanofi Diagnostics Pasteur (Chaska, MN) in April 1997.

Prostate Acid Phosphatase (PAP)

Prostate acid phosphatase (PAP) is a prostate-specific protein tyrosine phosphatase regulated by androgen. PAP, an inexpensive popular test before the advent of PSA, is currently being investigated as a prognostic marker. Although PAP does not appear to predict pathological stage or margin status, it may be a predictor of recurrence after radical prostatectomy (Moul JW, etal, T Urol, Mar 1998, 159(3):935-40).

ProstaScint

ProstaScint (capromab pendetide), an *in vivo* imaging radioimmunoconjugate developed by Cytogen (Princeton, NJ), was approved in November 1996 for imaging newly-diagnosed biopsy-proven prostate cancer at risk for occult disease, and suspected occult cancer recurrence in post-prostatectomy patients. ProstaScint uses a MAb (7E11-C5 or CYT-356) linked to indium-111 which targets prostate specific membrane antigen (PSMA), a protein expressed by prostate cancer cells and, to a lesser extent, by normal prostatic epithelium. The MAb used in ProstaScint is exclusively licensed to Cytogen.

Based on its approved indications, the USA market opportunity for ProstaScint consists of the approximately 20% of newly-diagnosed prostate cancer cases at high risk for metastatic disease and 40,000 to 60,000 patients previously treated for prostate cancer who develop symptoms of recurrent cancer that has not yet progressed to the point of skeletal involvement. Therefore, approximately 75,000 to 100,000 prostate cancer cases are candidates for ProstaScint scan each year in the USA and as many or more abroad.

ProstaScint was launched in the USA in February 1997, priced at $719 per course. It is being co-marketed by the Bard Urological Division (Covington, GA) of C.R. Bard (Murray Hill, NJ) under an agreement negotiated with Cytogen in August 1996. Bard Urological markets ProstaScint to urologists while Cytogen trains the medical imaging community through its Partners in Excellence (PIE) program. Both companies collaborate in marketing ProstaScint to managed care organizations. Sales of ProstaScint were $4.1 million in 1997. Cytogen, through Targon (Princeton, NJ), its joint venture with Elan (Dublin, Ireland), is developing Prostatec, a second generation version of ProstaScint.

Angiogenesis

Angiogenesis may also be an indicator of prostate cancer stage, prognosis and tumor progression. Bard Diagnostic Sciences (Redmond, WA) created the BioStage service which quantifies tumor angiogenesis (optimized microvessel density analysis) from a needle biopsy taken from tumors.

Cytokine Overexpression

Cytokine overexpression may be a hallmark of metastatic prostate cancer. Highly metastatic cells, growing in culture, constitutively and uniformly express higher levels of basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), type IV collagenase (72-kd and 92-kd), and multidrug resistance (mdr-1) mRNA transcripts than parental PC-3M cells or low metastatic cells, which display a heterogeneous pattern of gene expression. Human prostate cancer cells, implanted subcutaneously in nude mice at an ectopic site, expressed lower levels of epidermal growth factor receptor (EGFr), mdr-1, bFGF, IL-8, and collagenase type IV, than those implanted orthotopically in the prostate, indicating that the expression of these genes was dependent on the organ environment. Highly metastatic cells growing in the prostate expressed higher levels of EGFr, bFGF, type IV collagenase, and mdr-1 mRNA than low metastatic parental cells in the same site.

Exhibit 2
Total PSA Tests Performed in all Men and in Men Over Age 65 Between 1990 and 1997 in the USA

	Total Male Population		Total Male Population Over 65 Years of Age			
Year	PSA Tests		PSA Tests[2]	Total Cost per Procedure	Average Cost	Cost Covered by Medicare
	(#)	Rate[1]	(#)	($)	($)	($)
1997	28,850,500	22,107	4,819,237	297,494,832	61.73	55.00
1996	26,386,927	20,327	3,958,039	235,827,447	59.58	53.43
1995	30,636,207	23,935	4,595,431	272,111,595	59.21	54.30
1994	30,225,560	23,751	4,533,834	263,446,329	58.11	56.22
1993	27,892,093	22,157	4,211,706	243,902,737	57.91	63.75
1992	23,121,362	18,570	3,514,447	201,852,242	57.43	29.56
1991	14,658,469	12,055	2,125,478	120,065,655	56.49	28.74
1990	6,600,243	5,428	924,034	50,881,241	55.06	29.13

[1] Number of PSA tests per 100,000 males
[2] Diagnostic tests only
Source: HCFA and New Medicine estimates

However, although cytokines may serve as angiogenesis markers, both normal and malignant prostate epithelial cells express cytokines shown to possess angiogenic properties such as vascular endothelial growth factor (VEGF), bFGF, transforming growth factor-α (TGF-α), transforming growth factor-β1 (TGF-β1), IL-8, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF); therefore expression of these cytokines is intrinsic and not acquired during oncogenesis (Campbell CL, etal, AACR98, Abs. 364:53).

There appears to be a direct correlation between expression of several metastasis-related genes and the metastatic potential of human prostate cancer cells in nude mice. Therefore, multiparametric *in situ* hybridization analyses may be used to identify the metastatic potential of individual patients' prostate cancers (Greene GF, Am J Pathol, May 1997, 150(5):1571-82).

NOVEL MOLECULAR MARKERS

Factors associated with the etiology and pathogenesis of prostate cancer were discussed in considerable detail in FO, pp 284-286. Exhibit 5 lists and describes some of the key markers proven or assumed to be linked to the presence or status of prostate cancer. New markers are being discovered on a daily basis. For instance, a repeated differential display polymerase chain reaction (PCR) assay combined with Northern and slot blot hybridization, identified 10 uncharacterized genes that were either expressed differently in malignant and benign cells or were expressed at significantly higher levels in metastatic cell lines (Jing C, AACR98, Abs. 3089:454).

Among significant genetic changes in prostate cancer are gains for chromosomes 8q (identified in 80% of prostate cancer specimens), 11p and 3q, aneusomies of chromosomes 7 and 8; and allelic losses at chromosome regions 5q, 7q31.1-31.2, 8p12-21 (incorporating a putative tumor suppressor gene), 10q23-24, 13q and 16q22.1-24 (Dong JT, etal, Curr Opin Oncol, Jan 1997, 9(1):101-7). A tumor suppressor gene may also reside at 5q11 (Ozen M, etal, AACR98, Abs. 2316:339).

Various genomics companies are searching for prostate-cancer associated genes. Genset (Paris, France) has located genes on chromosome 1 as well as other loci, as part of a collaboration with Synthelabo (Paris, France) entered in April 1996, to discover prostate cancer-associated genes (see FO, p 312). In November 1996 Genset applied for patents relating to findings of genes associated with prostate cancer. Genset is collaborating in this effort with Centre de Recherche pour les Pathologies Prostatiques (CEREPP) based at Hospital Saint-Louis (Paris, France). In April 1998, Genset and Synthelabo extended their two-year collaboration for another year.

In 1997, Schering AG (Berlin, Germany) established a new subsidiary, metaGen (Berlin-Dahlen, Germany), to conduct application-oriented basic research into the molecular genetics of multifactorial diseases with current emphasis on identifying the molecular basis of spontaneous forms of breast and prostate cancer. Researchers at UroCor (Oklahoma City, OK) have also identified genes that are involved in prostate cancer metastases (An G, etal, AACR98, Abs. 1418:208). Gene Logic (Columbia, MD) is

using its restriction enzyme analysis of differentially expressed sequences (READS) approach to quantify gene expression levels in normal and malignant prostate cells, study patterns of gene regulation and identify key genes. Other genomics companies seeking prostate cancer genes are Myriad Genetics (Salt Lake City, UT), Millennium Pharmaceuticals (Cambridge, MA) and Human Genome Sciences (Rockville, MD).

Among important markers being sought are those that determine oncogenesis, invasion, metastasis and immortalization. Although possible markers are being constantly identified, their function still remains obscure. Interestingly, independent cell type (prostatic epithelial cells or fibroblasts)-specific mechanisms may be involved in the immortalization of different tumor cell types (Naimi B, etal, AACR98, Abs. 853:125).

Markers Associated with Familial/Hereditary Prostate Cancer

Unlike other major malignancies such as breast and colorectal cancer, no direct hereditary link has been conclusively identified in prostate cancer. However, recently familial clustering and Mendelian inheritance patterns of some prostate cancer has been confirmed. A segregation analysis study conducted in Sweden, involving a random sample of 2,857 nuclear families, designed to understand the nature of familial aggregation of prostate cancer, showed that the clustering of this cancer is caused by a high risk dominant allele with a population frequency of 1.67% and a lifetime penetrance of 63% (Grönberg H, etal, Am J Epidemiol 1997 Oct 1 146:7 552-7).

Analysis of the results of a literature search conducted between January, 1956, and October 31, 1994, which included case-control studies involving incidence of prostate cancer and relative risk (RR) of such cancer in the families of men with this disease compared with a control group, indicated that lifetime risk of prostate cancer is 9.5% and of death from prostate cancer is 2.9% for a man 50 years-of-age. For first-degree male relatives of men with prostate cancer, the calculated RR ranges from 1.7 to 8.73. Based on these findings, it appears that hereditary prostate cancer accounts for an estimated 43% of early-onset disease, affecting men <55 years-of-age, but only for 9% of all prostate cancers in men between 55 and 85 years-of-age (McLellan DL and Norman RW, CMAJ, 1995 Oct 1, 153(7):895-900).

Androgen receptor gene mutations involving repeats in the polymorphic (CAG)n and (GGN)n regions within the androgen receptor gene, have also been linked to prostate cancer risk. Associations between repeat lengths and risk of prostate cancer and the effects of confounding and modifying factors such as age, family history of prostate cancer, and body mass index, were evaluated in a population-based case-control study of prostate cancer in middle-aged (40-64 years) Caucasian men. DNA from 301 cancer cases and 277 controls was evaluated for repeats. The overall age-adjusted relative odds (RO) of prostate cancer associated with the number of CAG repeats as a continuous variable, was 0.97, suggesting a 3% decrease in risk of prostate cancer for each additional (CAG) repeat.

Further analyses identified several subgroups at increased risk. RO was 1.47 in men <60 years-of-age with fewer than the median number of CAG repeats (<22), 1.59 if they had an affected first-degree relative and 2.21 if they were relatively thin. Although only the latter result was statistically significant, these findings are provocative and support the hypothesis that (CAG)n array length is a predictor of prostate cancer risk. Similar analyses of (GGN)n showed that with the exception of men with a family history of prostate cancer and those in the highest quartile of body mass index, men with ≤16 repeats had a higher risk than did men with >16 repeats. Overall, men who had ≤16 repeats had a significant elevation in risk (RO=1.60). When both repeat lengths were considered jointly, the subgroup with two short repeats (CAG <22 and GGN ≤16) had a 2-fold elevation in odds (RO=2.05) relative to those with two long repeats (CAG≥22 and GGN >16). These data suggest that determination of both androgen receptor repeats within germ-line DNA may be useful in assessing an individual's risk of developing prostate cancer (Stanford JL, etal, 15 Mar 1997, Cancer Res, 57(6):1194-8). The number of androgen receptor gene mutations now stands at 309 (Gottlieb B, etal, Nucleic Acids Res, 1 Jan 1998, 26(1):234-8).

BRCA1 or BRCA2, in addition to breast cancer, may confer increased risks of ovarian, and prostate cancer. Carriers of the 185delAG and 5382insC mutations in BRCA1 and the 6174delT mutation in BRCA2 were identified in collected blood samples from 5318 Jewish subjects who had filled out epidemiologic questionnaires. Risks of breast and other cancers were estimated by comparing cancer histories of relatives of carriers of the mutations with those of noncarriers. The estimated risk of the 125 carriers of a BRCA1 or BRCA2 mutations, by age 70, was 56% for breast cancer, 16% for ovarian cancer and also 16% for prostate cancer (Struewing JP, etal, NEJM, May 15, 1997, 336:20 1401-8). For additional information on BRCA1 and BRCA2, see FO, pp 498 and 361-362.

Hereditary prostate cancer 1 (HPC1), a locus recently identified on chromosome 1q24-25, has been associated with familial cancer. Families carrying HPC1 are characterized by multiple cases of prostate cancer, younger age at diagnosis (63.7 versus 65.9 years-of age), higher tumor grade (Grade 3 cancer occurrence was 41% versus 31%), and a higher incidence of advanced-stage disease. Therefore, there is definite clinical importance of this gene for early detection of prostate cancer (Grönberg H, etal, JAMA, 278(15): 1251-5 1997 Oct 15). Proof of the existence of HPC1 originally came from researchers at the National Human Genome Research Institute (NHGRI; Bethesda, MD), formerly known as the National Center for Human Genome Research (NCHGR), Johns Hopkins University (Baltimore, MD) and Universitet i Umeå (Umeå, Sweden). Location of a cancer susceptibility gene on chro-

Exhibit 3
PSA Tests on the Market and/or in Development

Company	Product	Type of PSA Test ❑ Methodology	Comments
Abbott Laboratories (Abbott Park, IL)	AxSYM	Total PSA ❑ microparticle enzyme PSA immunoasay (high-volume)	On the market in the USA and, as of 1995, abroad
Abbott Laboratories	IMx PSA	Total PSA ❑ medium volume immunoassay	On the market
Abbott Laboratories	PSA test	Total PSA	On the market
Abbott Laboratories ❑ EG&G Wallac (Turku Finland)	Free PSA and free-to-total PSA ratio		Received a USA patent in March 1996 and European patent in February 1996
AMDL (Tustin, CA)		Total PSA ❑ microwell PSA EIA	Available outside the USA (Europe)
AMDL	Q Check PSA Test	Total PSA	Available outside the USA (Europe)
AMDL	Q Stick PSA Test	Total PSA	Available outside the USA (Europe)
Bayer Diagnostics (Tarrytown, NY)	Bayer Immuno 1 Assays	Total PSA	On the market
Bayer Diagnostics	Bayer Immuno 1 Assays	Complexed PSA	May replace the free to total ratio PSA; in development
Bayer Diagnostics	Bayer Immuno 1 Assays	Free to total PSA	In development
Behring Diagnostics (San Jose, CA)	Opus PSA	Total PSA	On the market in the USA
Biomerica (Newport Beach, CA)	EZ PSA	Total PSA	Home test for PSA; not available in USA
Chiron Diagnostics (was Ciba Corning Diagnostics; Norwood, MA)	ACS PSA2	Total PSA ❑ direct chemiluminescence immunoassay test as a component of the ACS:180 system	On the market; received FDA approval in August 1994
Cholestec (Hayward, CA)	L•D•X	PSA	In development
CIS biointernational	RIACT	Free PSA	In development
Diagnostic Products Corporation DPC; (Los Angeles, CA)	Immulite	Total PSA	Third generation PSA assay; on the market
DPC Bierman (Germany)		Free PSA	Available outside the USA
Diagnostic Systems Laboratories (Webster, TX)	ACTIVE PSA (DSL-9700)	Free and total PSA ❑ IRMA	Available outside the USA
Diagnostic Systems Laboratories	ACTIVE PSA (DSL-10-9700)	Free and total PSA ❑ ELISA	Available outside the USA
Diagnostic Systems Laboratories	ACTIVE PSA, Ultra-Sensitive (DSL-10-9900)	Free and total PSA ❑ ELISA	Available outside the USA
Dianon (Stratford, CT)	PSA Test	Free and total PSA	Next generation test, received second patent; in development
EG&G Wallac	Prostatus	fPSA/tPSA ratio ❑ autoDELFIA System	
Horus Therapeutics (Rochester, NY)	ProstAsure		Clinical information processing software system based on artificial neural networks and other pattern recognition technologies; established through a proprietary mathematical algorithm that uses a patient's age and serum levels of PSA, PAP, total CK or CK-BB, CK-MB, and CK-MM (see FO, p 290)
Hybritech (Beckman Coulter; San Diego, CA)	Tandem-R PSA	Total PSA ❑ equimolar dual MAb RIA	On the market
Hybritech	Tandem-E PSA	Total PSA ❑ equimolar dual MAb EIA	On the market
Hybritech	Tandem-MP	Total PSA ❑ equimolar solid phase immunoassay	Introduced to Europe in 1996 by Hybritech Europe SA
Hybritech	Free PSA Test	Free PSA	Approved by the FDA on March 10, 1998

— continued on next page

Iatron Laboratories (Tokyo, Japan)	LPIA ACE PSA	Total PSA ❑ latex turbidimetric immunoassay	
Immuno-Biological Laboratories (Hamburg, Germany)	PSA IRMA 100	Total PSA ❑ IRMA	Available outside the USA
Immuno-Biological Laboratories	Free PSA IRMA 100	Free PSA ❑ IRMA	Available outside the USA
Immuno-Biological Laboratories	PSA ELISA 96	Total PSA ❑ ELISA	Available outside the USA
Immuno-Biological Laboratories	fPSA ELISA 96	Free PSA ❑ ELISA	Available outside the USA
MediCorp (Montreal, Quebec Canada)	Free/Total PSA Ratio	Free and total PSA	Distributed in USA by Dianon, co-owner of PSA diagnostic patent with Dianon (Diagnostic Decisions)
Merck KGaA	MAGIA	Total PSA ❑ ELISA	
Morningstar Diagnostics (Roseville, CA)	One Step PSA Test	Total PSA	Available outside the USA
Quantimetrix (Redondo Beach, CA)	Imusure	Total PSA	Approved by the FDA; about to be launched
Roche Diagnostic Systems/ Boehringer Mannheim (Indianapolis, IN)	Elecsys PSA	Equimolar detection of both free and total PSA ❑ immunoassay	Available outside the USA
Roche Diagnostic Systems/ Boehringer Mannheim	Enzymun-Test	Free and total PSA	Available outside the USA
Roche Diagnostic Systems	Cobas Core PSA		On the market
Tosoh		Total PSA	Uses Hybritech MAb assay in an automated system
UroCor (Oklahoma City, OK)	Free/Total PSA Ratio	Free and total PSA	Used in an investigational-basis only byUroCor in conjunction with their UroScore Biopsy
Yang Laboratories (Bellevue, Washington)	Pros-Check	Total PSA ❑ polyclonal competitive radioimmunoassay	On the market

mosome 1q was also confirmed by others (Cooney KA, etal, JNCI, 1997, 89:955-959). Several groups are working to clone HPC1.

Other Markers

With the exception of PSA, few molecular markers exist or are being developed that are diagnostic or prognostic of prostate cancer.

Insulin-like growth factor-I (IGF-I) has also been associated with prostate cancer. A multicenter study conducted by Dr. Michael Pollak and colleagues at McGill University (Montreal, Canada) has found that men with levels of circulating IGF-I in the top 25% are 4.3 times more likely to develop prostate cancer than men with lower levels of IGF-I. Also, a significant linear trend was identified that indicated that 100 ng/ml increase in IGF-I doubles the risk of developing prostate cancer. The study was based on 152 cases and 152 controls from the Physician's Health Study (Chan JM, etal, AACR98, Abs. 2488:365). This finding may adversely effect programs using IGF-I as a treatment for neurological conditions.

Nuclear matrix proteins form a three-dimensional framework within the cell nucleus, to help organize active genes in the nucleus and, therefore, play a fundamental role in determining cell type and cell function. Although the specific mechanisms of action are not yet fully understood, Matritech (Newton, MA) and others have demonstrated that there are differences in the types and amounts of NMPs found in cancerous and normal tissues and also among different types of normal cells.

In collaboration with clinicians at Johns Hopkins University Medical School, Matritech scientists have identified a nuclear matrix protein, NMP-23, present in elevated amounts in the cells of prostate cancer patients and absent, or present in low amounts, in normal and benign disease. The company has developed monoclonal antibody (MAb), PRO4:216, to this protein which was tested by Johns Hopkins scientists using prostate biopsies (Partin AW, etal, Urology, Nov 1997, 50(5):800-8) and found to be clinically useful in differentiating prostate cancer cells from their normal and benign counterparts. Matritech has confirmed that this NMP is released into the blood and is presently evaluating other antibodies to this protein using a macromolecular biological interaction analysis instrument, as a first step in developing a clinical fluid-based assay for use in the management of prostate cancer.

Matritech also has a research agreement with the University of Pittsburgh to identify specific nuclear matrix proteins for predicting prostate cancer metastasis. Pittsburgh scientists have identified these types of proteins

and have isolated and obtained partial peptide sequences. Matritech intends to produce specific MAb: to these proteins and develop a blood and/or tissue-based prostate cancer test. The company has retained an option to obtain worldwide manufacturing and marketing rights for any prostate cancer test resulting from the Pittsburgh NMPs.

P53 mutations have also been detected in prostate cancer. Sporadic p53 mutations or allele losses are usually associated with advanced disease. A phase I clinical trial using an adenoviral vector to insert wild-type p53 *in vivo* in the tumors of prostate cancer patients, sponsored by Introgen Therapeutics (Austin, TX) in collaboration with Rhône-Poulenc Rorer, is ongoing. Also, Cascade Oncogenics (Portland, OR) is using its targeted inverted repeat DNA amplification (TIRA) technology to identify p53-regulated genes related to DNA repair and the cell cycle.

In order to understand why prostate cancer exhibited an exceptionally higher incidence in American males than in Japanese males, the frequency and spectrum of p53 gene mutations in a series of 90 lesions in Japanese males, were examined using polymerase chain reaction (PCR)-single-strand conformation polymorphism (SSCP) analysis. Patients' mean age was 69.3 years (range=57-87) and median Gleason score was 7.9. Mutations in exons 2-11 of the p53 gene were found in 11/90 cases (12%) and were significantly associated with advanced disease stage [0 of 5 with clinical Stage A, 1 of 25 with Stage B (4%), 3 of 35 with Stage C (9%) and 7 of 25 with Stage D (28%) cancers]. Six transversions (55%) and four transitions (36%) were found. Previous reports comparing the p53 gene mutational spectrum in prostate cancer in Japanese males with those reported for American and European subjects, showed that, in Western societies, 33% were transversions and 61% were transitions. This data suggests that different factors are responsible for carcinogenesis of the prostate gland in Japan and the USA, and provides an explanation as to the recent increase in prostate cancer incidence with the westernization of lifestyle in Japan (Watanabe M, etal, Carcinogenesis, Jul 1997, 18(7):1355-8).

Prostate specific membrane antigen (PSMA) is a unique antigen expressed by prostatic epithelial tissue. PSMA, a glycoprotein that reacts with MAbs 7E11.C5 and 3F5.4G6, is highly specific for prostatic epithelial cells but overexpressed in prostate cancer (Murphy GP, etal Prostate, Apr 1996, 28(4):266-71). It is overexpressed in primary prostate cancer, but it is expressed most highly in the more aggressive forms of prostate cancer, including those that do not express PSA and those that do not respond to hormone therapy. When compared to various PSA tests (PSAD, tPSA, fPSA), PMSA demonstrated the best correlation with primary tumor stage; elevated PMSA values correlated best with poor prognosis (Murphy GP, etal, Cancer, 15 Aug 1996, 78(4):809-818).

PSMA was extensively studied and the gene for PSMA first cloned at Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY). In July 1996, a patent entitled "Prostate-Specific Membrane Antigen" was issued to Sloan-Kettering Institute for Cancer Research, an affiliate of MSKCC. An assay based upon detection of the gene for PSMA was able to detect circulating prostate cancer cells in the blood of patients at high risk for prostate cancer metastases. In November 1996 Cytogen obtained an exclusive, worldwide license to this technology and PSMA is the antigen targeted by the 7E11-C5 (CYT-356) MAb used in ProstaScint.

Hybritech and Pacific Northwest Research Foundation (Seattle, WA) have developed a test that uses a second MAb which targets the end of the molecule opposite the region which expresses the 7E11.C5 epitope, to form a sandwich assay. This assay may be able to measure PSMA in tissue fluids, including serum, and would be potentially useful in monitoring patients who are risk of metastatic prostate cancer.

In January 1997, Cytogen issued a non-exclusive option for the PSMA technology to Boehringer Mannheim (now merged with Roche Holdings) in the area of *in vitro* diagnostics, including reverse transcriptase-polymerase chain reaction (RT-PCR) assays to detect circulating prostate cancer cells in the circulation. This agreement provides Cytogen with an up-front fee and royalties payable if and when products come to market. Cytogen is continuing discussions with other companies interested in this aspect of the PSMA technology.

Prostate stem cell antigen (PSCA) appears to be upregulated only in prostate cancer and not in benign conditions, making it a tumor marker candidate. According to Dr. Robert Reiter of the University of California, Los Angeles (UCLA), the PSCA gene produces a protein that is found more prevalently on the cell surface of prostate cancer cells than normal prostate cells. The study further demonstrated that there was moderate to strong PSCA expression in 111 out of 126 (88%) prostate cancer specimens, including high-grade PIN and both androgen-dependent and androgen-independent tumors. PSCA could also have usefulness as a cell surface target site for antibody or other therapeutic intervention. UroGenesys (Santa Monica, CA) has obtained exclusive commercial rights to the PSCA gene.

MEETING COVERAGE

REPORT FROM THE SIXTH INTERNATIONAL CONFERENCE ON GENE THERAPY OF CANCER

SPONSORED BY THE SIDNEY KIMMEL CANCER CENTER NOV 19-22, 1997, SAN DIEGO, CA

Gene therapy strategies for treating cancer have had a difficult time moving from theory to clinical application. In the past few years there has been considerable skepticism about the clinical feasibility of this approach as a cancer therapy. But progress toward development of successful anticancer approaches is now evident, particularly in using gene therapy as part of an overall strategy which includes other interventions. One such strategy involves introducing genes into cells to render them more sensitive to more traditional chemotherapies.

Many gene therapy protocols have moved beyond the laboratory and are now being tested in humans. There are presently 190 clinical trials underway using gene therapy strategies in the USA alone, including 93 trials using immunotherapy approaches, 35 targeting gene markers, 31 attempting to enhance drug sensitivity, 21 focused on tumor suppressors and antisense strategies, and 10 to reverse drug resistance.

APOPTOSIS AND GENE THERAPY

A review of current understanding of mechanisms leading to apoptosis and their relationship to cancer therapeutics was presented by John Reed of the Burnham Institute (La Jolla, CA). Inhibition of the apoptotic cell suicide pathway is one of the most common biochemical defects found in tumors. When the process is intact, it helps to regulate tissue cell numbers in order to keep cells at homeostatic levels. In fact, daily, 50-70 billion cells are produced and eradicated in each human being. An imbalance, such as an increase in the rate of cell division or a decrease in cell death, causes an abnormal accumulation of cells and results in the formation of tumors. Therefore, finding ways to restore a functional apoptotic pathway is an important goal for treating many types of cancers.

All anticancer drugs commonly used in the clinic, as well as radiation therapy, depend on triggering this apoptosis pathway. The mechanism of apoptosis induction by these interventions, as well as by normal genotoxic stress events, involves several biochemical pathways. One such pathway involves p53 and is counter-regulated by proteins such as Bax and Bcl-2. Another pathway, the jun kinase (JNK) pathway, interacts with and activates the "death receptor" fas (CD95). The third pathway leading to apoptosis uses lipid second messengers such as ceramide, which also appear to activate the cell death receptor. Defects in these pathways play an important role in making tumor cells resistant to chemotherapy by preventing apoptosis.

Endogenous biochemical stimuli that trigger apoptosis include tumor suppressors, cytotoxic lymphokines, tumor necrosis factor (TNF), fas, calcium oxidants, growth factor deprivation, and even certain viruses. In all cases, there is activation of a family of caspases that are cysteine proteases that incorporate an Asp in the P1 position. These proteases degrade a variety of substrates, including cytoskeletal proteins, other protein kinases, U1 snRNP, etc. It is the proteolytic events, such as DNA degradation, collapse of microtubules, and changes in ion flux, which cause the phenotype associated with apoptosis.

The step which commits a cell to activate caspases involves the family of Bcl-2/Bax-type proteins. It is the imbalance in the relative ratios of these proteins that leads to survival or death. The Bcl-2 family can be divided into three groups characterized by their tendency to promote or inhibit apoptosis as well as their ability of forming homodimers or heterodimers with other groups. Group I includes Bax and Bak, and these proteins promote apoptosis and form homodimers or heterodimers with members of group II. The second group includes Bcl-2, Bcl-XL, Mcl-1, A1 (BFL-1) and Bcl-W. These proteins inhibit apoptosis and they can form homodimers or heterodimers with either group I or III. Among the members of group III are Bcl-Xs, BAD (a raf-1 kinase) and Bik/Nbk-1. Like group I, this group also promotes apoptosis, but the members do not homodimerize and they can only heterodimerize with members of group II. Group III probably serves to sequester Bcl-2 to prevent cell death, so they are transdominant inhibitors of Bcl-2.

An imbalance in the Bcl-2 family of proteins is often found in tumor cells. For instance, a number of cancer types overexpress Bcl-2 itself. In prostate cancer, progression to a hormone-refractory stage is associated with an up-regulation of Bcl-2. A decrease in levels of Bax has also been found in certain tumors and, in others, there may be an altered regulation of one of the proteins by, for example, a change in their post-translation modification.

Any gene that induces cell death is a potential tumor suppressor gene. Bax is considered to be a candidate tumor suppressor because mutations inactivating Bax are often found in tumors. In some cases these mutations involve a homopolymeric stretch of AGs that happens to lie in the coding region of the gene that is prone to frameshift mutations in tumors that have defects in the DNA mismatch repair pathway. Such inactivating frameshift mutations in Bax genes have been identified in colon and gastric cancers which are also characterized by microsatellite instability, i.e., an expansion or deletion of repeat elements that occurs when the mismatch repair process is defective. Further evidence for the role of Bax as a tumor suppressor is the predisposition of Bax knockout animals to developing tumors.

Heterozygosity and single copy loss of Bax may confer tumor cells a selective survival advantage because the ratio of Bcl-2 and Bax is critical; thus Bax, unlike other tumor suppressor genes, may not follow the Knudsen two-hit

mutation theory that proposes that a cell does not become a tumor cell until both alleles are inactivated. Decreased levels of Bax are known to occur frequently (35%) in breast cancer in later stages of progression, and this characteristic is associated with a poor response to chemotherapy and shorter survival. This may paradoxically occur with a loss of p53, suggesting that loss of p53 might downregulate Bax as well. Thus, a possible therapeutic strategy would be to turn on the apoptosis pathway downstream of p53 by transfecting tumor cells with the bax gene or somehow increasing its activity.

Exhibit 4
Worldwide PSA Markets in 1997

Type of Test	USA (#)	Price ($)*	Revenue ($ mil.)	Outside the USA (#)	Price ($)*	Revenue ($ mil.)	Total Market ($ mil.)
Diagnostic tPSA	7,500,000	5.50 (range= 2.50-9.00)	41.3	7,500,000	7.50	56.3	97.5
Screening tPSA	21,350,500	3.75 (range= 2.50-5.00)	80.0	13,649,500	5.00	68.2	68.2
Total	28,850,500	4.20	121.3	21,149,500	5.90	124.5	245.8

** Price is estimated at the manufacturer's level. Actual cost of the test at the end-user's level depends on the testing volume performed to allocate costs of controls, etc. The more tests performed the lower the cost.*

Although the function of the Bcl-2 family of proteins is not yet fully understood, they are known to be integrated into intercellular membranes, typically the outer mitochondrial membrane as well as the endoplasmic reticulum and the nuclear envelope. There are two possible ways Bcl-2 proteins may function to impact cell survival. One is the formation of pores in cytoplasmic membranes, which might promote transport of proteins capable of initiating the cell death pathway. The other is an interaction of Bcl-2-type proteins with other proteins that trigger this pathway. In this function Bcl-2 could serve as an adapter or docking protein to recruit other proteins to it and mediate their functional activation.

Understanding the function of the Bcl-2 family of proteins has been facilitated by analogy to its homologs in *Caenorhabditis elegans* death (CED) proteins. CED-3 and CED-4 are necessary for cell death to occur, while CED-9 protects the cell from death by inhibiting CED-4. Mammalian CED-4 counterparts are the caspase family of proteases (formerly known as interleukin-1β-converting enzyme, or ICE family). The prototype mammalian CED-9 protein is Bcl-2, while the mammalian homolog of CED-3 is not yet known and, indeed, may not exist. In *C. elegans* CED-4 appears to function as an ATPase. The cytosolic form of CED-4 binds and activates CED-3/caspases, initiating cell death. Activation involves an autocatalytic process whereby the caspases self-cleave. CED-9/Bcl-2 bound to the mitochondrial membrane can tether the CED-4/CED-3 complex and inhibit activation of CED-3/caspases, thus preventing cell death. CED-9/Bcl-2 may also form homodimers or heterodimers with Bax, which displace the CED-4/CED-3 complex and promote cell death.

Release of cytochrome c (Cyt c) from mitochondria appears to be critical for triggering cell death. Cyt c is found in the intermembrane space between the inner and outer membranes and is released into the cytosol shortly after induction of apoptosis. Once released into the cytosol, Cyt c recruits and activates caspases. Bcl-2/Bax appears to play a role in this. Complexes containing this family of proteins are located in the outer membrane of mitochondria and enriched at sites in which the outer and inner membrane are in contact. When Bax expression is upregulated by transfection, it induces release of Cyt c, suggesting that it might form a channel that allows Cyt c to escape. By contrast, transfection of Bcl-2 suppresses Cyt c release and caspase activation following an apoptotic stimulus. Thus, Bcl-2 seems to control activation of caspases.

In its function as a protein-binding death activator, Bcl-2 can serve as a membrane adaptor or docking protein for at least 5 proteins, Bax, Apaf-1, MRIT, Raf-1 and calcineurin. All these proteins bind members of the Bcl-2 family, in a region near their N-termini. MRIT may be a functional homolog of CED-4. When Bcl-2 forms a complex with BAD and Bcl-2 family group III member, the complex can be phosphorylated by Raf-1. In its non-phosphorylated form BAD promotes apoptosis by inhibiting Bcl-2; but BAD is released into the cytosol after it is phosphorylated, leaving Bcl-2 free to function in its cell survival capacity. Another kinase that can phosphorylate BAD is Akt, a protein kinase C (PKC) which has been implicated in a number of growth factor signal transduction pathways and is known to play an important role in regulating cell survival. Akt is a PKC-related serine-threonine protein kinase which contains a Src homology-2 (SH2)-like region. Most growth factors promote survival through a pathway in which the downstream kinase is Akt. Once phosphorylated by Akt, BAD is released into the cytosol and effectively neutralized. A phosphatase which binds to Bcl-2 is calcium-activated calcineurin (CnA/B). Its substrate is also BAD, but it activates it by dephosphorylation, allowing it to return to its interaction with membranes to neutralize Bcl-2/Bcl-X and induce cell death.

The mechanism of pore formation by Bcl-2 has been predicted by comparison with the known pore-forming domain of diphtheria toxin (DT) or bacterial colicins. When DT transports proteins, it does so by forming a membrane channel. Colicins form ion channels that are homol-

ogous to Bcl-2 homodimer channels. Liposome release of ions can be induced by adding colicin or Bcl-2. But Bax also induces a chlorine-efflux from KCl-loaded liposomes, so both Bcl-2 and Bax induce membrane transport in spite of the fact that they have opposite apopototic properties. One possible explanation for this is that they may form different types of channels. In models of channels formed by Bcl-2 and Bax, the hydrophilic amino acid residues lining the channel lumen would be acidic in one case and basic in the other.

There exists a multi-protein "mega" channel in the inner membrane which is enriched at the contact sites where the inner and outer membranes come together. This channel can open during apoptosis, leading to a depolarization of the mitochondrial membrane and uncoupling of oxidative phosphorylation. As a result, there is production of reactive oxygen species, rupturing of the outer membrane, release of Cyt c, and dumping of sequestered calcium into the cytosol. Bcl-2 might be involved in regulating this process either by directly interacting with the channel or by altering ion fluxes.

When recombinant Bax protein is added to isolated mitochondria, it induces a release of Cyt c and activation of caspases. Addition of Bcl-2 or Bcl-X suppresses this activation. Because Bax activation of Cyt c does not involve a swelling of mitochondrial membranes, which would be typical of calcium-activated Cyt c release, it probably functions by directly forming a channel through which Cyt c is transported. Once in the cytosol, Cyt c forms a complex with other proteins. One protein Cyto c interacts with is Apaf-1, which is a CED-4 homolog that binds ATP. Apaf-1 has an internal domain that is similar to the pro-domain in some caspases, and it can form a homophilic interaction with certain caspases. It also has a large regulatory domain involving WD repeats that is thought to bind to Cyt c. It is believed that this protein lies dormant in cells until activated by Cyt c release from mitochondria. Released Cyt c binds to and induces a conformational change in Apaf-1 that allows it to interact with caspases. Caspases are then activated by an energy-requiring mechanism provided by the ATPase activity of Apaf-1.

Release of Cyto c does not represent the point of no return in the decision to commit to cell death. There is a family of proteins that function as endogenous inhibitors of caspases called the IAP family of antiapoptotic proteins. They are characterized by presence of a common 16 amino acid domain called BIR (baculovirus IAP repeat) motif. IAPs have been reported to be overexpressed in nearly all tumor cell lines, thus their aberrant expression may be involved in tumorigenesis. IAPs work downstream of Cyt c to inhibit caspase and will inhibit activation of some of types of caspases by Cyt c in cell extracts *in vitro*.

It appears that there may be two parallel pathways that lead to activation of the downstream caspases (X-IAP, C-IAP-1, C-IAP-2). It is the downstream caspase group, the one activated by Cyt c, that is inhibited by the IAPs. Activation of the upstream caspases are induced by members of the TNF family of cytokine receptors which recruit caspases to the receptor. Upon activation, upstream caspases can then activate the downstream caspases, leading to apoptosis. Thus, there is a lot of crosstalk between these two pathways. In one such interaction, upstream caspases activate mitochondria and lead to release of Cyt c, thus activating the downstream caspases indirectly in addition to being able to activate them by direct cleavage. In addition, a number of these stimuli are now known to induce transcriptional upregulation of fas, TNF, TRAIL and other death receptors. These death receptors work by interacting with other proteins through a functional amino acid sequence called the death domain (DD). They recruit the adapter protein Fadd, which contains a death effector domain (DED). DED binds to a pro-caspase domain that triggers their activation by self-cleavage.

Regulation of this process occurs by participation of "pseudo-caspases". One of these, called FLIPS or Flames, is similar to pro-caspase 8 in that it contains a death domain, except that the sequence contains mutations that render it inactive in proteolysis. When FLIPS binds to caspase activators it blocks binding of the corresponding pro-caspase. In cancer cells FLIPS is often upregulated, making them resistant to Fas and inhibiting apoptosis.

The stress kinase pathway leading to apoptosis, can be inactivated in a similar manner. An adapter protein for that pathway has been isolated called Daxx, which contains the same death domain, and triggers a cascade of phosphorylation that activates the JNK pathway. Another regulator of this pathway is a protein tyrosine phosphatase called FAP-1 , which binds to the C-terminus of fas and prevents activation of cell death. This protein is overexpressed in certain colon cancers and probably contributes to a chemoresistant state in these tumors. Thus, there are several regulators of the various pathways leading to apoptosis, and the challenge is to find ways to overcome individual blocks and restore a cell's capability to respond to chemotherapy.

P53

The majority of human gene therapy clinical trials currently ongoing are attempting to interfere with the status of p53 *in vivo*. Extensive research in the status of p53 in normal and malignant cells has revealed that more than 52 distinct types of cancer are associated with p53 mutations and that such mutations are the single most common genetic aberration in over 50% of all human cancer cases.

Most gene therapy strategies covered in this session involved p53 gene replacement therapy using adenovirus as a delivery vector. Advantages of using adenovirus to deliver genes to cells stems from the fact that this group of viruses infect a broad range of cell types and they can replicate in quiescent as well as dividing cells. DNA does not integrate into the host chromosome and, therefore, cannot cause mutations as a result of a genetic insertional event. In some cases the virus is replication-defective and, therefore, does not kill the cell or spread. Thus, there is no viral-induced damage to non-tumor cells. One vector, ONYX-

Exhibit 5
Prostate Cancer Markers

Marker	Description
Androgen receptor	The androgen receptor, a cytosolic receptor protein, is the main target of hormonal therapy (see FO, pp 286 and 401 and the text). Contained within the androgen receptor gene is a repeating three-nucleotide sequence (CAG) with repeats occurring between 11 and 33 times in different men. The more the repeats, the lower the risk of prostate cancer
Anti-apoptotic proteins (Bcl-2, Bcl-X, and Mcl-1)	Studies have shown that levels of these proteins rise as prostate cancer progresses indicating that it may represent a phenotype of advanced prostatic carcinomas (Krajewska M, etal, AACR96, Abs. 3923:572-3). Expression of bcl-2 is highly correlated with cancer progression and androgen-independent prostate cancer phenotype.
Bombesin	See FO, p 286
Breast cancer gene 1 and 2 (BRCA1/BRCA2)	See FO, pp 498 and 361-362 and the text
c-MET	c-MET is a proto-oncogene encoding a receptor for hepatocyte growth factor (HGF), or scattering factor usually overexpressed in prostate cancer and, in a lesser degree, in breast cancer that may lead to invasiveness (Chau CM, etal, AACR98, Abs. 1444:211)
C-myc	Amplification and increased expression of c-myc is correlated with tumor progression in some human prostate cancer (Ashankyty IM, etal, AACR96, Abs. 1617:236)
Caveolin	Carveolin, the major constituent of caveolae, a differentiation marker for endothelial cells, can affect widespread gene activation. It is present in increased abundance as prostate cancer progresses (Thompson T, CaP CURE97).
Chondroitin sulphate (CS)-proteoglycan (versican)	Versican has shown to be associated with PSA progression. It may allow stratification of patients with early stages of prostate cancer for alternative therapies (Horsfall DJ, etal, AACR98, Abs. 1587)
DNA ploidy and S-phase fraction (SPF)	Aneuploid tumors were associated with higher progression rates and lower survival rates than non-aneuploid tumors in androgen-dependent prostate cancer; SPF was of no prognostic significance (Pollack A, etal, Prostate, 1 Apr 1997, 31(1):21-8)
E-cadherin expressed by a gene located in chromosome 16q	Decreased levels of E-cadherin, a calcium dependent cell adhesion protein, are commonly found in metastatic prostate cancer specimens (see FO, V2 #1, p 285)
Endothelins A and B and endothelin B receptor	See FO, p 287
Epidermal growth factor (EGF) and its receptor (EGFr)	See FO, p 286
Gonadotropin-releasing hormone (GnRH) and its receptor (GnRHr)	GnRH plays a role in the regulation of the male reproductive system. GnRH, but not its receptor, are expressed in normal prostate cells but both are expressed in tumors *in vitro* (Lau HL, etal, AACR98, Abs. 95:14)
Hereditary prostate cancer 1 (HPC1) gene located on chromosome 1q24-25	HPC1 is probably associated with cancer susceptibility (see text)
Heregulin/neu differentiation factor (HDF/NDF)	Studies suggest a deficiency of HDF may be involved in prostate cancer tumorigenesis (Lyne JC, etal, AACR96 Abs. 1660:243)
Human glandular kallikrein 2 (hk2)	Like PSA, hk2 is localized in the prostate and is also tumor-associated (Corey E, etal, Urology, Aug 1997, 50(2):184-8)
Insulin-like growth factor I (IGF-1) and IGF-II	According to one study, IGF-1 may be a better early-stage indicator of prostate cancer than PSA. Men with higher, but still normal levels of IGF-1 had more than four times the risk of prostate cancer (see FO, p 286)
IGF binding factors (IGFBP)	IGFBPs are circulating proteins that control the amount of IGF I and IGF II available for cell growth (see FO, p 286)
Interleukin-1 and IL-1 receptor antagonist (IL-1ra)	See FO, p 287
Interleukin-6 (IL-6)	IL-6 acts an an androgen receptor activator (Konwalinka G, etal, AACR98, Abs. 763:112) and has been shown to be more accurate at diagnosing prostatic carcinoma than PSA in patients with androgen-independent prostate cancer (Daliani D, etal, ASCO97, Abs. 1183:331a)
Kang ai 1(KAI1) located on chromosome 11p11.2-13	Kang ai (stands for anti-cancer in Chinese) is a tumor metastasis suppressor gene, that is down-regulated in prostate, breast, lung and pancreatic cancer, at or post-transcription. There is a direct relationship between KAI1 and p53 genes and loss of p53 function leads to KAI1downregulation (Mashimo T, etal, AACR98, Abs. 172:26). KAI1 is being developed by Centocor (Malvern, PA) under an NIH license.
Multidrug resistance-1 (MDR-1)	MDR-1 expression in prostate cancer cells is related with resistance to chemotherapeutic agents (Am J Pathol, 1997 May, 150(5):1571-82)

— continued on next page

Nkx-3.1 maps to chromosome band 8p21	Expression of Nkx-3.1, a gene homologous to the drosophila NK homeobox gene family, is largely restricted to the prostate gland in adults and is confined to epithelial cells. Nkx-3.1 mRNA levels decrease significantly in response to castration, suggesting that its expression is androgen-dependent. Nkx-3.1 protein appears to function as a transcription factor that plays a prominent role both in the initiation of prostate development and in the maintenance of the differentiated state of prostatic epithelial cells (Bieberich CJ, etal, J Biol Chem, 13 Dec 1996, 271(50):31779-82) and it is frequently deleted in prostate cancer (He WW, etal, Genomics, 1 Jul 1997, 43(1):69-77). This gene is being characterized by Human Genome Sciences.
Nuclear matrix protein (NMP) NMP-23 and others	See text and FO, p 286
P53 located on chromosome 17p	Progression to metastatic cancer may involve inactivation of the tumor suppressor gene p53 (See FO, p 285 and text)
PCA3 (DD3) mRNA marker, located on chromosome 9q21-22	PCA3 was overexpressed in prostate cancer. DiagnoCure (Quebec, Canada) is developing a reagent, DiagnoGene PCA3, which can detect this prostatic marker (Tamimi Y, etal, AACR98, Abs. 1600:234)
Platelet-derived growth factor receptor (PDGFr)-α	PDGFr-α is expressed in primary prostate cancer but not in normal tissue. It was present in 5 of 6 metastatic prostate cancer bone marrow samples (Bubley G, etal, AACR97, Abs. 368:54)
Pi-class glutathione S-transferase gene (GSTP1)	GSTPI is found in human carcinoma specimens but not in normal prostatic tissue or BPH (PNAS 91 (24):11733-7 1994 Nov 22); also see FO, p286
Prostate acid phosphatase (PAP)	Originally the standard of *in vitro* assessment of prostate cancer, PAP is currently finding a place as a prognostic marker (see text)
Prostate carcinoma tumor antigen-1 (PTCA-1)	PTCA-1 encodes a 35 kDa secreted protein that shares a 40% sequence homology with the N-amino terminal region of members of the S-type galactose-binding lectin (galectin) gene family which has been implicated in tumorigenesis and metastasis (Su ZZ, etal, PNAS USA, 9 Jul 1996, 93(14):7252-7)
Prostate specific antigen (PSA)	See Exhibit 2 and text
Prostate stem cell antigen (PSCA) located on chromosome 8q24.2	PSCA, a member of the Ly-6/Thy-1 cell surface antigens, is relatively prostate-specific (only a very small signal was detected in the placenta) and highly up-regulated in both androgen-dependent and independent prostate cancer. PSCA is expressed predominantly on the cell surface of prostate cancer cells (Reiter RE, CaP CURE97 and PNAS USA, 17 Feb 1998, 95(4):1735-40)
Prostate tumor inducing gene-1 (PTI-1)	PTI-1 is differentially expressed in prostate cancer versus normal prostate and BPH (Sun Y, etal, Cancer Res, 1 Jan 1997, 157(1):18-23). PTI-1 cDNA encodes a predominant approximately 46-kDa protein and multiple PTI-1 transcripts were identified in RNAs from human carcinoma cell lines derived from the prostate, lung, breast, and colon (Shen R, etal, PNAS USA, 92(15):6778-82 1995 Jul 18)
Retinoblastoma gene (Rb) on chromosome 13q	The retinoblastoma gene product is deleted or altered through point mutation in some human prostate cancer specimens (See FO, p 285)
Telomerase	Telomerase activity and telomere shortening may play an important role in the transformation of prostatic intraepithelial neoplasia (PIN) into malignancy (Koeneman KS, etal, AACR98, Abs. 147:22)
Thrombospondin-1 (TSP-1)	Thrombospodin, an extracellular matrix (ECM) protein, is involved with angiogenesis, wound healing and other extracellular mechanisms. TSP-1 is an inhibitor of angiogenesis and is upregulated by wild-type p53 (wtp53). Low TSP-1 expression may be related with tumor neovascularity and mutant p53 expression. TSP-1 in the ECM binds to cell surface receptors including integrin avb3, an interaction which may interfere with the endothelial cells' ability to form new blood vessels. TSP's interactions with its cellular receptors play a role in modulating platelet aggregation, blood coagulation, cell growth, tumor metastasis and malarial cytoadhesion (Grossfeld GD, etal, JNCI, 5 Feb 1997, 89(3): 219-27)
Thymosin-β 15	Thymosin-β 15 is found in more aggressive prostate cancers such as those with high Gleason scores (Zetter B, CaP CURE97)
Transforming growth factor-β (TGF-β) receptor and TGF-β1	Expression of TGF-βr is inversely correlated with either prostatic carcinogenesis or progression of prostate cancer, or both. Overexpression of TGF-β1 in expressed prostatic fluid may be indicative of advanced prostate cancer (Klein KG, AACR98, Abs. 4280:629)
UC325	More abundant as cancer progresses, UC325 outperforms other serum markers (Ralph D, CaP CURE97); in development by UroCor
Urokinase-type plasminogen activator (u-PA)	u-PA mediates angiogenesis and, therefore, prostate cancer invasion and metastasis. It appears that prostate cancer cells stimulate increased expression of u-PA by endothelial cells (Evans CP and Wood CG, AACR98, Abs. 285:42)
Uteroglobin (UG)	UG is a secretory protein that may suppress cellular invasiveness. UG expression is high in normal prostate glandular epithelium, diminished in PIN and absent in prostate cancer. Recombinant UG inhibits the *in vitro* invasion of human epithelial metastatic prostate cancer cell lines in a specific and dose-dependent manner. Loss of UG expression may facilitate prostate cancer invasion (Weeraratna AT, etal, AACR97, Abs. 3671:547). UG is development by Metastatin Pharmaceuticals (Rockville, MD) for the diagnosis of prostate cancer

Legend: AACR=American Association for Cancer Research
CaP CURE=The Association for the Cure of Cancer of the Prostate

015, replicates only in cells lacking a functional p53 gene, and specifically targets tumor cells in this manner.

Among concerns addressed by the preclinical and clinical trials reported here were:

- whether gene therapy is applicable to a broad range of tumor types
- the impact of the "bystander effect,"
- to what degree an immune response to the viral vector might interfere with the effectiveness of gene therapy
- the effectiveness of the administration route (intrahepatic, intravenous, intratumoral, or intraperitoneal)
- use of gene therapy as a surgical adjuvant
- use of gene therapy as a chemosensitizing approach in combination with standard drug therapies.

Interestingly, as more is known about p53 and cancer, it appears that gene therapy involving insertion of normal p53 genes into cancer cells is beneficial not only in cases when such genes are found to be missing or defective, but also in cases when a wild-type p53 gene is present. Because treatment appears to be non-toxic, it has been employed in cancer patients irrespective of their p53 status.

Introgen Therapeutics

Introgen Therapeutics (Austin, TX) has one of the most active clinical programs (see Exhibit 6) in *in vivo* gene therapy using an adenovirus-p53 (Ad/p53) construct (INGN 201). *In vitro*, INGN 201 expresses p53 over a period of 3 to 5 days followed by a rapid fall-off. To date, completed phase I/II clinical trials involved about 100 patients who were treated with more than 600 doses of INGN 201. Generally, treatment was not associated with any serious side effects.

The company has completed three clinical trials of p53-based gene therapy, in non-small cell lung cancer (nsclc) and head and neck cancer. Currently, a phase II clinical trial is ongoing in head and neck and lung cancer and a phase I clinical trial has been initiated in prostate cancer. All in all, Introgen, in collaboration with RPR Gencell (Rhône-Poulenc Rorer), plans to conduct over 20 clinical trials of INGN 201 in 10 different cancers, worldwide. Also, in April 1996, Introgen and RPR signed a letter of intent with the National Cancer Institute (NCI) for a CRADA agreement to compare INGN 201 against other treatments for breast, ovarian, bladder, liver and brain cancers.

In its gene therapy program, in addition to p53, Introgen is also targeting k-ras and p16 genes that have also been implicated in the development of cancer.

Phase I clinical trials in head and neck cancer were reported by James A. Merritt of Introgen Therapeutics. The head and neck clinical trial enrolled 45 patients with recurrent or chemotherapy-refractory cancer, who were not screened for p53 phenotype. In most cases the tumor was resectable, a selection criteria which permitted evaluations of gene therapy as a surgical adjuvant. The treatment regimen involved delivering the agent in 6 doses over a 2-week period, followed by tumor resection, if possible, with doses 7 and 8 delivered directly into the surgical wound, one during the operation and the other through the surgical drain 3 days later. Patients were then followed-up to evaluate safety and disease-free survival. Patients with non-resectable cancer were treated monthly after biopsy.

About half of tumors had p53 mutations and another half had abnormal p53 immunohistochemistry. Most had an incomplete concordance between the gene and the immunohistochemistry studies. Transgene expression was evaluated in 4 patients who were administered different doses; surgical resection of tumor and control tissue was performed in these patients specifically for this purpose. Messenger RNA for p53 was identified in only two of the patients administered higher doses, possibly because tissue was collected too soon after treatment. Protein expression from the vector was identified by immunohistochemical staining of the tumor material from a patient whose own p53 mutation (stop codon) rendered it non-immunogenic. A robust immunological response to the adenovirus vector was detected in all patients tested.

Gene expression from the Ad-p53 vector correlated with tumor necrosis in 3 out of 33 patients. There was one complete histological response and two partial responses (PR). However, extensive central necrosis which occurred in several tumors, did not qualify as a response. Some degree of tumor regression occurred in more than 50% of tumors. The patient who experienced a complete response (CR) was disease free at 3 years. Average survival among the nonresectable group was 5-6 months, which is typical in advanced head and neck cancer. In the resectable group the median survival time (MST) was over 1 year with 50% of the patients still alive at the time of this presentation. Thus, there is a clear improvement in survival in patients who are treated by surgical resection and gene therapy.

This phase I clinical trial showed that INGN 201 is a safe and well-tolerated therapy for head and neck cancer over a 7 month treatment period. Expression of p53 occurred despite an immune response to the vector, and therapy did not interfere with surgical wound healing.

Phase I studies of INGN 201 for treating non-small cell lung cancer (nsclc) in which the agent is being used singly or in combination therapy with cisplatin, are also underway. In the combination therapy trial, cisplatin is administered as a single dose 3 days prior to direct intratumoral injection of INGN 201. All enrolled patients had advanced refractory nsclc with p53 mutations. About 50% of those treated with INGN 201 had evidence of gene transfer in the form of expression of mRNA or protein. There were two PR in each group. It is encouraging to note that both PR patients who were treated with the vector-cisplatin therapy had failed prior cisplatin monotherapy. INGN 201 was well-tolerated in this trial. There were no Grade 4 adverse reactions, some Grade 3 reactions included fever and injection site pain and, in one case, there was nausea attributable to cisplatin. Plasma and serum levels of active vector peaked at 30-50 minutes after intratumoral

injection in spite of the presence of an active anti-adenovirus immune response; thus, the immune response did not interfere with therapy.

Ovarian cancer is a particularly appropriate target for p53 gene replacement because it is frequently associated with p53 mutations. Also, ovarian cancer tends to remain confined to the peritoneal cavity where it is accessible to treatment. To determine the types of p53 mutations associated with ovarian cancer, J.A. Lee of Introgen Therapeutics summarized results of genetic sequencing studies. Out of 99 ovarian cancer patients who were screened for p53 mutations, 72% had altered p53 genes; 44 of them were missense mutations and 27 were null mutations. There was an 80% chance that null mutations would be missed by immunohistochemical screening. In clinical follow-up studies, null mutations were of particular interest because they tended to be associated with metastasis rates that were 10 times higher than those of missense mutations or normal p53 status. Results suggest that patients chosen for p53 gene replacement must be thoroughly screened for p53 mutational status, because those with null mutations may have poor therapeutic response rates. Introgen Therapeutics plans to initiate phase I/II clinical trials in ovarian cancer in the near future.

Exhibit 6
Gene Therapy Agents in Development by Introgen Therapeutics

Indication	Product Name ❑ Number ❑ Administration Route	Status
Head and neck cancer	AD-p53 ❑ INGN 201	Phase II (o4/98) USA
	AD-p53 ❑ INGN 201+ surgery	Phase I (c97) USA
	AD-p53 ❑ INGN 201+ cisplatin	IND (f97) Europe
	AD-p53 ❑ INGN 201+ radiotherapy	Preclin (4/98) Europe
Non-small cell lung cancer (nsclc)	AD-p53 ❑ INGN 201	Phase I/II (c97) USA
	AD-p53 ❑ INGN 201+ cisplatin	Phase I/II (c97) USA
	AD-p53 ❑ INGN 201+ radiotherapy	Phase I/II (o4/98) USA
	RV-AS-K-RAS ❑ INGN 111	IND (a97) USA
	RV-p53 ❑ INGN 101	Phase I/II (c97) USA
Liver cancer (hepatoma)*	AD-p53 ❑ INGN 201 ❑ percutaneous	IND (a97) USA
	AD-p53 ❑ INGN 201 ❑ intra-arterial	Preclin (4/98) USA
Prostate cancer	AD-p53 ❑ INGN 201	Phase I/II (o4/98)
	AD-C-CAM ❑ INGN 231 ❑ percutaneous	Preclin (4/98) USA
Bladder cancer*	AD-p53 ❑ INGN 201 ❑ intravesical	IND (a97) USA
Ovarian cancer*	AD-p53 ❑ INGN 201	Preclin (4/98) USA
	AD-p53 ❑ INGN 201+ chemotherapy	Preclin (4/98) USA
Brain cancer (glioblastoma)*	AD-p53 ❑ INGN 201 AD-p53 ❑ INGN 201+ radiation	Preclin (4/98) USA Preclin (4/98) USA
Breast cancer*	AD-p53 ❑ INGN 201	Preclin (4/98) USA
	AD-p53 ❑ INGN 201+ chemotherapy	Preclin (4/98) USA
Malignant ascites	AD-p53 ❑ INGN 201	IND (a97) Europe
Colorectal cancer	AD-p53 ❑ INGN 201	
	AD-scFv-RAS ❑ INGN 212	IND (a97) Europe
Other cancers	p53 analog ❑ INGN 202	Research (4/98) USA
	AD-p16 ❑ INGN 221	Preclin (4/98) USA
	F42K ❑ INGN 301	Research (4/98) USA
	p53 bystander effector ❑ INGN 401	Research (4/98) USA
Solid tumors	AD-mda-7 ❑ INGN 241	Research (4/98) USA

Note: The p53 and K-RAS agents are being developed in collaboration with Rhône-Poulenc Rorer
** NCI CRADA*

Preclinical studies of p53 replacement gene therapy for pancreatic cancer using the Introgen Therapeutics construct Ad5/CMB/p53, were described by M. Bouvet of M.D. Anderson Cancer Center (Houston, TX). Experiments showed that delivery of p53 in an adenovirus vector resulted in induction of cell death and suppression of growth of 6 pancreatic cancer cell lines.

Pancreatic cancer is a likely candidate for p53 gene therapy approaches because 70% of pancreatic tumors have mutations in p53 and generally respond poorly to chemotherapy. Reporting on an investigation involving use of adenovirus as a vector in these cells, J.A. Lee of Introgen Therapeutics reported that efficiency of gene transfer varied when 6 pancreatic cancer cell lines were transfected by an adenovirus-βgal vector. When the same cell lines were then transfected with an Ad p53 vector, in spite of the difference in gene uptake efficiency, a reduced growth rate was observed in all. As the dose of Ad p53 increased, the frequency of apoptosis also rose. There was no toxicity. Ongoing studies will investigate use of this strategy for treating pancreatic tumor xenografts.

Tumor regression in vivo following p53 combination therapy was described by Ruth A. Gjerset from the Sidney Kimmel Cancer Center (San Diego, CA), based on

preclinical trials of p53 gene therapy combined with chemotherapy, performed with the support of Introgen Therapeutics. The purpose of these trials was to extend use of p53 gene therapy to different types of tumors, because so many are known to have defects in p53. Over 50% of lung and colon cancers, 25-40% of gliomas, 37% of head and neck cancers, and 25-40% of breast cancers have loss-of-function mutations in p53. And, of course, loss of p53 correlates with poor prognosis, poor response to therapy and resistance to chemotherapy. Thus, restoration of p53 function should also restore full response to therapy.

Some of the conventional cancer treatment modalities that become less effective in the presence of p53 loss are cisplatin, radiation, topoisomerase I inhibitors, and 5-fluorouracil. All of these agents are known to increase the degree of DNA damage in the cell; and, normally, DNA damage leads to apoptosis through a biochemical pathway mediated by p53. The protein binds to damaged DNA in the form of single stranded breaks, single-stranded ends, and abnormal structures known as insertion-deletion loops. It then promotes cell death, probably by activating the cell death receptor Fas. Genetic instability, which is a hallmark of tumor progression, may also promote p53 loss.

In order to test the effectiveness of p53 gene therapy and cisplatin chemotherapy, 9L Fisher rat glioblastoma cells were transfected *ex vivo* with either a vector containing the p53 gene or an empty vector. A rat glioblastoma model is an extremely fast-growing tumor that is typically resistant to therapy and closely mimics characteristics of human glioblastoma. The plasmid used contained a chlotamphenicol acetyltransferase (CAT) gene as well, which permitted confirmation of vector expression by CAT assay. Cisplatin sensitivity was assayed by an *in vitro* viability assay. Cells were plated at low density in 96 well plates, treated for 1 hour with varying concentrations of cisplatin, then grown in the absence of the drug for 7 days, and counted. When cisplatin-treated cell growth was compared to untreated cell growth, wild-type p53-modified 9L cells were more sensitive to cisplatin than control-modified cells.

The transfected glioblastoma cells were then implanted intracranially into normal Fisher rats that were treated with weekly doses of cisplatin. There was improved survival with combination therapy but not with gene therapy or cisplatin treatment alone. When cells were recultured from the tumors and tested for plasmid CAT gene expression, none was detected after several days of tumor growth, thus even limited p53 expression is sufficient to sensitize cells to cisplatin.

Two additional tumor models were analyzed for sensitivity to the combination p53 gene therapy and chemotherapy. One was a mouse subcutaneous colon cancer model, DLD-1 cells, which have a mutation in the p53 gene. These cells were sensitized to 5-FU *in vitro* when infected with the Ad p53 vector. Injection of subcutaneous tumors with the vector, followed by chemotherapy, resulted in growth stabilization as compared to empty vector-treated tumors. The second model was a FaDu head and neck cancer model. This cell line was sensitized to chemotherapy with cisplatin *in vitro*, and tumor growth in the animal model was also stabilized by the combination therapy.

Canji (Schering-Plough)

Schering-Plough Research Institute (Kenilworth, NJ) and Canji (San Diego, CA) are also actively pursuing clinical trials using p53-based gene therapy. Canji is currently evaluating its recombinant adenovirus encoding p53 (rAd/p53) construct (SCH 58500) in phase I clinical trials in various solid tumors using intrahepatic, intraperitoneal, and intratumoral administration. To date, 62 patients with colon cancer that has metastacized to liver, ovarian and head and neck cancer, melanoma and nsclc, have been treated with varying doses of SCH 58500 using various administration routes. In 30 of 57 cases treatment resulted in normal p53 expression, despite pre-existing antibodies to the adenovirus.

SCH 58500 is also being studied in the treatment of drug-resistant ovarian cancer and, delivered intravesically, in superficial bladder cancer. In addition, Canji is investigating rAd/p53 treatment as a means of purging peripheral cancer stem cell products and as a modulator of the immune system.

Phase I clinical trials of intrahepatic SCH 58500 were initiated to determine safety and dosage. One question addressed in preclinical studies was the relative efficiency of delivery of the vector via the intrahepatic artery (IHA) as compared to an IV route. It is known that liver tumors derive 80% of their blood supply from the hepatic artery and that chemotherapeutic agents are much more effective when delivered by IHA than by the IV route. When a single bolus injection was administered IV in the rat model, plasmid was found primarily in normal hepatic parenchymal cells. However, when the vector was delivered by IHA it was detected in tumor cells. Clinical trial enrollees had colorectal cancer metastatic to the liver or incurable hepatocellular carcinoma. They were required to have tumors with p53 mutations and anti-adenovirus antibodies at baseline. Results thus far indicate that there is no dose-limiting toxicity and, in spite of an increase in adenovirus antibodies at about day 7, all patients showed evidence of active vector expression in their tumors.

According to Alan Venook of the University of California, San Francisco who presented results of preclinical and early clinical trials, the p53 gene under control of a cytomegalovirus promoter, was delivered in a replication defective adenovirus vector containing a deletion in the viral E1A, E1B, pIX and E3 genes. Preclinical studies used a syngeneic rat model of hepatocellular carcinoma, MCA-RH777 cells, which were injected intrasplenically into Buffalo rats and grew into extensive multifocal tumors within a month. These cells contain a mutation in the p53 gene at codon 280.

Adenovirus-mediated p53 gene therapy synergizes with paclitaxel against human ovarian, mammary, prostate, head and neck, and liver cancer. Loretta L. Nielsen

of Schering-Plough Research Institute discussed the concept of synergy in combination chemotherapy and gene therapy using SCH 58500. There are three possible interactions between these two forms of therapy acting together. They can be additive, antagonistic, or synergistic in their combined effects. Synergy between two drugs is an empirical phenomenon in which the effect of a combination of agents is greater than that which would be predicted from each agent working along. Synergistic combinations of drugs lead to better clinical efficacy.

To determine synergy between Ad p53 gene therapy and a variety of anticancer drugs, a panel of cancer cell lines were screened for single dose sensitivity to individual chemotherapeutic agents in combination with different concentrations of rAd/p53 plasmid or taxol to examine the combined effects in a cell proliferation assay. Drug interactions were analyzed by statistical models, first by generating a three-dimensional model of the data fitted with a bivariant spline, and then by generating isobolograms. Goodness-of-fit of the models were determined, along with p values, and the isobolograms were analyzed by interaction index methodologies. ED_{50} values were taken from the cell proliferation assays and data from drug A was plotted on one axis while that of drug B was plotted on the other. An isobol line, created when the data falls on a straight line, indicates additive effects of the two drugs. If the data falls above the isobol line, there is antagonism between the effects of the two drugs. If it falls below the line there is a synergistic effect between them.

In tests on p53-null cancer cell lines, when the effects of paclitaxel (Taxol; Bristol-Myers Squibb) and p53 were compared, there was synergy between the two therapies. Because there have been reports that paclitaxel and p53 antagonize each other, these results were examined in more detail. In all cell lines, even at low concentrations of paclitaxel, there was synergy with p53 gene therapy. Taxol actually increases the transduction rate of adenovirus, and it does so at lower concentrations than those required for microtubule condensation. Thus, synergy between the two therapies has nothing to do with Taxol-induced microtubule collapse.

Onyx Pharmaceuticals

Onyx Pharmaceuticals (Richmond, VA) is developing ONYX-015, a genetically engineered E1B-deleted adenovirus that replicates in and kills cells containing mutant p53 (see FO, p 654). Replication of this virus kills the cell as a direct result of the infection, thus tumor cells which lack p53 are specifically killed by this strategy while those with wild-type p53 are spared. Preclinical trials of this agent showed that the virus has no effect on RKO cancer cells which have a fully functional p53 gene, but readily kills RKO cells which were genetically altered so that they fail to express a functional p53 protein. Parenthetically, the ONYX-015 approach cannot be described as gene therapy because there is no delivery of a novel gene to the cancer cell.

Phase I clinical trial of ONYX-015 were first undertaken in head and neck cancer because this type of cancer is frequently (45%-70%) associated with mutant p53 genes. In addition, head and neck cancer is easy to evaluate, inject, and biopsy; and recurrent disease is quite common. David Kirn of Onyx Pharmaceuticals reported on the clinical trial of ONYX-015 delivered by direct intratumoral injection. In these clinical trials, 32 patients were enrolled and effects of escalating dose of the agent were determined. All patients had histologically confirmed squamous cell carcinomas of the head and neck (HNSCC) which were recurrent or advanced and not surgically resectable. After a single injection of ONYX-015, virus was detected in 30% of the tumor cells. A repeat dose was given to some patients 4 weeks later. Tumors were biopsied at 0, 7, and 21 days after infection, and analyzed for viral sequences, neutralizing antibody and viral shedding.

Results of the trial indicated that there was no dose-limiting toxicity and the only adverse effects were mild transient flu-like symptoms (30% after a single dose and 60% after multiple doses), which would be expected for a mild viral illness. Multiple dosing was found to be superior to single dosing, with more patients (6/9) showing a partial response or remaining stable after 2 doses as compared with single dosing (3/9). Tumor necrosis was associated with clinical benefit. Although neutralizing antibodies were detected, the immune response did not prevent a clinical response, probably because neutralizing antibodies do not penetrate solid tumors. Thus, there was continued response in the face of high levels of neutralizing antibodies following multiple treatments.

A single-agent phase II clinical trial which began in July 1997, is treating HNSCC patients on a multi-day administration schedule. Another phase II clinical trial, initiated in October 1997, is testing a combination of ONYX-015 and two chemotherapeutic drugs in treating HNSCC. In a third phase II clinical trial which began in March 1998, ONYX-015 is administered daily for five days for two consecutive weeks. After a third week with no treatment, HNSCC patients are evaluated and may continue for another intensive treatment cycle or on maintenance treatment. This regimen is the most aggressive use of ONYX-015 to date, involving more frequent administration over a longer treatment cycle than either of the other phase II studies. This trial is being conducted by the Physician's Reliance Network (PRN; Dallas, TX) under the direction of John Nemunaitis, MD, and will be expanded to selected sites where the other phase II trials of ONYX-015 are already underway. This trial may enroll up to 30 patients.

Preclinical studies with ONYX-015 in combination with chemotherapy were reported by Carla Heise of Onyx Pharmaceuticals. Mode of delivery was tested in a mouse xenograft model of human tumors by comparing effects on tumor growth inhibition by intratumoral, intraperitoneal, or intravenous delivery. Prior experience indicated that intratumoral injection might be more effective in inducing

complete rejection, but response depends on the extent of disease progression.

Combination therapy is likely to be more efficacious than monotherapy because each agent exhibits a different mechanism of action and non-overlapping toxicities. Therapy with ONYX-015 targets p53 in cancer cells and chemosensitizes them. The viral ElA protein, which only acts upon entry into S phase, increases p53 expression, which in turn induces the apoptotic response to chemotherapy. When ONYX-015 was delivered IV, there was growth inhibition of the tumor but not complete rejection. Intratumoral injection resulted in complete rejection of 60% of the tumors, and the number of completely rejected tumors rose to 90% after intraperitoneal injection. The efficacy of the therapy correlated with intratumoral virus distribution.

In vitro studies involved several tumor types and several chemotherapeutic agents in combination with ONYX-015. When the HCT 116 colon cancer cell line was treated by infection with ONYX-015, in combination with 5-FU, there was a significant inhibition of cell growth as compared with either agent alone or no treatment. A similar effect was observed in the RKO p53-expressing colon cancer cell line infected with ONYX-015 and exposed to CPT-11 (Camptosar; Pharmacia & Upjohn).

For *in vivo* rials, mice implanted with tumor cells were treated IV by tail vein for the first week and intraperitoneally for the second week. The virus alone had little effect on tumor growth, 5-FU had some effect, but the combination had a much greater effect. Six out of seven of the animals experienced complete tumor regression. In this model, therefore, IV administration appears to augment the antitumor effect.

In mice with HLaC head and neck tumors, ONYX-015 was injected into the tumor followed by either 5-FU or cisplatin. Mice treated with this combination survived longer (40 days) than those treated with a single agent (20 days). Survival was further extended (60 days) when both cisplatin and 5-FU were administered after virus injection. By testing various combination therapy regimens, it became apparent that it is necessary to administer the virus before or simultaneously with chemotherapy to achieve optimal survival. Furthermore, chemotherapy had no effect on viral replication.

CELL CYCLE-BASED GENE THERAPY

For a comprehensive review of the cell cycle in cancer see FO, pp 591-600.

HepaVec

The cell cycle of cancer cells is commonly blocked at the G_1 to S phase transition because of mutations in the proteins involved in regulating this step. Regulators that are central to the G_1 to S phase transition that are commonly aberrantly expressed in tumors, are retinoblastoma protein (Rb), CDK4, cyclin D1, p16 and p53. Rb functions by forming an inhibitory complex with E2F that blocks transcription of other S phase genes. Active Rb, therefore, blocks cell proliferation. Rb is regulated by phosphorylation and mutations frequently render Rb non-functional, resulting in unregulated cell division. Therefore, restoration of functional Rb by gene therapy may enable a cell to control its own growth cycle.

CDK4 forms a complex with cyclin D1 which phosphorylates and inactivates Rb, promoting cell proliferation. It is now understood that all mitogenic signaling pathways lead to activation of CDK4 by induction of cyclin D1. Formation of the CDK4-cyclin D1 complex can, in turn, be inhibited by the kinase inhibitor p16, a protein which is activated by a number of negative growth regulators. P53 induces p21 which inhibits CDKs 4 and 2 and cell proliferation, in addition to activating the cell death pathway. If it is true that all regulatory factors act through this pathway, then it should be deregulated in every individual tumor type regardless of other mutations that may also occur.

Michael Strauss of Humboldt University (Berlin, Germany), in collaboration with HepaVec (Berlin, Germany), reported on a method to restore cell cycle function in tumor cells by targeting the deregulated cell cycle by adenoviral transfer of two complementary tumor suppressor genes. A number of tumors were analyzed for deregulators of the cell cycle pathway; 10% to 15% of tumors were found to lack a functional Rb, 20-25% overexpressed cyclin D1, 60-70% lacked functional p16, less than 10% had mutations in CDK4 and 10% simultaneously overexpressed cyclin D1 and failed to express a functional p16. Because p16- tumors are very common, it should be possible to restore the cell cycle pathway in all tumors which contain a functional Rb (85% of tumors) by overexpressing p16, in order to counteract overexpression of cyclin D1 and mutations in CDK4.

Overexpression of p16 in Rb+ tumors infected with an adenovirus-p16 (Ad-p16) construct lead to a reduction in Rb concentration. In p16-, but Rb+ cells, there was a block at S phase entry, but in p16+, Rb- cells, the cell cycle was normal. Overexpression of p16 inhibits the cell cycle and results in a reduction of Rb expression through inhibition of Rb phosphorylation. These events occur no matter what the p53 status of the cell may be. Thus, p16 has a co-apoptotic effect that requires some other major players in the apoptotic pathway.

Initial experiments clearly showed that overexpression of p16 could reduce the rate of tumor growth, but in no case was there a regression of tumors. When p16 and p53 expression was combined to enhance the apoptotic effect of p53 by the overexpression of p16, exit from G1 occurred and apoptosis was induced. All but 1% of tumor cells entered apoptosis at day 3 after transduction of both genes. This was not attributed to a cooperative effect of p16 and p21, which could have been induced by p53.

In a nude mice model, p16 transduction resulted in an inhibition in the outgrowth of tumors. With a p16 and p53 gene therapy combination there was an impressive inhibition of tumor outgrowth. Even with established xenograft

tumors, there was a significant reduction in tumor growth when p16 and p53 combination vectors were injected. However, in some animals, tumors resumed growing and this turned out to be related to the efficiency of gene transfer. There was complete inhibition of tumor growth and regression of some subcutaneous and liver metastases of various tumor lines so long as there was 60-70% gene transduction as monitored by cotransference of the βgal gene. However, when gene transfer was less than 60%, there was no significant tumor growth inhibition. Because only 60% transduction was sufficient for tumor regression, there must be some bystander effect.

These experiments have demonstrated that a combination of p16 and p53 drives various types of tumor cells efficiently into apoptosis. Most importantly, there is a synergistic effect between p16 and p53 that can lead to a significant reduction of virus load in the treatment protocol. Future clinical trials are being planned in the near future using a combination of the two genes in the same virus in a double gene therapy protocol.

Canji

Doug Antelman of Canji described results of preclinical trials using systemic delivery of retinoblastoma (Rb) gene replacement using lipid gene delivery for treatment of metastatic small-cell lung cancer (sclc) in immunodeficient mice. Like p53, Rb is commonly mutated in tumors, and almost all sclc have alterations in the Rb gene. Median survival of patients with sclc, a systemic metastatic disease with a poor prognosis, is less than 12 months. The 5-year survival is less than 5%, even with aggressive chemotherapy.

Cationic lipid gene therapy has several advantages over virus-mediated gene therapy. Liposome carriers can deliver more than one gene, are not limited in transgene capacity, do not induce an immune response, have a low toxicity, and can deliver genes to a variety of tissues. Historically, the chief disadvantage of this method has been its low gene transfer efficiency. Among cationic lipids used for systemic gene transfer are DDAB, cholesterol, DOGS, DOTMA and DOTAP-cholesterol. Therapeutic genes delivered by cationic lipids include G-CSF and p53. Commonly, the highest level of gene expression is usually seen in the lung where protein expression levels have been measured at 1 ng of protein per mg of tissue.

Canji is experimenting with Rb gene delivery using a lipid vector, GL67, that was developed by Genzyme (Cambridge, MA) for airway delivery of agents for treating cystic fibrosis. Gene transfer efficiencies associated with GL67 were reported to be 100 times higher than those usually observed using liposomes. GL67 is generally used with DOPE, a neutral lipid, in a 1:1 mixture. Its structure is a T-shaped carbamate linkage between a spermine moiety and a cholesterol moiety. GL67-Rb systemic gene therapy was first tested in an animal model for sclc which is characterized by frequent alterations in tumor suppressor genes, especially Rb.

The animal model used was H69 sclc in Beige-SCID mice. When H69 cells are injected by the tail vein, tumors develop at multiple sites within 6 to 8 weeks in a variety of organs. Virtually 100% of the animals develop tumors in the adrenals and ovaries, 80% in the heart, 80% in the kidneys, 10% develop neck tumors, but none develop lung cancer. All of the mice die 45-50 days after tumor cell injection.

Efficacy of gene transfer by injection of GL67-mediated genes was first confirmed using a GL67-CAT reporter plasmid. There was good expression in the lung, adrenals and ovaries 48 hours after injection but expression in other organs was lower. There was a dose-dependent expression of the reporter gene in the ovary, the adrenals, and the lung, in tumor-bearing animals. Interestingly, in control animals, gene uptake was restricted to the lung, suggesting that there was enhanced uptake when tumors were present. There were actually higher levels of expression in organs which contained tumors than in the lung.

The therapy agent GL67-Rb consisted of the Rb gene driven by a cytomegalovirus promoter and an adenovirus tripartite leader to enhance mRNA translation. In an RT-PCR assay to detect gene expression in an animal model, the Rb transgene was found in the same tissue that expressed CAT when the control vector was used. In an efficacy study, mice which had been injected with tumor cells 14 days previously were administered the GL67-Rb plasmid by tail vein injection. After 35 days of treatment, the mice were monitored for survival. MST was 42 days for untreated animals, 47 days for control vector-treated animals and 57 days for animals treated with the GL67-Rb plasmid. Thus, the therapy significantly improved survival in this model.

In December 1997, Genzyme Molecular Oncology (GMO; Framingham, MA) entered into a research and option agreement with Schering-Plough to combine GMO's proprietary lipid delivery systems with six of Schering's proprietary genes, including the p53 tumor suppressor gene, to develop gene therapy products. GMO could receive approximately $7 million in revenue in 1998 as a result of up-front payments, research funding and milestone payments for research progress on a lipid-based p53 tumor suppressor gene therapy. At any time during this initial one-year research period, Schering-Plough may exercise its option to exclusively license GMO's lipid vector technology for delivery of the p53 gene. If this option is exercised, Schering will have the option to exclusively license this vector technology for delivery of five additional genes. If milestones for all six product candidates are successfully achieved, revenue to GMO could exceed $80 million, excluding royalties.

BCL-2

Another approach to chemosensitize cancer cells is by inhibiting Bcl-2 using a protein knock-out strategy. Alain Piche of the University of Alabama (Birmingham, AL) reported on studies in ovarian cancer in which Bcl-2 was

blocked by single chain antibodies (sFvs). These antibodies consist of the antigen-binding domain of the parental antibody and are produced by expression of transgenes encoding the antibody fragment delivered to the cancer cell in a vector such as adenovirus. This method avoids many of the problems of the antisense gene knock-out approach, such as nonspecificity, insolubility, short half-life, and low cellular uptake. SFvs have already been used successfully to abrogate the function of several cellular and viral proteins.

Binding affinity of anti-Bcl-2 sFvs to Bcl-2 was determined in an ELISA assay. HeLa cells were transfected with the sFv vector and co-transfected with a Bcl-2 vector. The amount of Bcl-2 expressed was lower in the presence of the anti-Bcl-2 sFv vector. When ovarian, breast and prostate cancer cells were transduced with anti-Bcl-2-sFv, Bcl-2 levels were reduced. When ovarian cancer cells that overexpressed Bcl-2 were treated with Ad-Bcl-2-sFv and cisplatin, there was an increase in sensitivity to the drug (cell killing was 55% versus 35% in controls), while the effect on cell proliferation was unchanged. Intracellular expression of anti-Bcl-2-sFv reduced survival of tumor cell clones. While SW262 cells, which lack Bcl-2, were unaffected, PA-1 cells, which express functional Bcl-2, experienced reduced rates of proliferation. Thus, anti-Bcl-2-sFV expression can sensitize cells which express a functional Bcl-2 to chemotherapy, but has no effect on tumor cells lacking Bcl-2.

JNK/SAPK PATHWAY

Isis Pharmaceuticals

Another approach to sensitizing human tumor cells to chemotherapy and radiotherapy, and to inhibit tumor growth is by inhibiting the Jun kinase (JNK)/SAP kinase pathway. One of the steps in the JNK pathway is activation of the transcription factor c-jun by JNK itself, which phosphorylates two N-terminal serines in c-jun, increasing the AP-1 complex activity, and stimulating cell proliferation. Each step of the JNK pathway is a homolog of the MAP kinase pathway. Like the MAP kinase pathway, this pathway can also be activated by the ras pathway through CDC42 and RAC1. This pathway which is activated by a variety of pro-inflammatory cytokines, has also been implicated in apoptosis, thus it may have an important stress-mediating role, especially in certain immune responses.

The JNK pathway is also activated by DNA-damaging agents and other forms of genotoxic stress. Besides c-jun, the JNK pathway also phosphorylates the CREB-like factor ATF-2, giving rise to the c-jun/CREB heterodimer which activates a different set of genes than the usual AP-1 genes and those that are involved in DNA repair of cisplatin-DNA adducts. Cisplatin forms intrastrand crosslinks that are repaired by nucleotide excision repair in conjuction with the set of genes activated by the jun/ATF-2 heterodimer. The drug is a strong activator of the JNK pathway. By contrast, transplatin, the chemically-identical isomer, has no effect on the JNK pathway.

Among cells that express dominant-negative inhibitors of the JNK, one, TAM 67, contains a c-jun protein that lacks the N-terminal phosphorylation sites. It can still complex with other partners, but is less active in driving transcription. Another cell contains a mutation which replaces the crucial serines of c-jun with alanine. Expression of the dominant-negative c-jun in human tumor cells sensitizes them to cisplatin. If wild-type c-jun is overexpressed, however, there is no change in viability after exposure to cisplatin.

When cells are exposed to cisplatin prior to isolation of genomic DNA there is a reduction in the ability of the DNA to serve as a template for replication by Taq polymerase. If the cells are allowed to recover for 5 hours after exposure to cisplatin, replication competence of the genomic DNA is restored, indicating a repair process has occurred during that time. The same experiment conducted with cells expressing a nonphosphorylated c-jun resulted in failure of the genomic DNA to recover its replication competence. These results indicate that c-jun is involved in repairing cisplatin-DNA adducts which could confer resistance to cisplatin chemotherapy.

According to Daniel Mercola of the Sidney Kimmel Cancer Center (San Diego, CA), in an attempt to to make a soluble drug that could inhibit the JNK pathway, a series of antisense oligonucleotides which bind to various regions of the JNK gene (ISIS 12539, 12548 and 12554 which target JNK-1, and 12558, 12560 and 12569 which target JNK-2) were created in collaboration with Isis Pharmaceuticals (Carlsbad, CA) and tested for their ability to eliminate JNK mRNA. JNK-1- or 2-targeting oligonucleotides specifically lower levels of the corresponding JNK mRNA. Human lung cancer cell lines, which express large amounts of JNK pathway activity, proliferate in response to epidermal growth factor (EGF). When the dominant-negative c-jun genes were expressed in these cells, their ability to be activated by EGF was nearly inhibited. Likewise, when the anti-JNK oligos were expressed the same result was obtained. Thus, JNK inhibition may be a useful therapy for lung cancer and treatment of animals models using these oligos combined with cisplatin are underway.

OTHER GENE THERAPY APPROACHES

Inhibition of Nude Mice Xenografts Using Growth Inhibitory Gene Mda-7

Paul B. Fisher of the College of Physicians & Surgeons at Columbia University (New York, NY) described work that has been supported by GenQuest (Seattle, WA) to develop differentiation therapy of human cancer. Initial efforts were directed toward identifying genes from terminally differentiated cancer cells by subtractive hybridization. A temporally spaced cDNA library from human melanoma cells was constructed and mixed with a library from proliferating cells to identify genes that are uniquely overexpressed by cancer cells. The rationale of this approach is that many cancer cells have defects in genes controlling differentiation and that, by identifying these

genes, it may be possible to selectively block their expression and, thus, prevent the cell from becoming malignant. The focus of Dr. Fisher's research efforts has been on human melanoma. A number of years ago a series of compounds were identified that, when used in combination, could arrest melanoma growth. The combination used was interferon β and mezerein which induced reversible growth arrest, or markers of differentiation in melanoma, and resulted in the terminal differentiation phenotype.

Substractive hybridization identified 73 overexpressed cDNAs, 23 of which were differentially expressed; 11 of them were novel genes. Four classes of these melanoma differentiation associated (mda) genes were identified, i.e., genes that were upregulated by either the single inducer or the combination, those induced by the second inducer or the combination, those induced by all three, or, most interestingly, those that were induced only by the combination. One of the latter class, mda-6, encodes p21, the cell cycle protein that is induced by p53.

A novel mda gene is mda-7, which is induced within the first 24 hours of terminal differentiation induction and which remains transcriptionally active throughout differentiation. Antibodies developed to the protein it encodes recognize a 23.8 kDa protein which is found in a complex with a 90-110 kDa protein. When mda-7 is tranfected into normal cells, it has growth- and tumor-suppression properties. It dramatically reduces colony formation when overexpressed in every cancer cell so far examined. The mda-7 gene reduces tumor growth when it is delivered to tumor cells by adenovirus vector.

Mda-7 inhibits tumor growth by inducing apoptosis, which can be detected by a ladder DNA assay within about 2-3 days of transfection. In normal cells, mda-7 interacts with chromatin, and its overexpression results in upregulation of Bax. Furthermore, overexpression of Bcl-2 nullifies the growth-suppressing effects of mda-7, suggesting that mda-7 plays a significant role in the control of the apoptotic pathway. The gene maps to the long arm of chromosome 6 and is unrelated to other known proteins. It does not contain a nuclear localization signal, thus is presumed to be transported to the nucleus by a chaperone.

Anti-papillomavirus Ribozymes

Papillomaviruses (PV) are responsible for many oral cancers and virtually all cases of cervical cancer. E.J. Shillitoe of the State University of New York (Syracuse, NY) described an antisense ribozyme (RZ) approach mediated by adenovirus vector delivery to control PV infection This antisense approach uses synthetic phosphorothioate oligonucleotides which bind to the sense strand of target RNAs and either inhibit binding of functional proteins or serve to deliver a ribonuclease in the form of a ribozyme which cleaves the target RNA, rendering it nonfuntional. PVs are small DNA viruses which replicate in the nucleus and produce several viral-specific mRNAs. E6 and E7 genes are associated with viral transformation properties. Three ribozymes have been developed which cut the E6 and E7 viral mRNA transcripts and prevent production of the transforming viral proteins. The most effective of these is RZ 309, which has been shown to cut the target RNA *in vitro* and in model systems *in vivo*, and to prevent production of viral proteins responsible for transformation. In HeLa cells, which are cervical cancer cells containing PV gene sequences, RZ 309 expression inhibits cell growth and focus formation, as well as other markers of transformation.

The adenovirus vector used to deliver RZ 309 is pAVS6A, produced by Genetic Therapy (Gaithersburg, MD). The insertion site of this vector links the transgene with a Rous sarcoma virus (RSV) promoter linked to the translation-enhancing adenovirus tripartite leader sequence. One construct containing the RZ 309 sequences was altered to replace the RSV-adenovirus leader with the mouse mammary tumor virus promoter. A control vector, AV1LacZ4, was constructed which contained the lacZ gene, and another contained an antisense sequence which lacked catalytic activity.

The lacZ expression vector was used to determine the multiplicity of infection that would maximize transduction efficiency and minimize non-specific growth inhibition. When the RZ was expressed from the vector with the RSV promoter, there was no effect on focus formation of HeLa cells. However, when it was expressed from the MMTV promoter, it reduced the number of HeLa cell foci by 66% relative to the control vector, which is consistent with what one would expect from delivery of the RZ gene by a plasmid. When tested on the growth of an oral cancer cell line 1483 which contains PV DNA and is susceptible to inhibitors when they are expressed from plasmid, the Ad-RZ vector was ineffective; neither HeLa cells nor 1483 cells were growth inhibited by the Ad-RZ vector. The lack of effect on HeLa cells was not expected because RZ inhibits growth of HeLa cells when expressed in a plasmid vector. Repeat experiments showed that untransfected HeLa cells or cells transfected with vector only, survive G418 selection better than cells transfected with the vector-RZ combination.

To determine if there was something about adenovirus vectors that makes them less effective than plasmids in delivering the functional RZ, a different cell line, HOK-18A, was obtained which was derived by immortalizing normal cells by infection with PV. RZ 309 expression resulted in cleavage of the target RNA transcripts but growth inhibition of these cells was less than 50% at best.

One of the effects of PV is to reduce p53 protein levels, but RZ expression failed to bring these levels back up to normal. This could be related to the fact that RZ has its main effect on the spliced RNA while p53 is regulated by the E6 protein, which is mainly produced by the unspliced transcript.

Results indicated that adenovirus delivery of an anti-PV RZ inhibits focus formation, but not growth, of HeLa cells and inhibits growth of PV-immortalized cells. The adenovirus MMTV promoter was more effective than the GTI RSV promoter with adenovirus tripartite leader sequences.

The utility of this vector for clinical trials is not yet clear, but it can be used to answer certain questions about a ribozyme approach to therapy. Ribozymes should be capable of releasing from the target RNA after cleavage is mediated, must have a sufficiently long half-life and must be distributed in intracellular compartments to access the target RNA.

Anti-ras and Anti-raf Approaches

Motoya Takeuchi and Naoto Senmaru of Hokkaido University School of Medicine in Japan reported that, in preclinical studies, a dominant-negative H-ras mutant, N116Y, in an adenovirus vector (AdCEA-N116Y), inhibited growth of pancreatic and esophageal cancer cell lines carrying k-ras mutations. H-ras mutants block conversion of GDP-ras to its active GTP-ras form, thereby blocking ras-mediated cell signaling and arresting cells in the G0 to G1 phase of the cell cycle. Pancreatic cell lines which express carcinoembryonic antigen (CEA) were targeted by inserting a CEA promoter upstream of the N116Y gene (AdCEA-N116Y).

In work supported by the Canadian Breast Cancer Foundation, Aghdass Rasouli-Nia of the University of Alberta (Edmonton, Canada) reported that an antisense strategy to inhibit expression of c-raf could enhance paclitaxel chemotherapy in cervical tumors that had become resistant to the agent.

Apoptin

Apoptin is a recently discovered protein derived from chicken anemia virus which has powerful anticancer properties. According to Alexandra M. Pietersen of Leiden University in the Netherlands, apoptin induces apoptosis in a number of tumor cell lines, but not in non-transformed lines. Transfection of the apoptin gene in an adenovirus expression vector results in induction of apoptosis by a mechanism which is independent of p53 but stimulated by overexpression of Bcl-2. The protein is found in the nucleus of normal cells, but it is cytosolic in transformed cells. This difference in localization pattern may be related to its ability to induce apoptosis in one but not the other type of cell. In preclinical studies in animals the protein had no apparent toxic effects. Further studies are underway to investigate its use as a cancer therapeutic agent.

Interleukin-1β Converting Enzyme (ICE)

Interleukin-1β converting enzyme (ICE), a key inducer of apoptosis in mammalian cells, was harnessed for therapeutic use in gene therapy strategies for hepatocellular carcinoma (HCC). O.J. Qian of the Tumor Immunology and Gene Therapy Center (Shanghai, P.R. of China) reported results of preclinical studies which showed that apoptosis could be induced in an HCC cell line by direct transfection with a retroviral vector containing the ICE gene. In animal studies, intratumoral delivery of the vector resulted in suppression of tumor cell growth in mouse xenografts. Researchers plan to combine ICE gene therapy with chemotherapy in future studies.

SUICIDE GENE THERAPY STRATEGIES

In "suicide" gene therapy cancer cells are selectively transfected with suicide genes that confer a vulnerability to a certain drug that is non-toxic to unstransfected cells. In this manner, a suicide gene may be introduced either intratumorally, or systemically in a form that is taken up or expressed selectively only by tumor cells. A toxic drug is then introduced systemically that affects only the transfected tumor cells.

Target Selectivity

While suicide genes sensitize cells to a prodrug, an anticancer strategy also requires selectivity in order to avoid producing intolerable cytotoxicity. Approaches to selectivity include intratumoral delivery in localized but lethal types of cancer, such as brain cancer, ovarian cancer and mesothelioma. In this case the suicide gene is introduced directly into the tumor by injecting it in an expression vector, and the prodrug is administered systemically. Another approach uses systemic administration of a gene in a form which is taken up or expressed selectively by tumor cells. This requires either expression of the suicide gene under control of a tumor cell-specific promoter or use of some targetable characteristic of the tumor cell. This approach is possible if the degree of selectivity surpasses that obtained with standard chemotherapy, which commonly targets cancer cells by having a greater effect on cells that are undergoing rapid growth. Suicide gene strategies with selectivity have been used for treatment of hepatomas and melanomas.

Pre-emptive Suicide Gene Strategies

There are two essential conditions for success of suicide gene therapies, targetability and a sufficient level of expression. Targeting tumors, however, generally requires that the malignancy is localized. Most cancers may be inherently incurable once they have spread because they become virtually impossible to target. Therefore, it is reasonable to consider a third suicide gene therapy option, a pre-emptive strategy involving introduction of the suicide gene into cells before a cancer arises. Because cancer cells are clonal, a tumor that develops from a suicide gene-bearing cell will carry the gene as a clonal property.

A pre-emptive suicide gene strategy must surmount a number of challenges. One is the need for persistence of gene expression because, as a rule, trandsuced genes eventually undergo downregulation or mutational loss. A second issue is transduction efficiency because of the need to introduce the gene to all cells in the tissue. Another important consideration is whether the tissue is non-vital or vital. In some situations elimination of all of one type of tissue, such as lung tissue, would be life-threatening. However, when a tumor arises from non-vital tissue, as in the case of prostate cancer, complete ablation would be possible without serious consequences. Maintenance of vital tissues may require a mosaic use of two or more suicide genes.

Several suicide gene strategies for cancer therapy have an imminent prospect for clinical use. The first is suicide

gene transduction of certain cells in order to protect the patient from the eventuality that they may develop some kind of aberrant behavior. One example is allogeneic bone marrow transplant, a procedure that may be associated with severe morbidity caused by graft-versus-host reactions. Introduction of a suicide gene permits rapid elimination of transplanted stem cells if they begin to stimulate a systemically destructive immune response. A second example involves introduction of a suicide gene into autologous stem cells used to treat chronic myelogenous leukemia, in the event they undergo blastic transformation.

Another potential type of *ex-vivo* use of suicide gene therapy is a pre-emptive fail-safe strategy in cells cultured *in vitro* for eventual reconstitution in a patient. Introduction of a suicide gene may provide protection against eventual development of aberrant behavior such as malignancy, immunopathology or some other kind of malfunction. Cells could be protected against a mutation in p53 which might occur in a transgene introduced in p53 gene therapy. By introducing a vector containing an additional suicide gene, the patient is protected against clonal expansion of cells in which a random p53 mutation occurs.

Longer term prospects include use of suicide genes in animals that are a source of xenografts, to enhance safety. Another would be use of suicide genes by *in vivo* transduction into tissues at risk for cancer, such as the breast in individuals known to harbor predisposition genes. However, such strategies would require a major improvement in transduction efficiency.

One simple experiment of the pre-emptive suicide gene strategy was performed with the TM4 cell line which is a line of preneoplastic murine mammary epithelial cells. When injected into syngeneic mice, TM4 cells sometimes develop into mammary carcinomas. When this cell line was transfected with the HSV-tk gene and injected into mice and ganciclovir (GCV) treatment was initiated when carcinomas arose, there was a marked retardation of tumor growth compared with untreated controls. Thus, insertion of a suicide gene into a cell that is not yet malignant can facilitate later intervention.

Herpes Simplex Virus Thymidine Kinase (HSV-tk) Gene Combined with the Prodrug Ganciclovir (GCV)

One of the original suicide gene therapy strategies involved the herpes simplex virus thymidine kinase (HSV-tk) gene combined with the prodrug ganciclovir (GCV). Transfected cells which express HSV-tk will activate GCV to its phosphorylated derivatives which inhibit DNA synthesis. Because mammalian tk cannot use GCV as a substrate, only cells expressing the viral tk can activate the prodrug, which has no effect on non-transduced cells.

Phase II clinical trials of GLI 328 HSV-Tk gene therapy in recurrent glioblastoma were described by Mitchel S. Berger of the University of California, San Francisco, in collaboration with Genetic Therapy and Novartis (Basel, Switzerland). The agent delivers the HSV-tk gene by means of a retrovirus vector. For details about a phase II multicenter trial that was designed to determine safety and efficacy of repeated intratumoral injections of GLI 328 in patients with recurrent glioblastoma, see FO, p 750.

A series of phase I clinical trials of adenoviral-based gene therapy for malignant mesothelioma with concomitant immunosuppression was described by Daniel H. Sterman of the University of Pennsylvania Medical Center (Philadelphia, PA). This phase I trial, which was supported by the National Institutes of Health (NIH), began in November of 1995 to determine dosage of an E1/E3-deficient adenovirus containing the HSV-tk gene under control of an RSV promoter. The protocol involved initially draining the pleural fluid of patients through a chest tube placed in the intrapleural space. The following morning the Ad-HSV-tk vector was injected through the chest tube, and biopsies from the pleural cavities were taken after three days to determine the extent of gene transfer. GCV treatment was administered IV twice a day for 14 days.

There was evidence of tk gene transfer in the pleural cavity biopsies. Adverse reactions included an acute febrile response, initiated 6 hours post-injection, that lasted for up to 48 hours and increased in severity with dose. There was an elevation in transaminases after 12-15 hours and again 24 hours after GCV treatment. Eleven out of 26 patients developed a cutaneous vesicular bullous reaction around the chest tube site which resembled pemphigus vulgaris on biopsy. This was most likely caused by chemotherapy because it resolved by the end of GCV therapy and no vector sequences were detected in the lesions. Humoral, cellular and cytokine-mediated reactions occurred in all patients. Anti-adenovirus antibodies rose and persisted for a year. A pleural inflammatory reaction was observed which correlated with better gene transfer. There was an increase of mononuclear cells in the peripheral blood and of cytokines IL-6 and TNFα in the pleural space.

Among 26 enrolled patients, disease stabilized in some, but 11 died and several experienced disease progression. Assessing tumor progression is challenging because this type of tumor is difficult to quantify. For instance, there were no deaths among Stage I patients and a few of those with early-stage disease at the beginning of treatment survived for a prolonged period of up to 2 years. In more advanced disease, however, treatment had no significant effect on survival.

The role of the immune response is an important issue in gene therapy. On one hand, an immune response may augment antitumor mechanisms in the form of a "vaccine effect." On the other hand, an immune response limits gene uptake and transfer. In animals studies, immune suppression during Ad-tk gene therapy was beneficial, helping to inhibit virus-neutralizing antibodies and cytotoxic T cell production, and improving overall response to therapy. An additional phase I trial was, therefore, initiated to test the effect of transient immunosuppression induced by supple-

mental treatment with corticosteroids. This treatment eliminated the acute febrile response to gene therapy and chemotherapy, and mitigated the intrapleural inflammatory reaction. A few of the complications of steroid treatment were seen, including "steroid psychosis". Gene transfer was equivalent, but not superior, to the results of the trials without steroids. Most importantly, there was no significant blunting of the anti-adenovirus immune response or of the mononuclear cell proliferation response to adenovirus proteins in patients who were treated with steroids.

In conclusion, this vector is safe at dose levels of 10^{12} pfu, although inflammatory reactions are present. In future trials, an attempt may be made to decrease the immunogenicity of the vector and to enhance viral binding to the cell. Gene therapy will be initiated after tumor debulking to get better viral to cell ratios in order to increase the delivered gene dose.

Noninvasive imaging of adenoviral-mediated HSV-tk gene transfer and expression was discussed by Juri Tjuvajev of Memorial Sloan-Kettering Cancer Center (New York, NY). The HSV-tk gene was used in these studies as a noninvasive imaging marker together with the radiolabeled substrate FIAU, an analog of thymidine that can be incorporated into DNA without ill effect. FIAU is a good substrate for HSV-tk but not for mammalian kinases; thus, when it enters a cell expressing HSV-tk, it is phosphorylated and becomes trapped intracellularly. Cells expressing HSV-tk can then be imaged by positron emission tomography (PET). Gene expression, measured as radioactivity per gram of cells, was similar to that of lacZ gene co-expression from a bicistronic expression vector as determined by standard methods.

To develop a technique for monitoring therapeutic gene transfer and expression by measuring expression of a different marker gene using imaging methods, a bicistronic retroviral vector was prepared containing a gene for nerve growth factor receptor (NGFr) and HSV-tk. Expression of NGFr, a membrane-bound cell surface protein, can be quantified by FACS analysis. Expression of HSV-tk was measured as incorporation of radiolabeled FIAU. A retroviral vector was used to ensure reproducibility of results by creating stably transduced cells. Results demonstrated that both the NGFr and HSV-tk genes were proportionately co-expressed by transduced cells over a wide range of expression levels. Thus, HSV-tk can be used as a marker gene to monitor efficacy of transfer of a second clinically therapeutic gene. Similar multigene expression vectors might also be useful for noninvasive monitoring of movement and survival of transduced blood stem cells in patients.

Many clinical trials are now using adenovirus-mediated gene transfer under conditions in which gene transfer and expression are not easily monitored. To determine clinical utility of noninvasive imaging of adenovirus-mediated HSV-tk gene transfer and expression to monitor effectiveness of gene therapy, the HSV-tk gene was inserted into a replication defective adenovirus vector, DL-312, under control of a CMV promoter. This vector was then used in suicide gene therapy to treat an animal model of liver cancer metastasis. Liver tumors were produced in mice by injection with the colon carcinoma cell line MCA-26. The viral vector was injected intratumorally seven days later, followed by I-131 FIAU 18 hours later. The animals were then imaged 24 hours later with a gamma counter. Some of the animals were also treated with GCV for 6 days. Gamma counter images of the animals showed that radioactivity was present in the tumor itself, and in a punctate pattern in nontumor-infiltrated parts of the liver in animals injected with the Ad-HSV-tk vector, but not the control vector. The punctate radioactivity was attributed to uptake by the cholangial duct epithelium that had been transfected by the virus. Tumors disappeared after treatment with GCV. Thus HSV-tk levels were sufficient for suicide gene therapy as well as for imaging to monitor therapy. This work was supported by the NIH.

Other Prodrug Activating Gene Therapy

According to Frederick Moulton of Edith Norse Rogers Memorial Veterans Hospital (Bedford, MA) many prodrug-activating gene combinations are currently being tested for clinical use. One such approach is activation of 5-fluorocytosine (5-FC) by the *E. coli* cytosine deaminase (CD) gene which converts the prodrug 5-FC into 5-fluorouracil (5-FU). Another strategy involves conversion of 6-thioxanthine to the corresponding toxic ribonucleotide by the gamma-glutamyltranspeptidase (GGT) gene product. Cyclophosphamide, the prodrug for cytochrome P450-2B1, converts it to an alkylating agent, phosphoramide mustard. Although the P450-2B1 gene is normally expressed in the liver, when it is expressed by tumor cells it sensitizes them to cyclophosphamide by allowing them to perform the conversion intracellularly. Gene therapy with the gene for purine nucleotide phosphorylase results in the conversion of normal purines to 6-methyl purines. Overexpression of deoxycytidine kinase sensitizes tumor cells to ara-C by converting it to a monophosphate. Expression of the *E. coli* nitroreductase gene activates the prodrug CB 1954. Expression of a fusion protein consisting of Fas and FK506-binding protein, activates the death receptor and induces apoptosis by promoting Fas aggregation through binding of the fusion protein to FKNP.

Mutant HSV-1 tk combined with acyclovir (ACV) instead of GCV, was described by Margaret E. Black of Darwin Discovery (Chiroscience; Bothell, Washington). While GCV is effective as a prodrug in suicide gene strategies, its dose must be limited because of its tendency to be immunosuppressive. By contrast, ACV, which is commonly used to treat HSV infections, is not immunosuppressive and is non-toxic at high doses. But ACV has not been used as a prodrug for HSV-tk gene therapy because it is a relatively poor substrate for the enzyme as compared to GCV. The approach taken to improve ACV's usefulness for gene suicide therapy was to find mutant HSV-tks that have a higher specificity for this prodrug.

Mutant HSV-tk clones were generated by a random sequence mutagenesis approach and screened in a tk- strain of *E. coli* to identify those that had a higher specificity for ACV, or GCV, or both. Selected clones were expressed in mammalian cells to determine their ability to confer prodrug sensitivity. The resulting panel of clones all had multiple mutations in tk, which generally were 4-6 amino acid changes at the active site. Two clones, 30 and SR39, were selected for further characterization and when tested in a mouse xenograft model *in vivo,* both caused tumor growth arrest in combination with either ACV or GCV. Also, both mutants were more active *in vivo* and *in vitro* when compared with wild-type tk. Thus, ACV can function as a prodrug in suicide gene therapy when used with one of these mutant HSV-tk genes.

New enzyme/prodrug combinations for use in gene-directed enzyme prodrug therapy (GDEPT) are being investigated by Caroline J. Springer of CRC Centre for Cancer Therapeutics (Surrey, UK) in collaboration with Glaxo Wellcome, to identify enzyme prodrug combinations for use in gene suicide therapies. One combination tested with positive results was the *E. coli* nitroreductase (NR) gene and the nitrogen mustard-based prodrug CB 1954 whose active form crosslinks DNA interstrandedly. When NR+ and NR- cells were mixed in a one-to-one ratio and treated with CB 1954, a plot of cell viability versus the presence of cells expressing NR, showed much greater cell killing than expected if only the NR+ cells were affected. There was total cell killing when only 20% or less of the cells expressed NR.

Another gene therapy combination studied was bacterial carboxypeptidase G2 (CPG2) with the prodrug CMDA, a nitrogen mustard bifunctional alkylating agent. Expression of CPG2 improved the sensitivity of mammalian liver cells to CMDA by 11 to 95 times that of controls; 100% cell killing was achieved when only 4-12% of the cells expressed the enzyme. To improve the effectiveness of this combination, the CPG2 enzyme was mutated to a form that was expressed on the surface of the cell by a molecular tether. This enabled the enzyme to activate the prodrug outside the cell, which was expected to improve efficiency of the bystander effect by allowing activated drug to diffuse to neighboring cells more readily. Total cell kill occurred when 2%-19% of the cells expressed the enzyme. Cells expressing the cell surface form of the enzyme were significantly more sensitive to CMDA than controls, but only slightly more sensitive than cells expressing the internal form of the enzyme. Thus, gene therapy with either form of the CPG2 gene is likely to be effective in GDEPT with the prodrug CMDA.

THE BYSTANDER EFFECT

By definition, the bystander effect occurs when only a fraction of the tumor expresses a transfected gene, such as p53, and yet a larger portion of the tumor exhibits properties, such as apoptosis, induced by the expression of that gene. The effect can be demonstrated by mixing tumor cells expressing p53 with an equal number of cells lacking the functional gene. *In vitro*, only cells expressing p53 undergo apoptosis when a chemotherapeutic agent is added to the culture medium. Thus, in a 50:50 mix i*n vitro*, 50% of the cells die. When this cell mixture is implanted into an animal, however, 80% of the cells undergo apoptosis, a phenomenon attributed to the bystander effect, which is presumed to be mediated by production of cytokines. In another example, in HSV-tk/GCV gene therapy, a bystander effect is possibly caused by diffusion of the active drug from one cell to another.

The bystander effect *in vivo* is a complex event. Killing tumor cells by strategies which depend on a bystander effect involves three steps, chemosensitization of the tumor; induction of hemorrhagic tumor necrosis; and alteration of the microenvironment in the form of an immune response. It is believed that the combination of these three factors *in vivo* leads to the bystander effect, a biological process that does not occur *in vitro.*

Evaluation of Adenovirus p53-mediated Bystander Effect *In Vivo*

Research on the bystander effect conducted at Louisiana State University School of Medicine (New Orleans, LA) was presented by Scott Friedman. The bystander effect phenomenon was first noted when therapeutic effects of p53 gene therapy *in vivo* were found to exceed that expected, given the dose of the gene delivered.

To determine whether cytokines were involved in the bystander effect an intraperitoneal tumor model was used in which mice were injected with p53-transfected F6 tumor cells or lacZ control-transfected tumor cells and then with tk cells and GCV. Surprisingly, the tk cells actually homed to the intraperitoneal tumors. When tumors were removed and analyzed for cytokine expression, those from animals treated with the p53-transfected cells expressed TNF, IL-1, IL-6 and IFN-γ and had undergone extensive hemorrhagic necrosis.

The extent of the bystander effect depends on the gene used. The p53 gene exhibits a relatively weak bystander effect, HSV-tk an intermediate effect, but an antiviral nucleotide analog has a strong bystander effect *in vivo*. The overall conclusion is that in the case of p53 transduction, a therapeutic bystander effect occurs only *in vivo* and is associated with pro-inflammatory cytokine production.

Bystander Effect in HSV-Tk Suicide Gene Therapy

Scott M. Freeman of Tulane University (New Orleans, LA) presented studies investigating involvement of the bystander effect in HSV-tk gene suicide therapy for treating chemotherapy-resistant tumors. Because 50% of all cancers relapse, it is theorized that resistant cells are present in about 50% of all tumors. Combating treatment resistance requires special strategies to ensure that both resistant and sensitive cells are killed simultaneously. This may be possible using the bystander effect associated with suicide gene therapy.

The bystander effect does not simply arise from a cell-to-cell interaction. For instance, there is no bystander effect *in vitro* with HSV-tk/GCV treatment. When cells expressing HSV-tk are mixed in culture with cells lacking the gene, only those cells expressing the gene are eventually killed by GCV. *In vivo*, however, a tumor containing 50% tk-expressing and 50% non-expressing cells totally regresses upon GCV treatment as a result of the bystander effect. One possible explanation for this phenomenon is an immune response that involves production of a cascade of pro-inflammatory cytokines as a result of activation of GCV which in turn leads to recruitment of CD3 cells to the tumor and causes hemorrhagic tumor necrosis.

A phase I clinical trial of tk-GCV suicide therapy for relapsed Stage III ovarian cancers was initiated involving 15 patients to study the bystander effect in the clinical setting. Ovarian tumors are accessible to intraperitoneal administration of the gene therapy vector because in 80% of cases they are confined to the peritoneal cavity. Relapses are uniformly fatal and refractory to treatment because of chemotherapy resistance. Relapses are easily detected early by imaging, and after debulking by surgery about 70% of relapsed tumors are less than 2 cm in size, so that there is minimal disease early after relapse. In dose escalation trials, tk-positive cells were injected intraperitoneally which tended to home to micrometastases in the peritoneal cavity. Adverse effects of this protocol were minimal and limited to fever, nausea and abdominal pain. Median survival was improved from an expected 9-10 months to 12 months. Three of the patients were cured and five remained stable with flat CA-125 levels.

Adverse effects of the protocol were minimal and limited to fever, nausea and abdominal pain. Median survival for these patients was improved from an expected 9-10 months to 12 months. Three of the patients were cured and five remain stable with flat CA 125 levels.

A second trial has been initiated for relapsed ovarian cancer with an amended protocol involving immunization with a cell line expressing Her2/Neu followed by treatment with the HSV tk-GCV chemosensitizing strategy. Patients treated with this approach experience prolonged survival. Immunization results in an initial infiltration of the tumor with lymphocytes, which appear to contribute to subsequent tumor necrosis caused by the bystander effect induced by the HSV tk-GCV strategy.

IMPROVING DELIVERY APPROACHES

Enhancing Nonviral Delivery

P.T. Jain of Virginia Commonwealth University Medical College of Virginia (Richmond, VA) discussed methods of enhancing nonviral gene therapy using several vehicles, including dimethyl sulfoxide (DMSO), polyethylene glycol (PEG) and combinations of ethanol and PEG. Results indicated that DMSO concentrations of less than 1% were most effective and that a critical characteristic of the vehicle is its ability to solubilize the drug.

Vaccinia Virus

T. Timiryasova of Loma Linda University School of Medicine (Loma Linda, CA) discussed studies in which vaccinia virus (VV) was tested for its ability to serve as a delivery vehicle for p53 gene replacement therapies. VV can infect a wide variety of cancer cell lines, including rat glioma cells which were selected to test a VV-p53-lacZ construct. These cells contain a mutated p53 gene and form tumors in athymic mice. Increased rates of cell death were observed when cells were infected with either VV-p53 or a control virus lacking the p53 gene, but the effect was more pronounced with the p53-containing virus. To determine the virulence of VV, a reporter construct was made that contained the genes for firefly luciferase and β galactosidase (lacZ). Reporter gene expression was found only in skin and kidneys of rats or nude mice one to two weeks after inoculation with the virus, and none of the animals showed signs of viral disease. When the viral constructs were used to transfect mouse xenografts, tumor growth was significantly retarded, whether or not the virus contained the p53 gene. Thus, the virus alone has some oncolytic potential and the addition of p53 expression enhances its anticancer effect. These studies aim to provide a new treatment strategy for malignant brain tumors.

Tumor-specific Ligand-liposome Gene Delivery

Tumor-specific ligand-liposome delivery of genes for cancer therapy improves gene transfer and, therefore, the success of gene therapy outcomes. Esther H. Chang of the Vincent Lombardi Cancer Center at Georgetown Medical Center (Washington, DC), described optimization of a ligand-liposome delivery system to treat radiation-resistant squamous cell carcinoma of the head and neck by p53 gene replacement. In an animal model, when p53 encapsulated in ligand-liposomes was delivered intravenously, preexisting xenografts regressed dramatically after radiotherapy or chemotherapy. Tail vein or intratumoral injection of ligand-liposomes with a reporter gene resulted in improved gene delivery as compared to injection of liposome-DNA or naked DNA, and the ligand-liposome system was shown to result in tumor-specific gene uptake.

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