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STATE-OF-THE-ART IN THE MANAGEMENT OF CANCER

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MEETING COVERAGE

GENE THERAPY OF CANCER — PART II

MECHANISM-BASED AND OTHER TREATMENT OPTIONS FROM THE 9TH INTERNATIONAL CONFERENCE ON GENE THERAPY OF CANCER, SPONSORED BY SIDNEY KIMMEL CANCER CENTER, SAN DIEGO, CA, DECEMBER 7-9, 2000

STATE-OF-THE-ART IN THE MANAGEMENT OF CANCER

BLADDER CANCER — PART IV

PROGNOSIS AND TREATMENT OF BLADDER CANCER BY STAGE AND GRADE

In the USA, bladder cancer is the fourth most commonly diagnosed malignancy in men and the eighth in women (FO, pp 1231-32 and 1234-36). Approximately 93% of bladder cancers are transitional cell carcinomas (TCC) originating in the inner lining of the bladder composed of transitional cells; 5% are squamous cell tumors, and 2% are adenocarcinomas or tumors of mixed lineage. Bladder cancer is not a single condition but a spectrum of diseases, ranging from superficial, well-differentiated tumors, that do not significantly impact survival, to aggressive malignancies with a poor long-term survival outlook. As a result, bladder cancer, primarily TCC, may be viewed as four separate disease entities, superficial TCC, accounting for 80% of all first diagnoses of bladder cancer, carcinoma *in situ* (CIS) which requires a more aggressive treatment course, muscle-invasive disease accounting for 15%, and metastatic disease, accounting for 5% (Exhibit 1). Each of these disease entities and subcategories within them are treated differently, depending on patient age or health status, patient or physician preference, quality of life issues, and cost. Exhibit 2 lists some of the treatment options available for different stages and grades of bladder cancer and comments on outcomes.

Eventually, the way tumors are classified will rely less and less on their histologic appearance and more and more on their molecular constituents (see FO, pp 1242-1244). Management will rely on prognostic assessments based on the tumor's molecular characteristics and how it is expected to respond to various treatment options. This new classification approach implies greater complexity in the staging and treatment of bladder cancer, but molecular-based identification of bladder cancer patients at greatest risk for progression may ultimately improve clinical management (Lee R and Droller MJ, Urol Clin North Am, Feb 2000;27(1):1-13, vii).

Although bladder cancer is rare, with about 54,193 Americans diagnosed with the disease for the first-time every year, it tends to recur at a high rate, and also progress to invasive and metastatic disease during its course (Exhibit 1). Therefore, it is estimated that in the USA, there are over 450,000 bladder cancer survivors, many under doctor's care to prevent recurrence and, in a small number of cases, to treat progressive disease. Also, a small but significant proportion of these patients needs to be retreated at some point because of recurrence, nearly doubling the number of bladder cancer patients seeking treatment every year (Exhibit 1). This situation has created a rather unique market opportunity in prognostic and monitoring services and prophylactic treatment using immunotherapy and/or chemotherapy.

TREATMENT OF BLADDER CANCER

Available treatment options for bladder cancer by stage and grade and their potential outcome are described in Exhibit 2. Management of bladder cancer differs significantly from that of other malignancies because this disease occurs mostly in the elderly, almost always presents in early stages, and most of the time recurs as early-stage disease rather than progressing to an invasive/metastatic stage. Also, it is currently recommended that an immunotherapy/vaccine approach be used in more aggressive early disease to prevent or at least lengthen the interval between recurrences.

Although a minimally invasive approach, transurethral resection (TUR), suffices in treating early disease, aggressive early tumors and later-stage disease may require removal of the bladder. Although reconstruction is possible, often the age and physical condition of patients make such procedures unfeasible. Currently, there is no satisfactory intervention for muscle-invasive and metastatic bladder cancer.

Superficial Bladder Cancer

Effective approaches for the management of superficial bladder cancer are fundamental in ensuring the ultimate success of treatment for this disease, because early bladder cancer comprises 80% of all newly diagnosed cases and the majority of cases of recurrent disease.

Superficial bladder cancer presents as a heterogeneous group of tumors with variable biologic potential. They are classified as ≤ Stage I malignancy confined to the urothelium such as papillary tumors (Ta), papillary tumors invading the underlying lamina propria (T1), and CIS, flat, reddened lesions with high-grade histologic features. Although early-stage tumors can usually be removed by TUR, they recur in over 70% patients within 5 years. Each tumor carries a risk of progression to invasive and potentially metastatic disease and, therefore, patients are stratified into low-or high-risk for recurrence and progression. For instance, patients with high-grade Ta tumors have a lifelong risk of disease stage progression and death from bladder cancer similar to those with T1 tumors (Herr HW, J Urol, Jan 2000;163(1):60-1; discussion 61-2). Risk of recurrence after initial resection of Ta or T1 tumors is estimated at 80% as indicated by a retrospective study involving 176 (evaluable=175) patients with primary Stage Ta and T1 bladder cancer, treated in Sweden between 1963 and 1972, who were followed until death or for at least 20 years (Holmang S, etal, J Urol, Jun 1995;153(6):1823-6; discussion 1826-7). According to this study, in 1993, 13 (7.4%) patients were disease-free, 39 had died of bladder cancer and 123 had died of other causes. Of 77 patients with a primary noninfiltrating tumors and 99 with a primary lamina propria invasive tumors, 9 (11%) and 30 (30%), respectively, died of bladder cancer.

To determine the natural history of TCC of the bladder, and to identify factors which place patients at lifelong risk of developing progression and dying from bladder carcino-

Exhibit 1
Estimated Incidence and Prevalence of Bladder Cancer by Stage in the USA in 2000

Stage	1st Diagnosis (#)	Total (%)	Patients with Recurrent/ Active Disease (#)	Incidence All Diagnosis (#)	Estimated 5-year Survival (%)	Deaths (#)	Prevalence of ever Diagnosed Bladder Cancer (#)
Superficial[1]	43,354[2]	80.0	30,000	73,354	95.0		
Grade I	21,677	50.0					
Muscle-invasive	8,129	15.0	7,500	15,629	50.0[3]		
Metastatic	2,710	5.0	2,000	4,710	10.0[4]		
Total	54,193	100.0	39,500	93,693		12,095	450,000

[1] Includes carcinoma in situ (CIS)
[2] Approximately 15% progress to muscle-invasive
[3] Survival ranges from 75% for low-grade Stage II tumors to 20% to 40% for Stage III tumors
[4] The 3-year survival rate is about 20%; median survival is approximately 1 year following treatment with traditional platinum-based regimens.

ma, scientists at the Diakonissehjemmets University Hospital (Bergen, Norway) evaluated retrospectively the long-term outcome of 231 patients presenting with superficial TCC between 1981 and 1986, based on a median follow-up of 108 months. Of 231 patients, 217 (94%) were initially treated by TUR or segmental resection. Recurrence developed in 141 of 217 (65%) patients. The recurrence-free interval was significantly shorter for patients with initial Grade III than Grade I tumors, and for those with T1 compared with Ta disease, with such differences being statistically significant. Disease progressed in 42 of 231 (18%) patients. In 27 of 231 (12%) patients, TCC of the bladder was the cause of death, while 118 (51%) died from unrelated causes. There were no deaths among patients with initial Ta/Grade I tumors, compared with 10 of 26 (38%) deaths in those with T1/Grade III disease at presentation. Long-term prognosis is good for patients with Ta Grade I tumors, while T1 Grade III is a potentially aggressive disease (Haukaas S, etal, BJU Int, Jun 1999;83(9):957-63).

There have been major strides in preventing early recurrence of superficial TCC. BCG immunotherapy has been confirmed to be highly effective in reducing tumor recurrence in the treatment of residual papillary TCC and the treatment of CIS. The response rate in the treatment of the papillary disease averages 55%, and for CIS 73%. In the prevention of tumor recurrence the relative benefit of BCG is 45%. In a direct prospective randomized comparison of BCG and intravesical chemotherapy, BCG was significantly superior to thiotepa, doxorubicin and mitomycin C when only patients with intermediate and high risk for recurrence were treated. However, in clinical studies in patients with low recurrence risk, there was no advantage with BCG immunotherapy and it was not superior to chemotherapy in preventing progression to ≥T2 (Kurth KH, etal, Eur Urol 2000;37 Suppl 3:1-9).

In this study, conducted by the EORTC Genitourinary Group at 18 institutions, the incidence of recurrence at 3 months (3RR) after complete TUR of all visible lesions, by year of entry, for single tumors, ranged from 21.0% to 43.8% during 1975-1978, 6.3% to 12.7% during 1984-1986, and 3% to 5.3% during 1987-1989. For multiple tumors it ranged from 50.0% to 61.5% during 1975-1978, from 20.2% to 27.3% during 1979-1983, and from 14.4% to 24.6% during 1984-1986. Throughout the period, and by institution, for single tumors, the 3RR varied from 0% to 36%, and for multiple tumors from 7% to 75%. Overall, the 3RR by number of tumors was 8.7% for single tumors, 21% for 2-5 tumors and 32.2% for >5 tumors. One of the key contributors to reduced recurrence was administration of BCG. A single early instillation of BCG within 6 hours after TUR in patients with a solitary Ta/T1 Grade I/III bladder tumors, would reduce the per year recurrence rate by nearly 50%. Optimal results were achievable by initiating treatment early (within 24 hours after TUR) and for a duration of 6 months, and maintenance (>6 months) for patients with a delayed first instillation (>7 days after TUR).

Because of the high likelihood for recurrence, the American Urological Association (AUA) issued guidelines in 1999 urging physicians to consider using intravesical immunotherapy or chemotherapy as adjuvant therapy following TUR for non-muscle-invasive bladder cancer (Smith JA Jr, etal, J Urol, Nov 1999;162(5):1697-701; comment in J Urol, Jun 2000;163(6):1890-1). However, although the data examined by the AUA panel shows that intravesical agents decrease bladder cancer recurrence rates, there was no evidence that they affect long-term progression, and they may not be appropriate in all cases.

Although a Southwest Oncology Group study (SWOG 8507) demonstrated increased efficacy of a BCG maintenance program consisting of 3 weekly treatments at 3 months, 6 months, and every 6 months thereafter, for 3

years following 6 weekly instillations with BCG as compared to no maintenance consisting of 3 weekly treatments at 3 months and 6 months, the remarkable results from the maintenance arm were unfortunately accompanied by Grade 3 or 4 toxicity in 26% of cases. In fact, only 16% of the patients in the maintenance arm were administered BCG at each of the 7 prescribed courses.

Low-risk superficial bladder cancer refers to early-stage superficial disease diagnosed for the first time, or after a long interval without recurrence. Low-risk tumors are of a papillary configuration, ≤3 cm in size, have not invaded the lamina propria (Ta), and are well or moderately differentiated (Grade I or II). More than 90% of patients with Stage Ta, Grade I TCC have a benign form of bladder neoplasm, and few have truly malignant tumors. Further distinctions between tumors may also have treatment implications.

In 255 (37.5%) patients with Stage Ta, Grade I TCC, tumors were further classified as papillary neoplasm of low malignant potential in 95 and low-grade papillary carcinoma in 160. During a mean observation time of 60 months, these patients underwent 1,858 negative cystoscopies and 577 operations for recurrences. Risk of recurrence was 35% in patients with papillary neoplasm of low malignant potential compared to 71% in those with low-grade papillary carcinoma, and was higher in patients with multiple tumors at first diagnosis as well in those with recurrence at the first follow-up after 3 to 4 months. Stage progressed in 6 patients (2.4%) with low-grade papillary carcinoma at diagnosis. Subgrouping of Grade I bladder tumors as papillary neoplasm of low malignant potential and low-grade papillary carcinoma seems to add valuable prognostic information (Holmang S, etal, J Urol, Sep 1999;162(3 Pt 1):702-7); no papillary urothelial neoplasm of low malignant potential progressed in stage (Holmang S, etal, J Urol, Apr 2001;165(4):1124-8; discussion 1128-30).

Although Ta Grade I TCC is considered a benign form of TCC, it recurred within 5 years in many series and may also advance in grade and stage. Among 152 patients with initial Ta Grade I TCC, followed for a mean of 76 months (range 6 to 241), tumor recurred in 83 (55%); within 12 months of follow-up in 38 (46%), between 12 and 24 months in 11 (13%), between 24 and 60 months in 22 (27%), and in 12 (14%) more than 60 months after the first tumor. Among these 83 patients, disease progressed in grade in 31 (37%), including 21 to Grade II and 2 to Grade III disease; CIS was diagnosed in 3 patients and muscle-invasive disease in 5. Progression occurred more than 24 months after initial diagnosis in 20 patients, and more than 60 months after first tumor event in 12. Ta Grade 1 bladder TCC has a high recurrence rate and grade progression is not uncommon (Leblanc B, etal, J Urol, Dec 1999;162(6):1946-50).

TUR is the standard treatment for low-risk superficial tumors. BCG may be used for prophylaxis in selected patients considered at risk for recurrences.

High-risk superficial bladder cancer refers to more advanced disease diagnosed for the first time, or one that underwent multiple recurrences within a short period of time. High-risk tumors are >3 cm in size, appear to be less papillary (sessile) in configuration, cannot be completely resected because of technical problems, or exhibit diffuse bladder involvement. High-grade tumors and/or tumors invading the lamina propria are classified as T1 lesions. CIS associated with papillary tumors, is also an adverse prognostic sign.

Significant variations are also observed within tumor subclasses. Among 121 primary Stage T1 tumors diagnosed in western Sweden between 1987 and 1988, analyzed with respect to the depth of invasion in relation to the lamina muscularis mucosa, the progression rate among those with Stage T1b Grade III cancer was 58% versus 36%, and the risk of dying of bladder carcinoma was double (45% versus 23%) compared to those with Stage T1a Grade III disease (Holmang S, etal, J Urol, Mar 1997;157(3):800-3; discussion 804; comment in J Urol, Nov 1997;158(5):1922). There is at least a 70% risk for recurrence and a 20% risk for stage progression in patients with high-grade carcinoma (Holmang S, Semin Urol Oncol, Nov 2000;18(4):273-9). Recurrence developed in 73% of patients with Ta Grade II/III TCC and disease progressed in 45% of the patients with Grade III TCC compared to 20% with Grade II. Grade II tumors in patients that progressed in stage years later seem to have different immunohistochemical and molecular marker profiles compared to those in matched controls (Holmang S, etal, J Urol, Apr 2001, *ibid*).

BCG is the standard prophylaxis for high-grade, superficial tumors. Patients who fail BCG are candidates for a subsequent treatment with other intravesical agents. Although these patients have a low risk of progression, the major clinical challenge is multiple recurrences. Among patients with high-grade tumors and CIS who fail a first course of BCG, 50% will respond to a second course of BCG. Patients who fail this second course of treatment should undergo cystectomy as they are at a high likelihood (30%-60%) of developing invasive or metastatic disease. IFN-α and valrubicin may be used in patients who decline cystectomy, or are not candidates for surgery. Presence of T1 tumor or persisting CIS after a 6-week course of BCG at the first 3-month cystoscopy is associated with a high risk of muscle-invasive disease and, therefore, requires a more aggressive management policy (Herr H and Jakse G, Eur Urol 1991;20:1-8).

In a prospective, non-randomized trial, 37 patients with high-risk, superficial bladder cancer (rapidly recurring Grade II or III Ta, T1 or CIS) were administered one or two 6-week induction courses of intravesical BCG, followed by monthly maintenance for 12 months. Entry criteria were identical to those of SWOG 8507. Within a mean follow-up interval of 40.7 months (range=13-101 months), 28/37 (75.7%) patients remained free of disease recurrence

Exhibit 2
Treatment of Bladder Cancer by Stage and Grade

Treatment	Prognosis/Survival
	≤ Stage I; any Grade
TUR + intravesical chemotherapy (mitomycin C) and/or immunotherapy using BCG, or IFN-α	Intravesical prophylaxis after TUR was recommended by the 1999 AUA Bladder Cancer Cancer Guidelines (Smith JA Jr, etal, J Urol, Nov 1999;162(5):1697-701; comment in J Urol, Jun 2000;163(6):1890-1)
TUR + BCG	Treatment may delay progression to muscle-invasive disease in Ta/T1 tumors; however, BCG is not well tolerated, because it induces chemical cystitis in over 90% of patients, with systemic symptoms such as fever, malaise, and nausea
TUR+ intravesical epirubicin (20 mg), *q* second week for 4 months, and then once-a-month, or *q* 2 weeks for next 8 months	RFS rate was 76.1% and 52.3% at 2 and 5 years after TUR, respectively (Kondo T, etal, Int J Urol, Apr 1999;6(4):178-83)
Full dose external beam radiotherapy (EBRT)	EBRT was associated with high local recurrence and serious complications; it is questionable whether elderly patients actually benefit from full dose EBRT (Holmang S, etal, (J Urol, May 1997;157(5):1642-6; comment in J Urol, May 1997;157(5):1647-8)
	Ta/N0/M0; Grade I/II
TUR + ≥1 courses of BCG	Among 23 patients with Grade II disease, the 15-year PFS rate was 95%; none died of the disease (Herr HW, J Urol, Jan 2000;163(1):60-1; discussion 61-2)
	Ta/N0/M0; Grade III (high risk)
TUR + intravesical immunotherapy	Among 605 patients with Stage Ta Grade III primary bladder tumors, treated in France from 1982 to 1996, 32 (5.3%) were treated with Pasteur strain BCG (75 mg/50 ml saline), weekly, for 6 weeks; at a follow-up of 2 to 13 years (mean=58.4 months), 9 (28%) patients responded positively to BCG without recurrence, while disease recurred as Stage Ta in 8 (25%) and T1 in 7 (22%), and progressed to muscle layer infiltration in 8 (25%); 4 (12%) died of bladder cancer (Lebret T, etal, J Urol, Jan 2000;163(1):63-7; comment:79-80)
TUR + ≥1 courses of BCG	Among 125 patients, 15-year PFS and disease-specific survival rates were 61% and 74%, respectively (Herr HW, *ibid*)
	T1/N0/M0; any Grade
BCG (27 mg) weekly for over 6 weeks	Among 111 treated patients (group A), tumors recurred in 39% compared to 71.7% of untreated patients (group B); progression of tumor stage was observed in 6.3% in group A and 10.9% in group B but significant differences were found in the number of recurrences, in those with Grade I (28.5% versus 69%) and Grade II (47% versus 72%) tumors, but not Grade III (53% versus 77%); because results were not as good as those reported in the literature, this approach is not recommended for Grade II/III disease, but may be used for Grade I disease (Moyano Calvo JL, etal, Arch Esp Urol, Sep 1999;52(7):760-8)
	T1/N0/M0; Grade II/III
Weekly BCG during the first month, *q* 15 days during the 2nd and 3rd months, and once monthly until completion of a 12-month regimen	Among 67 patients with Stage T1 bladder cancer, at an average follow-up of 51.3 months, 17 (25.4%) relapsed (Grade III=33% and Grade II=27%); monthly maintenance dose helps maintain immunity (Rivera P, etal, Actas Urol Esp, Oct 1999;23(9):757-62)
	T1/N0/M0; Grade III
TUR + ≥1 courses of BCG	Among 73 patients 15-year PFS and disease-specific survival rates were 44% and 62%, respectively (Herr HW, *ibid*); after a median follow-up of 85 months, among 51 patients, 32 (62.7%) remained progression free, 9 (17.6%) progressed, 8 (15.7%) died of other causes, and 2 (3.9%) were lost to follow-up; risk of disease progression was significantly higher for patients with a tumor measuring ≥3 cm or more and those with CIS and for patients with recurrent tumors, solid tumors, or early recurrence after BCG; at 5 years 34 patients (66.7%) were alive; 8 (15.7%) had died of other causes and 7 (13.7%) of bladder cancer, and 2 (3.9%) were lost to follow-up; disease-specific survival was 86.3% (Hurle R, Urology, Aug 1999;54(2):258-63)

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TUR alone	Among 34 patients (solitary tumor=47% and multiple tumors=53%), followed for a median of 40 months, disease recurred in 50% with a DFS of 9.6 months; progression of the primary tumor was observed in 23.5% of patients; ORR was 73.6% and the cancer-specific survival estimate was 88.2% at a mean 36 months of follow-up (Zungri E, etal, Eur Urol, Nov 1999;36(5):380-4; discussion 384-5)
Radical cystectomy	Patients treated with radical cystectomy had excellent survival regardless of the depth of invasion in the lamina propria (Holmang S, etal, J Urol, May 1997, *ibid*)
T1b/N0/M0	
TUR alone	Prognosis is poor in patients with deep lamina propria invasion treated with TUR alone (Holmang S, etal, J Urol, Mar 1997;157(3):800-3; discussion 804; comment in J Urol, Nov 1997;158(5):1922)
RT	RT was associated with poor survival in Stage T1b disease
Tcis/N0/M0;CIS	
TUR, in combination with intravesical chemotherapy/immunotherapy (BCG is the first agent of choice)	TUR preserves QoL; cystectomy may still be performed if recurrence occurs after TUR
Radical cystectomy	Although, because recurrence is high in CIS, cystectomy appears to be the optimal choice, after adjusting for age, it does not offer a significant survival advantage (Cheng L, etal, Cancer, 1 Jun 1999;85(11):2469-74)
T2/T3a, N0, M0 (Muscle-invasive)	
Radical cystectomy with pelvic lymph node dissection (PLND)	In T2 and T3a, lymph node-negative disease, 5- and 10-year RFS were 89% and 87% and 78% and 76%, respectively; probability of recurrence was much higher in patients with non-organ-confined T3b, or T4; 5-year survival was 82% for T2, 71% for T3a, 45% for T3b, 74% for T4a (ducts), 51% for T4a (stroma), and 26% for T4b (Stein JP, J Clin Oncol, 1 Feb 2001;19(3):666-75)
Radical TUR	Among 133 patients with invasive bladder cancer with tumor clinically limited to the muscular layer and all biopsies of the periphery and depth of the tumor bed showing muscular tissue negative for tumor cells, at 5 and 10 years of follow-up, cause-specific survival rates were 80.5% and 74.5%, and bladder preservation rates were 82.7% and 79.6%, respectively; initial presence of associated bladder CIS was the only independent progression predictive factor; patients with initial CIS should be treated by BCG and followed closely (Solsona E, etal, J Urol, Jan 1998;159(1):95-8; discussion 98-9)
TUR followed by 2-4 cycles of methotrexate, cisplatin, and vinblastine, followed by RT (50 Gy to the bladder and 40 Gy to the regional lymph nodes) in responders	Survival was similar to that with radical cystectomy; RT, in conjunction with concurrent platinum-based chemotherapy, controls most urothelial bladder tumors with histologically-proven CR rates of macroscopic tumors (unresectable by TUR) in the range of about 70%; after radiochemotherapy, a histologic response evaluation with repeated TUR is recommended and patients with residual tumor require salvage cystectomy; those with CR who maintain their bladders must be closely followed; the risk of severe late-radiation sequelae is low, in the range of <5%; about 75% of long-term survivors maintain a normally functioning bladder (Dunst J, Semin Surg Oncol, Jan-Feb 2001; 20(1):24-32)
External beam RT (EBRT) only	After a median follow-up of 7.5 years, 19/83 (23%) patients were alive (Moonen L, etal, Int J Radiat Oncol Biol Phys, 1 Apr 2001;49(5):1305-10)
T2/N0/M0	
Partial cystectomy	Survival rate is estimated at 29%-80%
TUR alone	Overall survival is estmated at 57%-70%
Visibly complete TUR, and CR after induction chemoradiotherapy	The ideal candidate for bladder preservation has primary clinical Stage T2 tumor and no associated ureteral obstruction (Shipley WU, etal, J Urol, Aug 1999;162(2):445-50; discussion 450-1)
Primary RT	Overall 5-year survival rate is about 40%, with a local control rate of 40%-50%; distant metastasis develop in 10% of patients
T3a/N0/M0	
TUR alone	Overall survival is 14%-57%
T3b/N0/M0	
TUR alone	Overall survival is 2%-7%

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T3/N0/M0	
Partial cystectomy	Survival rate is estimated at 7%-33%
Primary RT	The 5-year survival rate is approximately 20%, and the local recurrence rate ranges from 50%-70%
T2/T3/N0/M0	
Neoadjuvant MVAC and TUR or partial/radical cystectomy	Among 60 CR patients [T2=38 (63%) and T3=22 (37%)], overall survival at 10-year follow-up was 70% in the TUR arm and 65% in the radical cystectomy arm; the majority of patients with invasive bladder tumors who achieve T0 status after neoadjuvant MVAC chemotherapy preserve their bladders for up to 10 years with bladder-sparing surgery; the bladder remains at risk for new invasive tumors and cystectomy salvages the majority, but not all, of relapsing patients (Herr HW, etal, J Clin Oncol, Apr 1998;16(4):1298-301)
Advanced/metastatic disease	
Neoadjuvant chemotherapy with/ without bladder preservation	Chemotherapy is employed to downstage tumors and eradicate systemic micrometastases before surgery
Primary RT	The 5-year survival rate is 10%
Surgical resection of metastatic disease	Among 25 patients with metastatic urothelial cancer, a complete resection of all gross disease was performed in 96%; the most frequently resected location was lung in 20 (80%), followed by brain in 2 (8%), and distant lymph nodes in 3 (12%); MST from time of metastasectomy was 23 months; MTP following metastasectomy was 6.5 months but 3 patients remained free of disease for more than 5 years; results in this highly selected cohort, with 35% alive at 5 years post metastasectomy, suggest that resection of metastatic disease is feasible, and can sometimes contribute to long-term disease control (Siefker-Radtke AO, etal, ASCO01, Abs. 709:178a)
MVAC	MVAC in metastatic disease is associated with an ORR of 50% to 60%, a CR rate of 10% to 15%, and MST of 1 year
High-dose MVAC with methotrexate (30 mg/m^2) on day 1 + vinblastine (3 mg/m^2) + doxorubicin (3 mg/m^2) + cisplatin (70 mg/m^2) on day 2, *q* 14 days + G-CSF on days 4 to 11	Overall response rate was 73%, including 24% CR; a favorable PFS rate was seen with this regimen but no difference in MST was observed at the time of this analysis (Sternberg CN, etal, ASCO00, Abs. 1292:329a)
T4/N0/M0	
Partial cystectomy	Survival rate is estimated at 0%-20%
Radical cystectomy + MVAC	Operable tumor
MVAC or gemcitabine + cisplatin followed by radical pelvic RT	Inoperable tumor
T2/T3/T4, N1/3, M0	
Radical cystectomy with pelvic lymph node dissection (PLND)	Among 246 patients (24%), 5- and 10-year recurrence-free survival was 35%, and 34%, respectively; 5-year overall survival was 52% for <T3b, 17% for T3b, 33% for N1, 22% for N2, and 0% for N3; 10-year survival was 83% for T1, 74.8% for T2, and 64.7% for T3a (Stein JP, etal, J Clin Oncol, 1 Feb 2001;19(3):666-75)
Recurrent superficial TCC (Ta, T1 or CIS)	
BCG salvage for recurrent high-grade superficial bladder tumors post-RT	Among 10 patients previously treated with RT for T2-T4 infiltrating bladder cancer that recurred as a high-grade superficial tumor were treated with BCG, 4 were alive and disease-free with a preserved bladder within a 2-8 years follow-up, 4 others who required cystectomy for persistent or progressed tumor, were alive and disease free at 3-7 years follow-up, and 2 who were not amenable to major surgery died from the disease more than 2 years after treatment with BCG; BCG was well-tolerated by 70% of the patients with the rest experiencing minor complications (Sanchez-Martin FM, etal, Arch Esp Urol, Sep 1999;52(7):749-58)
Intravesical and percutaneous BCG as a 6-week induction course and also each week for 3 weeks administered 3, 6, 12, 18, 24, 30 and 36 months from initiation of induction therapy	Among 243 treated patients, estimated median RFS was 76.8 months, compared to 35.7 months in the no maintenance group; overall 5-year survival was 78% in the no maintenance compared to 83% in the maintenance arm (Lamm DL, etal, J Urol, Apr 2000; 163(4):1124-9)

Legend: ***DFS=****disease-free survival* ***MST=****median survival time* ***MTP=****median time-to-progression* ***ORR=****overall response rate* ***RFS=****recurrence-free survival*

and only one patient progressed to muscle-invasive disease. Only 1 (2.7%) patient experienced Grade 3/4 toxicity. In this single institution clinical trial, freedom from recurrence was significant with the monthly maintenance protocol, and Grade 3 or 4 toxicity was dramatically lower than reported in SWOG 8507 (Flanigan RC, etal, Urol Oncol, 15 Dec 2000;6(1):16-19).

Carcinoma *in situ* (CIS)

Although CIS is usually classified as a superficial TCC, its aggressive nature resembles that of invasive cancer. Patients with CIS of the bladder are at significant risk of cancer progression and death from bladder carcinoma. CIS is associated with the development of invasive cancer in 50% to 80% of the cases. Because of these attributes, historically CIS has been treated with radical cystectomy. The introduction in the late 1970s of intravesical BCG has enabled organ preservation and has made this therapy the gold standard in the management of CIS.

According to comprehensive literature review of the current status of management of CIS, complete and durable response rates were reported in more than 70% of patients with CIS treated with intravesical BCG. However, although an optimal therapeutic regimen has not been established, extended periods of treatment beyond the originally described 6-week course were not shown to improve CR rates and were also associated with adverse side effects. Various prognostic indicators of recurrence and progression exist that may identify a subset of CIS cases unlikely to respond favorably to a conservative approach, including CIS with associated Stage T1 bladder lesions, diffuse and multifocal CIS, multiple recurrences with intravesical therapy and extravesical involvement. Cases that are refractory or resistant to BCG therapy are a management dilemma with various available treatment options, including intravesical chemotherapy, combined immunochemotherapy, and radical cystectomy. Intravesical valrubicin and oral bropirimine have been shown to induce a CR rate of 21% to 50%, although data on long-term follow-up is not available. Radical cystectomy remains effective therapy for aggressive CIS (Kim JC and Steinberg GD, J Urol, Mar 2001;165(3):745-56).

Among 138 patients diagnosed with CIS at the Mayo Clinic between 1972 and 1979, none had previous or coexisting invasive urothelial carcinoma at the time of diagnosis. CIS usually was multifocal (50%) with a predilection for the trigone, lateral wall, and dome. At a mean follow-up after surgery of 11.0 years (range=0.7-25 years), actuarial progression-free, cancer-specific, and all-cause survival rates were 63%, 79%, and 55%, respectively, at 10 years, and 59%, 74%, and 40%, respectively, at 15 years. The mean interval from the time of diagnosis to cancer progression was 5 years. Patient age at diagnosis was significant in predicting progression-free and all-cause survival. Cystectomy performed within 3 months after the initial diagnosis was associated with improved all-cause survival. After controlling for age, there was no difference in survival between patients who underwent immediate cystectomy and those did not (Cheng L, etal, Cancer, 1 Jun 1999;85(11):2469-74).

Muscle-invasive Disease

Nearly 90% of patients with muscle-invasive tumors present with *de novo* disease (no prior history of bladder malignancies). With the violation of the smooth muscle layer, the cancer gains access to vascular and lymphatic channels. Tumor invasion into the muscularis propria involves numerous biological processes, including neovascularity, connective tissue breakdown (proteolysis), increased tumor cell motility, tumor cell embolization, and breakdown of the immune system. The overall 5-year survival of patients presenting with ≥Stage T2 bladder cancer is about 50% to 60%. Muscle-invasive progression has a poor prognosis with a survival rate of 20% to 50%. Despite having no evidence of metastases at presentation, >50% of patients with clinically invasive disease will die of dissemination within 3 years, despite either radical RT or radical cystectomy with pelvic lymph node dissection (PLND), the standard treatment of invasive bladder cancer; other options include RT, chemotherapy, or multimodality therapy.

Among 680 patients initially diagnosed with bladder cancer in the 1987-1988 period in Western Sweden, followed until 1994, 107 had Stage T2 to T3 and 41 had Stage T4 disease. Treatment was based on age with younger patients treated with radical cystectomy. Among those with Stage T2 to T3 disease, 30 underwent radical cystectomy, 33 full dose RT, and 44 nonradical therapy (mainly TUR); the 5-year crude survival rates were 33%, 15% and 14%, respectively. Among those with Stage T4 disease, 6 underwent radical cystectomy, 9 full dose RT and 26 nonradical therapy; all except 1 patient died of disease within 4 years. More than 60% of the patients in the cohort were considered unsuitable for radical cystectomy, and their survival was poor, whether treated with full dose RT or TUR alone (Holmang S, etal, J Urol, Aug 1997;158(2):389-92; comment in J Urol, Aug 1997;158(2):406-7).

Recurrent Disease

Recurrent early disease is the bane of early-stage bladder cancer. Annually, as many patients as newly diagnosed cases return for treatment for recurring disease. Also, although much of recurrent disease has the same outlook as newly diagnosed TCC, recurrent superficial high-grade Ta TCC has a lifelong risk of stage progression and death from bladder cancer similar to T1 disease. Among 148 patients with recurrent Ta tumors (high-grade=125 and low-grade=23) and 73 with T1 multiple, recurrent papillary bladder tumors, treated with complete TUR and 1 or more courses of BCG therapy, at a minimum follow-up of 15 years, the PFS rate was 95% in the 23 patients with low-grade Ta tumors and none died of disease. PFS and disease-specific survival rates were 61% and 74%, respectively, in 125 patients with high-grade Ta tumors compared to 44%

and 62%, respectively, in 73 with T1 tumors (Herr HW, J Urol, Jan 2000;163(1):60-1; discussion 61-2).

Recurrence and progression continue to occur in patients with superficial bladder cancer even after long periods of dormancy. Among 100 consecutive patients with superficial bladder cancer (Stage Ta and T1) who remained tumor-free for longer than 4 years after initial treatment, 24 (24%) recurred within 15 years after the initial treatment. The 10- and 15-year recurrence-free rates were 76.0% and 59.6%, respectively. Tumor progression occurred in 5 patients. Four variables including presence of multiple tumors, involvement of the bladder neck, positive urine cytology, and intravesical chemotherapy were found by univariate analysis to be significant risk factors for late progression. Among these factors, initial presence of multiple tumors (≥3) was determined by a multivariate analysis to be an independent risk factor for late progression (Fujii Y, etal, Eur Urol, Oct 1999;36(4):309-13).

Upper Urinary Tract TCC

Survival of patients with upper tract recurrence is poor, with a median of 10 months (Balaji KC, etal, J Urol, Nov 1999;162(5):1603-6). Although incidence of upper tract recurrence rates following radical cystectomy for bladder cancer is low, ranging from 2.4% to 3.3%, the risk for upper urinary tract disease in patients with primary superficial bladder cancer, treated with TUR and BCG, persists for up to 15 years after treatment. Among 86 patients, 18 (21%) had upper tract tumors after a median interval of 7.3 years (range=1 to 15). Tumors occurred within 5 years of follow-up in 6 cases, between 5 and 10 years in 7, and between 10 and 15 years in 5. The majority of cancers were invasive and 7 patients died of upper tract tumors. However, among those who developed progression and required cystectomy, only 2 patients developed upper-tract tumors (Herr HW, etal, J Urol, Oct 1996;156(4):1286-7). These two series suggest that radical cystectomy provides a protective affect from upper tract tumors.

The prostate is a frequent site of tumor relapse in patients with superficial bladder tumors followed for 15 years. Tumor relapse in the prostate may be classified as noninvasive (prostatic urethra and ducts), or invasive (stroma) with intraurethral or direct prostatic invasion. Among a cohort of 186 men with superficial bladder tumors followed for 15 years, 72 (39%) relapsed in the prostate after a median follow-up of 28 months (range=3 to 216), including 45 (62%) with noninvasive prostatic tumor, and 27 (38%) with stromal invasion. The survival rate was 82% in patients with prostatic urethra or duct involvement compared to 48% with stromal invasion. Intraurethral stromal invasion was associated with a 75% 15-year survival rate versus 9% for extravesical prostatic stromal invasion. Bladder tumor stage and prostatic stromal invasion were independent prognostic variables of survival (Herr HW, and Donat SM, J Urol, Jun 1999;161(6):1854-7).

Metastatic Bladder Cancer

Although metastatic bladder cancer is rare as a first-diagnosis, in spite of aggressive treatment many patients with muscle-invasive disease fail as a result of the presence of occult metastases at the time of clinical onset. The vast majority of patients with invasive bladder cancer who die of the disease do so from systemic metastases and not from local recurrence; even locally advanced lesions (>Stage T3b) recur locally in the pelvis only in approximately 10% to 12% of cases. Therefore, implementation of adjuvant chemotherapy in muscle-invasive disease is an area of extensive clinical research because of the significant failure rate despite radical cystectomy.

More than 90% of patients with metastatic bladder cancer die of their disease. However, a gradual improvement in the management of metastatic bladder cancer has been noted in the past 20 years, with MST doubling to 12 months and the 3-year survival rate increasing from <5% to 15% to 20%, mostly because of novel treatment approaches evaluated in clinical trials. MST of untreated patients with metastatic bladder cancer who are managed only with supportive care is 4 to 6 months, but rises to 7 to 8 months in those treated with conventional single-agent chemotherapy, and doubles to 12 months among those treated with platinum-based combination regimens, with a 3- year survival of approximately 20% to 25%.

Common sites of metastasis of bladder cancer include regional and distant lymph nodes, bone, lung, skin, and liver; metastases to abdominal viscera, brain, and meninges are seen less frequently. Sites of metastatic involvement correlate with response rate and survival and are important predictors of treatment outcome; survival of patients with lymph-node, lung, and soft-tissue metastases is better than with metastases to bone and liver. Also, metastasectomy may extend survival in selected cases.

In TCC, biopsies of distant metastatic sites are often consistent histologically with a TCC pattern, but exhibit a significant disparity with respect to growth parameters, ploidy, tumor markers, grade, and histologic features. Metastatic sites may also contain adenocarcinoma and/or squamous cell carcinoma, reflecting either the stem cell function of TCC, or emergence of second primary tumors. These characteristics contribute to additional variation in treatment outcomes.

PROGNOSTIC AND PREDICTIVE FACTORS AND DISEASE MONITORING

Numerous markers have been associated with bladder cancer (FO, pp 1242-44). However, there are currently no generally accepted molecular prognostic factors used to assess disease outcome or predictive factors that may indicate which patients will benefit from treatment. Rather, most prognostic/predictive approaches in this area rely on clinical parameters (Exhibit 3), generally arrived at empirically. Because of its propensity to recur, and the almost lifetime requirement for surveillance, the management of

bladder cancer would uniquely benefit from the availability of reliable prognostic and predictive markers. Therefore, there is ongoing concentrated effort to identify easily performed, reproducible molecular marker tests to aid in the chronic management of this disease.

Surveillance Requirements

Surveillance is mandatory in all patients treated for bladder cancer. It is at the heart of management of superficial bladder cancer treated with bladder preservation procedures. However, few guidelines exist as to the frequency and length of follow-up. Cystoscopy is the standard means of follow-up, which may be necessary for a patient's lifetime. For low-risk patients, cystoscopy at 6-month intervals for the ensuing 3 to 5 years usually suffices. Recommendations for follow-up for the least aggressive stage of TCC, Ta Grade I, vary. Some believe that because Ta Grade I TCC has a high recurrence rate and could progress in grade, close long-term follow-up is warranted, even when in some settings the trend is to discontinue follow-up after 5 years without any abnormal findings (Leblanc B, etal, J Urol, Dec 1999, *ibid*).

Others believe follow-up policies may be changed, because low-grade superficial tumors (Ta Grade I) almost always follow a benign course, so that patients with a single tumor at diagnosis and a negative cystoscopy at 3 months need only be examined 9 months later (Holmang S, Semin Urol Oncol, Nov 2000;18(4):273-9). Also, routine cystoscopy can possibly be discontinued in patients with low-grade, low-stage disease in whom there is a low risk of recurrence during follow-up (Haukaas S, etal, BJU Int, Jun 1999;83(9):957-63).

Lifelong endoscopic surveillance is mandatory for patients in whom new tumors are very active, at least for younger patients. For high-risk patients, cystoscopy is recommended every 3 months for the first year, every 6 months for 5 years, and yearly for 10 years. Regular follow-up urologic assessments should be continued until at least 15 years of tumor-free existence, especially in patients treated by intravesical chemotherapy or those initially having multiple tumors (Fujii Y, etal, Eur Urol, Oct 1999;36(4):309-13). However, follow-up cystoscopy may be discontinued 5 to 10 years after the last recurrence, at least in patients with a solitary low-grade primary tumor (Holmang S, etal, J Urol, Jun 1995;153(6):1823-6; discussion 1826-7). Recurrence must be detected early in Stage T1 Grade III disease using long-term follow-up with strict observance of surveillance protocols during a minimum 5-year tumor-free period (Lebret T, etal, J Urol, Jan 2000;163(1):63-7; comment:79-80).

A more complex procedure such as urography is recommended at initial diagnosis of bladder cancer, when tumor progression occurs, and when there is suspicion of upper urinary tract disease (Holmang S, etal, J Urol, Jul 1998;160(1):45-8), but routine follow-up urographic studies are neither cost-effective, clinically indicated, nor justified in patients with superficial bladder cancer.

Surveillance is also mandatory after radical surgery. Stage-specific surveillance protocol for monitoring patients after radical cystectomy is recommended that can reduce costly imaging studies while efficiently detecting recurrences and complications. In this protocol, surveillance for Stage T1 disease comprises a history, physical examination, chest x-ray and laboratory studies on an annual basis; for Stage T2 disease, the same studies are conducted at 6-month intervals for 3 years after cystectomy and annually thereafter; and for Stage T3 disease similar tests are conducted at the same frequency together with computerized tomography (CT) at 6, 12 and 24 months after cystectomy. This recommendation is based on a review of the records of 382 patients with TCC who underwent cystectomy between 1986 and 1994. Of 97 patients with metastases, 72 (74%) were asymptomatic, including 43 with metastases detected by routine chest x-rays (n=30) or blood tests (n=13). Surveillance CT identified isolated asymptomatic intra-abdominal metastases in 10 patients (10%), of whom 90% had Stage T3 disease. In addition, a radiographic study of the upper tract should be performed in all patients every 1 to 2 years to evaluate for recurrences and complications of the ileoureteral anastomosis (Slaton JW, etal, J Urol, Sep 1999;162(3 Pt 1):710-4).

Clinical Factors

Currently, prognosis in all stages/grades of TCC relies on clinical factors (Exhibit 3). In early disease, the most important prognostic factors are those determining risk of recurrence and progression. Clinical prognostic factors are also used to assess response to therapy in CIS as well as muscle-invasive and metastatic bladder cancer, and to predict survival.

In a prospective randomized clinical trial involving 207 patients with primary superficial bladder cancer followed over a period of 4.9 (range=3.7-6.0) years, based on univariate analysis, the stage, grade, papillary status, and proliferation indices MIB-1 (ki-67) and volume-corrected mitotic (M/V) index, were significant predictors of progression. Using multivariate analysis, MIB-1 score and papillary status were independent predictors of progressive disease and cancer-specific survival. Tumor grade was the only independent predictor of recurrence. Evaluation of tumor cell proliferation rate by M/V index, or by MIB-1 immunohistochemistry, and assessment of papillary status by light microscopy, are useful prognostic tools in tailoring treatment and follow-up schedules of patients with superficial bladder cancer (Liukkonen T, etal, Eur Urol, Nov 1999;36(5):393-400).

Chromosomal Abnormalities

Studies of urothelial tumors have identified structural abnormalities in a number of chromosomes. In a study designed to identify specific genetic changes associated with advanced urothelial cancers, 56 muscle-invasive bladder cancer tumors were screened with PCR, using 6

microsatellite markers, for loss of heterozygosity (LOH) at chromosomes 1p, 8p, 10p, 13q, and 17p. DNA was extracted after microdissection of the primary tumor and normal tissue from paraffin-embedded specimens. LOH findings were correlated with response to chemotherapy and survival. Allelic loss of specific markers was present in 26%-50% of the informative tumors. The most frequent LOH was observed at 17p, supporting the notion that this region may contain genes, such as p53, having importance to urothelial cancer progression. The overall rate of response to chemotherapy was 48%, and ranged from 40% to 56% according to specific LOH changes. MST of all patients from start of chemotherapy was 5.8 months and ranged from 5.3 to 7.9 months for patients with specific LOH changes. Response and survival of patients with no lost markers was the same as for those with one, two, or more lost markers. Specific genetic changes were detected in a significant number of tumors from patients with advanced urothelial cancer but were not predictive of response to chemotherapy, or the duration of survival (Sengelov L, Cancer Genet Cytogenet, Dec 2000;123(2):109-13).

Investigators have combined whole-organ histologic and genetic mapping with the human genome sequence database, in an attempt to identify genes potentially involved in early phases of bladder carcinogenesis (Kram A, etal, Lab Invest, Jul 2001;81(7):1039-48). In this approach, the evolution of allelic losses on chromosome 5 was studied in 234 mucosal DNA samples of 5 cystectomy specimens with invasive bladder cancer and preneoplastic changes in the adjacent urothelium. The frequency of alterations in individual loci was verified on 32 tumors and 29 voided urine samples from patients with bladder cancer. Also, deleted regions on chromosome 5 were integrated with the human genome contigs (contiguous clones) and sequence-based databases. Such analysis provided an accurate map of deleted regions with positions of 138 known genes and revealed several smaller gene-rich areas representing putative targets for further mapping.

Constitutional chromosomal abnormalities may exist in selected families with susceptibility to bladder cancer. Such a constitutional balanced translocation t(5;20)(p15;q11) was identified in a family with urothelial cell carcinoma (UCC). However, no aberrant chromosomal features were found by either classical or spectral karyotype analyses in 30 Dutch UCC families selected through an ongoing study on familial clustering of UCC, the largest study on this subject ever performed. This study included 1,193 new patients with UCC of the bladder, ureter, and renal pelvis, identified from the population-based cancer registries of the Dutch Comprehensive Cancer Centers East and South. Families were selected in which 2 or 3 individuals were affected, preferably diagnosed at a relatively young age. Blood samples were obtained from all probands, and routine cytogenetic analysis was performed on 30 patients and subsequent spectral karyotyping in 4 patients from families which were most suggestive for an inherited etiology (Aben KK, etal, Urology, Feb 2001;57(2):266-9).

Molecular Markers

Research to identify bladder cancer markers was spearheaded in the mid-90s by the Bladder Tumor Marker Network, an NCI funded cooperative, now folded into the International Bladder Cancer Network (IBCN). However, to date few molecular markers have demonstrated a prognostic/predictive value in bladder cancer. Also, one of the challenges in assessing the role of molecular markers is that there are no gold standards for most tumor marker assays.

In an effort to coordinate research in this area, IBCN, during its meeting in Ancona, Italy, in May 2001, proposed an international multi-institutional bladder cancer bank (IBCB) to serve as a database and virtual tumor bank to evaluate the biologic and prognostic significance of potential markers in bladder cancer. The consortium of the collaborating centers will provide prospectively collected, consistent data sets with long-term follow-up that are available and linked to specimens, at the time that the specimens are selected for analysis of new markers. As a start for this undertaking, a bladder cancer tissue microarrayer will be produced at two different sites and 20 institutions will provide the tissue (up to a maximum of 20 blocks) and adjacent data for each arrayer. This will enable a collaborating group to perform marker studies on a total of 800 different tumor samples. This project is intended to serve as a feasibility study and, by providing proof-of-principle that the consortium is "functioning," to obtain funding for establishing a larger-scale IBCB. Based on the gained experience, the tissue and the established infrastructure will be used for further projects.

Among molecular markers with potential utility in bladder cancer are:

- p53
- various apoptosis-related factors
- cell-cycle modulators
- integrins and cell adhesion molecules

p53, alone or in combination with other markers, has been the most evaluated potential prognostic marker in all stages of bladder cancer. Yet, despite numerous trials relating to its prognostic relevance, the impact of p53 accumulation on prognosis remains unclear. It should also be noted that, presently, there is no standardization of p53 measurement. Differences between studies with regard to the prognostic impact of p53 can be explained in most cases by differing study designs, and heterogeneous study populations. In addition, some of the varying results reported in the literature may be a result of inter-laboratory variation, differences in various antibody reagents used to determine overexpression, use of fresh frozen versus paraffin embedded blocks, varying cutpoints of positivity (5% versus 10% versus 20%), and so forth.

This problem was underscored by a study conducted by the NCI's Bladder Tumor Marker Network that evaluated the reproducibility of immunohistochemistry for measuring p53 expression in bladder tumors. Fifty paraffin blocks were chosen at random from among high-grade invasive primary bladder tumors at 5 institutions participating in the study. For overall assessments of p53 positivity, results demonstrated that intra-laboratory reproducibility was quite good. Concordance across the 5 participating laboratories was high for specimens exhibiting no or minimal nuclear immunostaining of tumor cells, or high percentages of tumor cells with nuclear immunoreactivities. However, there was a reduced level of concordance on specimens with percentages of stained tumor cells in an intermediate range. Discordancies were mainly attributable to staining differences in one of the 5 laboratories, and scoring differences in another. These results indicate that some caution must be used in comparing results across studies from different groups. Standardization of staining protocols and selection of a uniform threshold for binary interpretation of results may improve assay reproducibility between laboratories (McShane LM, etal, Clin Cancer Res, May 2000;6(5):1854-64), but use of a uniform threshold remains controversial.

A collaborative effort by the IBCN resulted in a large multi-institutional combined immunohistochemistry analysis of p53 as a prognostic marker in bladder cancer in the International Study Initiative on Bladder Cancer (ISBC) trial (Schmitz-Dräger BJ, etal, Proceedings of the Preparatory Meeting of the International Bladder Cancer Network (IBCN), Ancona, Italy, 11 May 2001). In this trial, 1,177 (69%) of 1,706 patients presenting with superficial tumors, i.e., Ta (n=726; 42.7%), CIS (n=12; 0.6%), or T1 (n=439; 25.7%), were followed for a median period of 11.1 years, while MST of the remaining 523 patients with advanced tumors (>T2) was 1.9 years. Of 1,272 patients assessable for survival, 299 died of bladder cancer, and another 199 from other causes. Cumulative survival analysis yielded no differences for sex and age but considerable differences were observed with regard to the distribution of tumor stage, multifocal tumor lesions and tumor progression. Tumor grade and tumor stage were clearly correlated with patient survival. Survival time differed significantly between the different centers, probably because of patient selection. The number of p53-positive tumor cells was lower in superficial as compared to advanced tumors. Using a breakpoint of 23% positive tumor cells, 170 of 684 (25%) assessable superficial tumors and 187 of 388 (48%) advanced tumors exhibited p53 accumulation. Cumulative analysis of this data shows that p53 accumulation is highly correlated with tumor stage and grade.

In many series, p53 overexpression was shown to be a strong and independent prognostic factor for recurrence and disease-specific survival, and several investigators have reported that overexpression of p53 in superficial, CIS, and invasive bladder cancer correlates with poor outcome and survival. Also, a high level of p53 expression is an independent marker predictive of a CR to chemoradiotherapy in muscle-infiltrating bladder cancer and could help clinicians to better identify patients who may be offered a bladder sparing treatment approach (Passalacqua R, etal, ASCO00, Abs. 1345:342a). However, prior studies have failed to observe a statistically significant impact on outcome of p53 nuclear accumulation in patients with locally advanced and node-positive bladder cancer.

p53 overexpression was shown to be a powerful predictor of survival in patients with muscle-invasive bladder cancer. Among 59 patients with TCC, serum p53 was detected in 14/59 and mutant p53 protein overexpression in tissue in 24/59 patients. All serum p53-positive patients had tissue p53-positive tumors, but some patients with tissue-positive immunoreactivity showed undetectable serum p53. None of the healthy controls had detectable serum p53. Titers of serum p53 were associated with stage and grade, and p53 overexpression was dependent on stage, grade, pattern of growth and focality. Serum p53 had a significant prognostic value for disease-free survival and life expectancy, and tissue p53 for life expectancy. Patients with serum-positive p53 showed a higher probability for a shorter survival (OR=6.38; range=1.77-22.99) than those with tissue-positive p53 (OR=4.00; range=1.31-12.8), or those who were negative for either measurement. Serum p53 may reflect p53 status and help in selecting bladder cancer patients with a worse prognosis (Sanchez-Carbayo M, etal, Anticancer Res, Jul-Aug 1999;19 (4C):3531-7).

Similarly, in another series, in invasive TCC, p53 protein was detectable in 61% of the tumors. For those treated only with external beam RT, local control was significantly better for tumors with wild-type p53 (Moonen L, etal, Int J Radiat Oncol Biol Phys, 1 Apr 2001;49(5):1305-10).

Overexpression of p53 may also be prognostic in early disease. Among 108 Stage Ta high-grade tumors (Grade II=95 and Grade III=13) that progressed in stage during follow-up, further analysis with immunohistochemical methods (p21, p53, ki-67 and Rb) was conducted. Disease progressed in 4% of patients with low-grade compared to 23% with high-grade carcinoma. In those with Grade III cancer, disease progressed in 45% compared to 20% with Grade II disease. At first diagnosis, p53 score was significantly higher among patients with Grade II carcinoma that later progressed compared to that in matched controls but there was no significant difference regarding the other markers. In contrast to Grade II, most Grade III carcinoma was aneuploid, had high mitosis frequency, high p53 and ki-67 scores as well as loss of retinoblastoma (rb) gene expression. A disadvantage is that the high-grade carcinoma group contained 2 subgroups with different progression rates and immunohistochemical marker profiles, corresponding to Grades II and III. Grade II tumors in patients that progressed in stage years later seem to have different immunohistochemical and molecular marker profiles com-

Exhibit 3
Clinical Prognostic and Predictive Factors

Factors	Comments
Age	Patient age is a clinicopathologic factor affecting response to intravesical instillation therapy with BCG (Tokyo 172 strain) for CIS of the bladder, which may point to a role for the reduced host immunocompetence in elderly individuals (Takashi M, etal, Int Urol Nephrol 1998;30(6):713-22)
Pathologic grade	Pathologic grade, based on cellular atypia, nuclear abnormalities, and the number of mitotic figures, is of great prognostic importance
Performance status (PS)	PS is of prognostic significances in invasive baldder cancer treated by TUR, chemotherapy and RT (Matos T, Int J Radiat Oncol Biol Phys, Jan 15 2000;46(2):403-9)
Histologic grade/CIS	High histologic grade and presence of concomitant CIS are poor prognostic factors
Stage	Stage is an important prognostic factor used routinely to determine therapy and assess outcome
Complete TUR	A complete TUR is the most important single prognostic factor
Depth of invasion in TUR specimens	In 83 consecutive patients with T1 bladder treated with TUR, after a median follow-up of 5.2 years, overall 5- and 7-year PFS rates were 82% and 80%, respectively; depth of invasion in the TUR specimens was associated with cancer progression (hazards ratio=1.6 for doubling of depth of invasion); 5-year PFS rate for patients with depth of invasion of ≥1.5 mm was 67%, compared with 93% for those with depth of invasion of <1.5 mm; no other variable, including age, sex, tobacco use, alcohol use, the presence of CIS, histologic grade, lymphocytic infiltration, or muscularis mucosa invasion, was associated with cancer progression (Cheng L, etal, J Clin Oncol, Oct 1999;17(10):3182-7); depth of tumor invasion was a significant independent predictor of progression in patients with T1 bladder cancer, suggesting that this measurement be included in the histopathologic report (Bernardini S, etal, J Urol, Jan 2001;165(1):42-6; discussion 46)
Obstructive uropathy	Obstructive uropathy is a good indicator of local spread of the disease, better than clinical T-stage in invasive baldder cancer treated by TUR, chemotherapy and RT (Matos T, etal, *ibid*)
Presence of aneuploidy	Aneuploidy is an indication of aggressiveness; among 108 Stage Ta high-grade tumors (Grade II= 95 and Grade III=13) that progressed in stage, most Grade III tumors were aneuploid and had high mitosis frequency (Holmang S, etal, J Urol, Apr 2001;165(4):1124-8; discussion 1128-30)
High proliferation rate (ki-67 immunostain-positive tumors)	In invasive TCC, high ki-67 index was found to be significantly associated with p53 expression and cyclin D1 overexpression (Moonen L, etal, Int J Radiat Oncol Biol Phys, 1 April 2001, 49(5):1305-10)
Low versus high S-phase fraction (SPF)	Tumor proliferation activity, based on SPF determination by flow cytometry, contributes prognostic information about tumor progression that is additive to tumor grade
Apoptotic index	In invasive TCC, local control rate after RT was significantly better in tumors with an apoptotic index above the median value (Moonen L, etal, *ibid*)
Tumor size and number	Tumor size and number influence annual recurrence rates; the largest the tumor and the number of lesions the shorter the disease-free interval; logistic regression analysis showed a relationship between tumor number and recurrence (RR=2.7), and identified tumor size as a characteristic of a high recurrence rate (RR=3.3) (Paez Borda A, etal, Arch Esp Urol, Apr 1999;52(3):229-34; discussion 234-5)
Recurrence interval	Recurrence >4 years after primary tumor removal is an ominous sign (Holmang S, etal, J Urol, Jun 1995;153(6):1823-6; discussion 1826-7); recurrence within 2 years of BCG treatment is a sign of poor prognosis (Leblanc B, etal, Can J Urol, Feb 2000;7(1):944-8)
Sessile, large (≥2 cm) and multiple tumors	In superficial bladder cancer, treated with epirubicin and TUR, these parameters conferred a significantly high risk for recurrence (Kondo T, etal, Int J Urol, Apr 1999; 6(4):178-83)

pared to those of matched controls (Holmang S, etal, J Urol, Apr 2001;165(4):1124-8; discussion 1128-30).

Among 149 cases of Stage T1 tumors diagnosed between 1973 and 1996, there were 94 in which the muscular layer was clearly identifiable and disease-free. Within a follow-up of 64.9 months (range=5 to 288), these 94 T1 bladder cancers were subclassified into two groups, with muscularis mucosa invasion (Stage T1b) or without (Stage T1a). Overexpression of nuclear p53 was detected in 37.2% of all tumors. Although univariate statistical analysis showed that p53 expression and tumor invasion depth significantly correlated with progression, on multivariate analysis only invasion depth and associated CIS remained independently significant as predictors of progression (Bernardini S, etal, J Urol, Jan 2001;165(1):42-6; discussion 46).

Among 33 patients with CIS, within a median follow-up of 124 months, disease progressed in 16 (48%) patients. The association between p53 nuclear overexpression, detected by antibody PAb1801 and immunohistochemistry, and tumor progression was assessed by multivariate analysis, controlling for possible confounding variables, such as patient age and sex, presence of associated stage Ta bladder tumor and adjuvant BCG therapy. Patients were stratified into 2 groups according to the percent of tumor cells displaying p53 nuclear overexpression, with group 1 consisting of 18 patients with <20% tumor cells positive, and group 2 of 15 patients with ≥20% tumor cells positive. Disease progressed in 3 patients (16.7%) in group 1 and in 13 (86.7%) in group 2. Detection of p53 nuclear overexpression in 20% or more tumor cells was the only independent marker of tumor progression in univariate and multivariate analyses. Death specifically from bladder cancer was also associated with this altered pattern of p53 expression. Therefore, p53 nuclear overexpression is an early event in bladder cancer, occurring in 48% of cases of CIS. These results also suggest that p53 nuclear overexpression offers significant clinical information and may be a useful tool in the selection of therapy for patients with CIS (Sarkis AS, etal, J Urol, Aug 1994;152(2 Pt 1):388-92).

Although disease progresses in the majority of patients with node-positive TCC of the bladder, a definitive subset is cured by surgery only. To establish the prognostic value of nuclear accumulation of p53 regarding disease progression in patients with node positive TCC, immunohistochemical analysis, using MAB1801, was performed on specimens of 59 patients with node-positive pathology treated with radical cystectomy between July 1988 and September 1999. In this cohort, overall median DFS was only 21 months, although 18% of patients were disease free at 5 years. There was evidence of p53 nuclear accumulation in 54% of cases and complete agreement of nodal status with bladder p53 nuclear accumulation. No significant baseline differences were noted in the covariates with respect to p53 nuclear accumulation. For stratum-specific DFS, univariate and multivariate analyses revealed that only pathologic and nodal status were significantly associated with prolonged DFS, while p53 nuclear accumulation was not. Despite credible evidence for p53 nuclear accumulation prognostication in patients with CIS and invasive TCC of the bladder, this marker is not predictive of DFS in node-positive disease (Fleshner N, etal, J Urol, Oct 2000;164(4):1177-82).

Induction of p53 gene expression has also been shown to be facilitated by prior exposure to cytotoxic agents such as cisplatin and mitomycin C. This altered expression of p53 may correlate with increased resistance to combination chemotherapy protocols, such as the MVAC regimen, and may be associated with previous intravesicular treatment. By contrast, response to paclitaxel-based chemotherapy regimens has been shown to be independent of p53 mutations in some studies.

Mutations in p53 may also be indicative of outcome. Mutations in exons 5-8 of the p53 gene were detected in 26 (14%) of 189 patients with urinary bladder neoplasms [82 (44%) neoplasms were low-risk (Ta, Grade I/IIa) and 106 (56%) high-risk (Grade IIb/IV or ≥T1)], with 30% of the samples exhibiting LOH for one or both of the p53 exogenic and intragenic repeat markers. Also, 31 (21%) samples showed LOH but were not mutated, suggesting other mechanisms than mutations inactivating p53. In addition, 4 mutations were found at codon 280 and 2 mutations were at codon 285, two previously reported hot spots for urinary bladder cancer. There was a boundary between Grade IIa and Grade IIb tumors concerning genetic events affecting p53 function; moderately differentiated (Grade II) tumors probably are genetically heterogeneous which supports the suggestion that they should not be grouped together but instead, be categorized as either low- or high-risk (Berggren P, etal, Br J Cancer, Jun 2001;84(11):1505-11).

To assess the prognostic relevance of p53 gene point mutations and LOH in tumor recurrence in superficial bladder tumors, polymerase chain reaction (PCR) was carried out with exons 5-8 in representative tumor tissue from 40 patients (Ta=18, T1=22; Grade I=7, Grade II=28, Grade III=5). Mutations were detected in 10 patients (2/18 Ta and 8/22 T1), LOH was detected in 11 patients, and both a mutation and LOH were detected in 3 patients. In 28/40 (70%) patients, p53 immunohistochemistry detected at least 5% positive nuclei. After a median follow-up of 26 months, disease recurred in 14 patients. Whereas DFS did not correlate with a mutation, LOH or a mutation in combination with LOH, a positive p53 immunoreaction was significantly associated with a short DFS. It appears that, in contrast to immunohistochemical accumulation, p53 gene alterations play only a minor role in tumor recurrence in patients with superficial TCC of the bladder, and that immunohistochemical accumulation of p53 protein has to be explained by mechanisms other than gene mutations (Friedrich MG, etal, Eur Urol, Feb 2001;39(2):159-66).

Different investigators have reported divergent results regarding expression of p53 and other markers. Human bladder tumors express both EGFr and TGF-α and their expression is closely correlated. In tumor biopsies obtained from 54 patients with primary bladder cancer (Stage T1=18 and Stage T2-T4=36), EGFr protein level was significantly increased in T2-T4 tumors compared with T1 tumors, while coexpression of TGF-α and EGFr proteins was significantly associated with muscle invasive tumors (T2-T4), and TGF-α protein level correlated significantly with EGFr protein expression; however, no correlation was observed between survival and the expression of EGFr and/or TGF-α (Thogersen VB, etal, Scand J Clin Lab Invest, Jul 1999;59(4):267-77).

In superficial and invasive bladder cancer, overexpression of EGFr has been related to several malignant characteristics, including invasive growth, high-grade histology, DNA ploidy, high proliferation rate, and prognosis. In the prospective randomized clinical trial involving 207 patients with primary superficial bladder cancer described above, based on univariate analysis, EGFr and p53 were significant predictors of progression (Liukkonen T, etal, Eur Urol, Nov 1999;36(5):393-400).

Among 144 patients with superficial TCC of the bladder, studied over a period of three years, EGFr expression was detected in 55 (38%) patients and p53 expression in 14 (9.7%). In this series, risk of recurrence was lower in bladder tumors that expressed EGFr than in those that did not. The difference in DFS was statistically significant, being 54.1 months in those with EGFr expression versus 30 months for those without. Also, when tumors expressing EGFr recurred, it was generally in the same site as the primary tumor, but those that did not express EGFr recurred in another or in multiple sites. In contrast, determination of p53 expression was not useful in assessing risk of recurrence or progression of superficial bladder tumors (Busto Castanon L, Arch Esp Urol, Jan-Feb 2001;54(1):13-21).

Among 59 patients with muscle-invasive, node-negative TCC of the bladder treated with four cycles of MVAC chemotherapy followed by locoregional treatment (either a radical cystectomy, partial cystectomy, or TUR), at a median follow-up of 153 months (MST=51 months), Bcl-2, Bax, and p53 overexpression were seen in 46%, 51%, and 54% of cases, respectively. Bcl-2/Bax ratio >1 was observed in 32% of cases. Prolonged survival correlated with pathologic downstaging after MVAC chemotherapy, lack of ureteral obstruction, and p53 <20% positive cells. Bax-positive cells of ≥20% and Bcl-2/Bax ratio >1 did not correlate with survival. MST of patients with Bcl-2 <20% positive cells was greater than with Bcl-2-positive cells ≥20% (70 months versus 36 months); however, this correlation did not achieve statistical significance. Although these findings may imply that Bcl-2 does not correlate with survival, it is also possible that because of the limited number of subjects, this study lacked sufficient statistical power to detect an association (Bajorin DF, etal, ASCO00, Abs. 1295:329a). In a further analysis, 5-year survival of patients with wild type p53 (no p53 overexpression) and no Bcl-2 overexpression was 65%, while it was 43% in patients with mutant p53 without Bcl-2 overexpression, and 22% in patients with both mutant p53 and Bcl-2 overexpression.

In July 2000, UroCor (Oklahoma City, OK) received a Phase II Small Business Innovative Research (SBIR) grant (2 R44 CA76823-02) of $693,828 for a two-year, multicenter trial to produce a second generation version of the company's p53 mutation assay for bladder cancer prognosis with enhanced technical performance. This grant follows an earlier Phase I NCI grant, which was completed in June 1999. UroCor also received development funding from the Oklahoma Center for Advanced Science and Technology (OCAST) Incentive Funding. The p53 urine test that has been available to urologists for the past two years, requires only patient urine, so no invasive biopsy is required. UroCor has developed the genetic test under a license from Ambion (Austin, TX), based on its NIRCA technology, a patent-protected method of screening for unknown point mutations (Goldrick MM, etal, BioTechniques 1996;21:106-112). NIRCA is available for licensing from Ambion for clinical reference labs and for companies developing SNP diagnostic kits for a wide variety of applications.

Cell cycle regulators p27(Kip1) and cyclin E in TCC of the bladder may also correlate with tumor grade and patient survival. These findings have clinical importance because they support a role for p27(Kip1) and cyclin E as novel predictive markers of the biological potential of bladder tumors and may enable identification of those tumors most likely to progress to muscle-invasive disease, and predict patient survival.

In a study evaluating the prognostic value of p27Kip1 in a series of 96 primary superficial (Ta) bladder carcinomas, high (>50% positive cells), moderate (25-50%), and low (<25%) p27Kip1 staining was observed in 39 (41%), 19 (20%), and 38 (39%) of tumors, respectively. No significant association was found between expression levels of p27Kip1 and tumor stage, but decreased p27Kip1 staining correlated with higher tumor grade. Interestingly, a significant association was observed between increased expression of p27Kip1 and positivity for p53 (>20% positive cells). A significant correlation was also observed between low expression of p27Kip1 and decreased disease-free and overall survival. Furthermore, on multivariate analysis, low p27Kip1 protein expression was an independent predictor of reduced DFS (RR=1.95) second only to tumor stage. These data indicate that p27Kip1 protein is frequently expressed at low levels in poorly differentiated tumors, and suggest that it may represent a useful prognostic marker for disease recurrence and overall survival in superficial bladder cancer (Sgambato A, etal, Cancer Res, 1 Jul 1999;59(13):3245-50).

To explore the relationship of cell cycle regulation in the p53 pathway, investigators studied 141 patients who underwent cystectomy for bladder cancer. Median follow-up was 21.9 months. Protein expression of p21 and mdm2 was detected by immunohistochemistry, while p53 gene mutations were identified by PCR-SSCP and sequencing. After adjusting for tumor stage, lymph node metastasis, vascular invasion and histologic grade, loss of p21 expression and presence of p53 mutations significantly reduced the survival rate. Also, although mdm2 overexpression was not significant by itself, when combined with loss of p21 expression and p53 mutations, it was found to be very significant in reducing survival with an adjusted relative risk of 3.0 (range=1.5-6.0). This study suggests that alterations of p21 and mdm2 expression, in combination with p53 mutations, may be of prognostic value in bladder cancer (Lu, M.L, etal, AACR99, Abs. 1835:277).

Expression of p53, mdm2, and p21 proteins and the value of the ki-67 index were analyzed in 244 tumors (Ta=194 and T1=50; Grade I=83 and Grade II/III=161) in a study to determine whether the expression of these markers may be predictive of superficial bladder cancer recurrence. The relative predictive power for tumor recurrence of a cell cycle index based on the number of abnormally expressed cell cycle markers was compared with a clinicopathologic index based on primary clinical tumor characteristics. The cell cycle marker index was created using the number of abnormally expressed cell cycle markers according to the following cutoff points, p53 (>5%), mdm2 (>20%), p21 (<5%), and ki-67 (>10%). The clinicopathologic index was created using adverse tumor characteristics such as Grade II/III, Stage T1, multifocality, and tumor diameter >3 cm. The clinicopathologic index was a strong, independent predictor of disease recurrence; risk of recurrence in tumors with three or four adverse characteristics at initial resection was fourfold that of tumors with no risk factors. A strong correlation was observed between ki-67 index >10% and overexpression of both mdm2 and p21 proteins. All in all, mdm2 was overexpressed in 106 (43%) tumors, p53 in 47 (19%), and ki-67 was >10% in 171 (70%) cases; 39 (16%) tumors were p21-negative. Risk of recurrence increased slightly with the number of abnormally expressed cell cycle markers, but when the clinicopathologic index was taken into account in multivariate analysis, the cell cycle marker index was not predictive of disease recurrence after initial resection of papillary superficial tumors (Pfister C, etal, Clin Cancer Res, Dec 1999;5(12):4079-84).

Cell-adhesion factors, such as E-cadherin and α6β4 integrin have a prognostic role in bladder cancer. The α6β4 integrin is a heterodimer integrin, and the main laminin receptor in epithelia (Rigot V, etal, Eur J Biochem, May 1999; 261(3):659-66). Weak expression of α6β4 in bladder cancer has been correlated with longer survival than for patients whose tumors exhibit either no expression or strong overexpression of α6β4 (Grossman HB, etal, Oncol Rep, Jan-Feb 2000;7(1):13-6).

Abnormal E-cadherin expression was significantly associated with disease recurrence, disease progression, and bladder cancer-specific survival, alone, and in multivariate analysis of CIS patients, with and without papillary disease of the bladder. It is therefore hypothesized that loss of E-cadherin expression in this setting predicts disease recurrence, disease progression, and bladder cancer-specific death, and that CIS with abnormal E-cadherin expression may represent a biologically more aggressive cancer, requiring early definitive therapy (Shariat SF, etal, Urology, Jan 2001;57(1):60-5).

MEETING COVERAGE

GENE THERAPY OF CANCER — PART II

MECHANISM-BASED AND OTHER TREATMENT OPTIONS FROM THE 9TH INTERNATIONAL CONFERENCE ON GENE THERAPY OF CANCER, SPONSORED BY SIDNEY KIMMEL CANCER CENTER, SAN DIEGO, CA, DECEMBER 7-9, 2000

Gene therapy in cancer is complicated by the fact that neither the genes involved in cancer development nor the mechanisms of disease action are well known or well understood. Also, cancer is a complex disease, having both genetic and acquired origins. This article continues the report on laboratory investigations, preclinical studies, and clinical trials of gene manipulation in human cancer therapy presented at the Ninth International Conference on Gene Therapy of Cancer (ICGTC00; San Diego, CA; December 7-9, 2000). Additional information on all agents in clinical/preclinical development included in this report may by found in NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), a subscription-based resource residing at www.oncologyknowledgebase.com.

The various mechanism-based approaches used for the gene therapy of cancer can be divided into several, sometimes overlapping, categories, including:

- mediation of oncosuppressor loss
- oncogene inactivation or modulation of other factors supporting tumor growth
- suicide gene and other means of effecting direct cytotoxicity
- enhancement of conventional therapeutic strategies
- and cell marking

Another role of gene therapy, potentiation of antitumor immunity, will be discussed in the third part of this series.

TUMOR SUPPRESSOR COMPENSATION/ENHANCEMENT/TARGETING

Certain genes have been identified as endogenous negative regulators of malignant transformation; absence or mutation of these tumor suppressor genes has been associated with cell-cycle defects that appear to contribute to

the pathophysiology of many cancers. Based on the function of tumor suppressor genes, one approach in cancer gene therapy is to slow or reverse the disease process through addition or insertion of a functional copy of an affected gene into the target cell. Also, genetic material coding for expression of a negative regulator of malignant transformation may be introduced to enhance tumor suppressor activity and prevent development (or progression) of cancer. In another strategy, replication-selective adenoviruses have been used to target a direct cytolytic action against tumor cells with defective expression of a particular tumor suppressor gene.

p53

The p53 tumor suppressor gene plays a central role in directing the repair of damaged DNA, or committing a cell to apoptosis. This gene is mutated, or otherwise dysfunctional, in the majority of human cancers.

Replacement of a defective p53 gene with a potentially therapeutic wild-type p53 (wtp53) gene in tumor cells is the target for various clinical protocols.

Introgen Therapeutics' (Houston, TX) RPR/INGN 201, developed in collaboration with Aventis Pharma (Frankfurt am Main, Germany), is an E1/E3-deleted, replication-defective adenoviral vector containing wtp53 cDNA under the control of a CMV promoter. A USA patent (#6,143,290) covering recombinant p53 adenovirus methods and compositions was issued to Dr. Wei-Wei Zhang and Dr. Jack A. Roth in November 2000, and exclusively licensed to Introgen by the University of Texas System. This patent covers the treatment of cancer with adenoviral vectors expressing wtp53, including use of the vector system in treatment regimens involving intratumoral injection, infusion, and intravenous administration.

Introgen's Lou Zumstein, PhD, noted that in preclinical studies, INGN 201 demonstrated a molecular mechanism consistent with the known activities of wtp53, inducing high level expression of p53 protein. INGN 201 also causes increased expression of p21, mdm2, TRAIL, Fas, DR5, Bax, Bak, and BAI-1 proteins as well as thrombospondin, and represses expression of VEGF. The most consistent and robust markers of INGN 201-induced p53 activity are increased levels of p21 and mdm2. INGN 201 consistently causes apoptosis in cancer cells, with minimal effects on normal cells. Evaluation of post-treatment tumor biopsies by PCR has demonstrated adenoviral vector sequences in 86% of patients, and vector-specific p53 mRNA sequences in 56% of patients. Apoptosis was demonstrated in 46% of post-treatment biopsies from patients treated with INGN 201 alone, and in 79% of those treated with a combination of INGN 201 and cisplatin.

INGN 201 is currently in phase III clinical trials in advanced head and neck cancer, phase II trials in lung cancer, and phase I trials in several other cancers (Roth JA, etal, ICGTC00, Abs. O-1:S1). According to Jack A. Roth, MD, of the University of Texas M. D. Anderson Cancer Center (UTMDACC; Houston, TX), who is collaborating with Introgen on adenoviral vector development, to date, over 600 patients have been treated in these trials, without the occurrence of significant toxicity, and with the observation of objective antitumor responses. INGN 201 is currently being tested at UTMDACC in the treatment of advanced non-small cell lung cancer (nsclc) that progressed on conventional therapy because p53 has been observed to be defective or mutated in 40% to 70% of nsclc. Among all patients treated with either INGN 201 alone (n=24), or a combination of INGN 201 and cisplatin (n=28), disease stabilized for prolonged (up to 24+ months) periods in 63% of patients, with PR in 8%; also 6/12 patients presenting with major airway obstruction, experienced opening of the airway. Actuarial survival at one year was 40% in patients treated with INGN 201 alone, which compares favorably to those treated with chemotherapy alone. For a detailed description of clinical activity involving INGN 201, consult nm|OK.

Schering-Plough's (Kenilworth, NJ) SCH58500 (formerly ACN53), a recombinant, E1-deleted, replication-deficient Ad5 vector expressing human wtp53 tumor suppressor under the control of a cytomegalovirus (CMV) promoter, has completed phase I clinical trials. Findings from these trials were discussed by Robert Warren, MD, of the University of California at San Francisco. In these trials, involving regional gene therapy in primary or metastatic liver tumors with p53 mutations, SCH58500 was administered by intrahepatic arterial infusion using an implanted pump (Warren R, ICGTC00, Abs. O-2:S1). All patients exhibited fever at all doses, and at higher doses, DLT was represented by chills, nausea and lowered blood pressure; one patient at the highest dose level experienced a dramatic, but treatable drop in blood pressure while 21/30 (70%) patients demonstrated elevated transaminases. Intrahepatic arterial infusion together with the hepatotrophic nature of adenovirus minimizes systemic exposure. However, while all patients presented evidence of active vector expression in their tumors, there has been some evidence of systemic "penetration." This, as well as observed increases in antibodies to adenovirus coat proteins, have raised concerns about the development of neutralizing antibodies diminishing transgene expression after multiple dosing.

High systemic concentrations of adenovirus have also resulted in significant hepatocellular insult (Nielsen LL, etal, Hum Gene Ther, 20 Mar 1998;9(5):681-94), and as Dr. Warren pointed out, although patients were being randomized to phase II trials that would have compared regional chemotherapy alone to regional chemotherapy in combination with regional gene therapy in primary and metastatic liver cancer, this activity was suspended following the 1999 death of a patient with hereditary liver disease treated intrahepatically with high doses of adenovirus (Marshall E, Science, 17 Dec 1999;286(5448):2244-5). This fatal toxicity may have been attributable to dendritic cell activation in the spleen. Phase I testing is continuing

to better establish the safety profile for this administration regimen.

Discussing the findings of a multicenter phase I dose-escalation study of intratumorally administered SCH58500 in patients with nsclc, Martin Schuler, MD, of the Johannes Gutenberg University (Mainz, Germany), also noted that neutralizing antibodies to the adenovirus developed within the first treatment cycle (Schuler M, etal, ICGTC00, Abs. O-4:S1). In this trial, SCH58500 was administered either by bronchoscopic intratumoral injection, or by CT-guided percutaneous intratumoral injection. The host immune response to adenoviral vectors has been shown to decrease transgene expression in preclinical models; however, when the effect of multiple dosing with SCH58500 by intraperitoneal administration was examined in patients with ovarian cancer, no diminishment of p53 expression was observed either in tumor biopsies or ascitic fluid (Wen SF, etal, ICGTC00, Abs. P-25:S7). Comprehensive reports from clinical trials of SCH58500 are presented in nm|**OK**.

Ontario Cancer Institute (Toronto, Canada) scientists, in an effort to selectively target adenoviral-mediated p53 gene transfer to nasopharyngeal cancer (NPC) cells, cloned the transcriptional cassette of the oriP element of the Epstein-Barr virus (EBV) genome in juxtaposition to the p53 gene in a noncytotoxic E1-deleted adenoviral vector (Li J-H, etal, ICGTC00, Abs. O-33:S10). NPC is universally associated with the presence of EBV, and all EBV-containing NPC cells express the transcriptional promoter EBNA1, which binds avidly to a family of repeats contained within the oriP region of the EBV genome. High levels of p53 expression were observed in EBV-positive C666-1 NPC cells transfected with this construct (adv.oriPp53), resulting in a 70% reduction in cell viability. By comparison, cell viability was only reduced by 10% in EBV-negative CNE-2Z NPC cells transfected with adv.oriPp53.

Charles L. Densmore, PhD, discussed how aerosol delivery of polyethyleneimine (PEI)-p53 complexes had been used at Baylor College of Medicine (Houston, TX) to inhibit growth of lung metastases in experimental murine melanoma, and in animal models of human osteosarcoma (Gautam A, etal, Mol Ther, Oct 2000;2(4):318-23, and Densmore CL, etal, ICGTC00, Abs. O-5:S2). Mice were injected with either SAOS LM-6 human osteosarcoma cells, or B16-F10 murine melanoma cells, and then treated with aerosol administration of PEI-p53 complexes twice a week for three weeks. More than 50% of the mice in the PEI-p53-treated group exhibited no visible tumor foci following treatment. Also, a highly significant reduction in lung weights of p53-treated mice was observed compared to control groups. Tumor burdens were significantly lowered in mice treated with PEI-p53 complexes, and a significant reduction in extrapulmonary metastases was observed in the groups treated with PEI-p53 complexes compared to controls, of which 50% exhibited metastasis to lymph nodes in the neck or abdomen. Treatment with PEI-p53 aerosol also led to about a 50% increase in mean length of survival.

Cytolytic agents targeting p53-deficient cancer cells represent a different approach to p53 tumor suppressor loss. Scientists at Onyx Pharmaceuticals (Richmond, CA) have constructed an E1B-deleted adenovirus, *dl*1520 (ONYX-015 or CI-1042), for use as a selective cytolytic agent. The first genetically engineered, replication-selective adenovirus to enter human clinical trials, ONYX-015, under development with Pfizer, has been well tolerated by various routes of administration (intratumoral, intraperitoneal, intra-arterial, and intravenous) in over 230 cancer patients (Kirn D, ICGTC00, Abs. O-26:S8). Common toxicities have included fever (typically Grade 2/3), asthenia, and injection site pain. Although clinically relevant hepatotoxicity has not been demonstrated, transient Grade 1/2 transaminitis was observed in some patients at dose levels above 10^{12} viral particles. Significant elevations of plasma neutralizing antibody titers have been observed, and induced serum cytokines have included IL-6, IL-10, TNF-α, and IFN-γ (Nemunaitis J, etal, ICGTC00, Abs. O-27:S8). However, although single agent-induced objective tumor regressions (15%-20%) have been demonstrated in head and neck cancer, no objective clinical responses were observed in pancreatic, colorectal, ovarian, or metastatic lung tumors; this lack of significant single-agent efficacy has lead Onyx to evaluate *dl*1520 as a neoadjuvant approach in RT and chemotherapy (Rogulski KR, etal, Cancer Res, 1 Mar 2000;60(5):1193-6, Ganly I, etal, Clin Cancer Res, Mar 2000;6(3):798-806, and Khuri FR, etal, Nat Med, Aug 2000;6(8):879-85). Numerous clinical trials have been completed, are ongoing, and are being planned with this construct, as described in detail in nm|**OK**.

mda-7

Melanoma differentiation-associated gene mda-7 derives its name from the fact that it is upregulated in actively proliferating normal human melanocytes versus primary and metastatic human melanomas, and in human melanoma cells treated to induce irreversible growth arrest and terminal differentiation. It is highly conserved and encodes a novel protein of 206 amino acids (Jiang H, etal, Oncogene, 21 Dec 1995;11(12):2477-86, Gopalkrishnan R, etal, AACR99, Abs. 4849;734, and Madireddi MT, etal, Oncogene, 2 Mar 2000;19(10):1362-8). The mda-7 gene is often referred to as a universal tumor suppressor, and the forced expression of mda-7 is growth inhibitory in a diverse range human tumor cells (including cells from breast, melanoma, central nervous system, cervix, colon, prostate, and connective tissue carcinomas) by selective induction of apoptosis, independent of p53, p16, ras or Rb mutational status (Madireddi MT, etal, Adv Exp Med Biol 2000;465:239-61, and Jiang H, etal, PNAS USA, 20 Aug 1996;93(17):9160-5).

Introgen's INGN 241 is an E1/E3-deleted adenoviral vector harboring the mda-7 gene under the control of a CMV promoter. Introgen has an exclusive worldwide

license to mda-7 for use in gene therapy from Corixa (Seattle, WA); Corixa obtained rights to mda-7 following its acquisition of GenQuest (Seattle, WA), which had exclusively licensed intellectual property rights (USA patent #5,710,137 issued in January 1998) to the gene from Columbia University (NY, NY), where the mda-7 gene was discovered in the laboratory of Dr. Paul B. Fisher (Jiang etal, Oncogene 1995;11:2477). INGN 241 has demonstrated proapoptotic tumor suppressor activity in a variety of tumor cell lines, including lines derived from breast, colorectal, and lung carcinomas (Saeki T, etal, Gene Ther, Dec 2000;7(23):2051-7, and Mhashilkar AM, etal, ICGTC00, Abs. O-6:S2).

Although mda-7 gene transfer has been shown to upregulate the death-promoting Bax gene, and to activate such key mediators of apoptosis as caspases 3 and 9, INGN 241-mediated growth inhibition has been observed in mda-7-transduced MCF-7 (breast) and DU-145 (prostate) cancer cell lines, which are devoid of caspase 3 and Bax, respectively. Exposure of cancer cells to INGN 241 treatment results in a G2/M cell cycle block in addition to induction of a sub-G0/G1 population, whereas growth inhibition is not observed in normal cell lines. Because mda-7 protein is actively released from cells treated with INGN 241, this agent may have a wide radius of effect on surrounding cancer cells, providing enhanced anticancer activity (Gopalkrishnan R, etal, *ibid*). In November 2000, Introgen initiated a dose-escalation phase I clinical trial of INGN 241 in patients with solid tumors, including breast cancer. The study, to enroll 15 patients, is being conducted by US Oncology (Dallas, TX). The protocol involves administration of a single intratumoral injection of INGN 241. John J. Nemunaitis, MD, director of clinical research at US Oncology, is the study's principal investigator.

pHyde

Investigators at the University of Tennessee (Memphis, TN), in collaboration with Genotherapeutics (Memphis, TN), are looking at prostate cancer growth inhibition by an E1/E3-deleted, replication-deficient recombinant adenovirus type 5, containing the pHyde tumor suppressor gene under the control of a truncated RSV promoter (Steiner MS, etal, ICGTC00, Abs. O-7:S2). The pHyde gene, initially cloned at the University of Tennessee by cDNA competitive hybridization from Dunning rat prostate cancer cell lines (Rinaldy AR, etal, Gan To Kagaku Ryoho, May 2000;27 Suppl 2:215-22), is comprised of 2713 nucleotides with an open reading frame of 1467 nucleotides coding for a protein of 489 amino acids. The pHyde gene product has been shown to upregulate the apoptotic pathway in rat prostate cancer cell lines, and the adenoviral vector, AdRSVpHyde, has demonstrated 77% and 83% *in vitro* growth inhibition of the human prostate cancer cell lines DU145 and LNCaP, respectively. *In vivo*, a single injection of AdRSVpHyde (5 x 10^9 pfu) reduced DU145 xenografted tumors in nude mice by 75% compared to untreated controls or noncoding vector-treated animals. AdRSVpHyde induced apoptosis in transduced cells, stimulating both p53 expression and caspase 3 activity (Steiner MS, etal, Cancer Res, 15 Aug 2000;60(16):4419-25).

p27

The cell-cycle inhibitor p27 (Kip1), a member of the universal cyclin-dependent kinase (Cdk) inhibitor family, is a putative tumor suppressor. Although p27 gene mutations are rarely observed in cancer, a decreased level of p27 protein expression is associated with tumor development and progression in several human malignancies, including bladder, laryngeal, colorectal, and lung carcinomas (Del Pizzo JJ, etal, Am J Pathol, Oct 1999;155(4):1129-36, Kudo Y, etal, Cancer Lett, 14 Apr 2000;151(2):217-22, Gunther K, etal, J Surg Res, Jul 2000;92(1):78-84, and Park K, etal, Lung Cancer, Mar 2001;31(2-3):149-55). The main inhibitory action of p27 is as a negative regulator of cyclin E-Cdk2 activity, that results in G1/S arrest (Tong W and Pollard JW, Mol Cell Biol, Feb 2001;21(4):1319-28).

At Seoul National University College of Medicine in Korea, researchers have investigated the antitumor effects of a recombinant replication-deficient adenoviral vector expressing human wild-type p27 (Ad-p27) on human head and neck squamous cell carcinoma (HNSCC) cell lines (Koh T-Y, etal, ICGTC00, Abs. PD-18:S5), as well as human lung cancer cell lines (Lee C-T, etal, ICGTC00, Abs. PD-19:S6). After transduction of the HNSCC cell lines SNU-1041, SNU-1066 and SNU-1076 with Ad-p27, a high level of p27 protein expression was observed, together with a significant decrease in S phase and an increase in G1 phase. Transduction of Ad-p27 resulted in statistically significant growth inhibitory effects in all three lines.

Similar results were observed upon transduction of several human lung cancer cells with Ad-p27. Because p27 is degraded by phosphorylation at Thr187 and ubiquitination-dependent proteolysis (Tsvetkov LM, etal, Curr Biol, 17 Jun 1999;9(12):661-4), the researchers also constructed an adenoviral vector expressing a mutant form of p27, Ad-p27m, in which the residues Thr187/Pro188 (ACGCCC) were mutated to Met187/Ile188 (ATGATC). Ad-p27m demonstrated stronger G1/S arrest and growth inhibition in human lung cancer cell lines than Ad-p27, and when injected intratumorally into nude mice bearing human lung cancer xenografts, Ad-p27m induced greater growth suppression of established tumors than did Ad-p27.

In a somewhat indirect approach to modulating p27-mediated G1/S cell cycle arrest in tumor cells, scientists at the Human Gene Therapy Research Institute (Des Moines, IA), in collaboration with Hokkaido University (Sapporo, Japan) and Hammersmith Hospital (London, UK), are investigating the possible use of the von Hippel-Lindau tumor suppressor gene, VHL, for cancer gene therapy (Kim M, etal, Biochem Biophys Res Commun, 30 Dec 1998; 253(3):672-7, and Seth P, etal, ICGTC00, Abs. O-9:S3).

Von Hippel-Lindau disease is an autosomal dominant syndrome characterized by predisposition for bilateral and

multicentric hemangioblastoma in the retina and central nervous system, pheochromocytoma, renal cell carcinoma, and cysts in the kidney, pancreas, and epididymis (Couch V, etal, Mayo Clin Proc, Mar 2000;75(3):265-72, and Hes F, etal, Hum Genet, Apr 2000;106(4):425-31). The VHL tumor suppressor gene associated with von Hippel-Lindau disease was cloned in 1993, and somatic mutations in this gene have been detected in the sporadic occurrence of up to 60% to 78% of the various tumors characterizing this syndrome (Koper JW and Lamberts SW, Eur J Clin Invest, Jun 2000;30(6):493-500, Meyer AJ, etal, Int J Cancer, 1 Sep 2000;87(5):650-3, Ashida S, etal, Clin Cancer Res, Oct 2000;6(10):3817-22, and Esteller M, Eur J Cancer, 1 Dec 2000;36(18):2294-300).

The transduction of various cancer cell lines endogenously expressing defective VHL with an adenoviral vector containing wild-type VHL cDNA (AdVHL), resulted in high levels of VHL mRNA and protein expression as well as G1/S arrest and cell growth inhibition. AdVHL-mediated cell cycle arrest is associated with an increase in p27 mRNA synthesis and induction of p27 protein, suggesting that VHL controls cell cycle progression by regulation of p27 at both the mRNA and protein levels.

INHIBITION/KNOCK-OUT OF PROMOTERS OF TUMOR GROWTH

A gene therapy strategy analogous to the replacement of defective or nonfunctioning tumor suppressor genes involves knock-out or inhibition of specific genetic lesions, e.g., oncogenes, implicated in carcinogenesis. Typically, this involves the creation of synthetic nucleic acid sequences (SNAS) that are capable of modulating the expression of targeted oncogenes by sequence-specific hybridization to cellular mRNA or genomic DNA. Alternatively, anticancer gene therapy may involve the suppression of tumor growth factors or alteration of the tumor microvasculature. A detailed review of the state-of-the-art in SNAS development in cancer was presented in FO, pp 1229-1247, 1269-1279, 1282-1319; also consult nm|OK.

Sex hormone-binding globulin (SHBG)

Sex hormone-binding globulin (SHBG) is a 45 kDa glycoprotein that is the plasma carrier for estradiol and androgens, which it binds with high specificity and affinity. In addition to breast tissues, SHBG is produced in the liver, endometrium, and prostate via E2 (17β-estradiol)-activated estrogen receptor (ER)α and is secreted into plasma, where it regulates the activity of bioavailable sex steroids and modulates cell growth regulation. Complexes of E2-SHBG have been shown to bind to the SHBG receptor (SHBGr) in breast cancer cell membranes, and to internalize through SHBGr-mediated endocytosis, resulting in the induction of intracellular cAMP and E2-responsive second messenger, causing estrogen action in breast cancer cells (Murayama Y, etal, Breast Cancer, 25 Oct 1999;6(4):338-43).

At the Tokyo Medical and Dental University (Tokyo, Japan), scientists have cloned the 3' portion of the shbg gene of MCF-7 human breast cancer cells, and identified steroid binding domains within exons 6 and 7. Antisense phosphorothioate oligonucleotides complementary to these exons were then constructed and used to treat cultured MCF-7 cells supplemented with E2 (Murayama Y and Sankichi H, ICGTC00, Abs. PD-16:S5). These antisense oligomers inhibited shbg expression and the synthesis of SHBG protein in a sequence-specific manner, resulting in approximately 88% of antisense-treated MCF-7 undergoing apoptosis, compared to 28% to 36% in control oligomer-treated cells. Nonspecific cytotoxicity was judged to account for only about 20% of observed apoptosis in the antisense-treated cells.

T-ag of Human Polyomavirus BK (BKV)

Researchers at the University of Tromso (Tromso, Norway) and Karolinska Hospital (Stockholm, Sweden) are studying antisense-mediated inhibition of the large tumor antigen (T-ag) of human polyomavirus BK (BKV) as a possible approach to the treatment of neuroblastomas (Jorgensen GE, etal, Med Pediatr Oncol, Dec 2000;35 (6):593-6, and Johnsen JI, etal, ICGTC00, Abs. PD-17:S5). Neuroblastomas have been shown to exhibit aberrant cytoplasmic localization of p53, which compromises the protein's suppressor function. In addition, BKV has been found to be present, and its T-ag expressed, in nearly all human neuroblastomas examined but not in normal adrenal medulla (Flaegstad T, etal, Cancer Res, 1 Mar 1999;59(5):1160-3).

When neuroblastoma cells lines containing BKV DNA and expressing T-ag are subjected to molecular analysis, T-ag and p53 are found to be co-localized in the cytoplasm, with T-ag forming a complex with the p53 protein. Upon treatment of these cells with a T-ag antisense construct, translocation of p53 from the cytoplasm to the nucleus occurs, accompanied by enhanced p21(waf1/cip1) expression, activation of the Bax promoter, and induction of apoptosis, indicating functional p53. Although not proof of causality, these findings demonstrate that virally encoded T-ag apparently plays an important role in the mechanisms of neuroblastoma cell survival, probably by binding to p53 and causing aberrant cytoplasmic localization of the protein, which renders the p53-mediated apoptotic pathway of the cell nonfunctional. The continuous treatment of nude rats bearing human neuroblastoma xenografts with anti-T-ag antisense oligonucleotides has resulted in a 40% reduction in tumor growth compared to control animals.

K-ras

The most common oncogenic mutations in pancreatic cancer are point mutations in the K-ras gene, which occur in 75% to over 95% of pancreatic tumors (Minamoto T, etal, Cancer Detect Prev 2000;24(1):1-12, Sakorafas GH, etal, Cancer Treat Rev, Feb 2000;26(1):29-52, and Lohr M, etal, Int J Pancreatol, Apr 2000;27(2):93-103). Scientists at

Tokai University School of Medicine (Isehara, Kanagawa, Japan) designed a hammerhead ribozyme (Rz) containing K-ras-specific nucleotides flanking the Rz catalytic core, which targets mutant codon 12 (GGT to GTT) of the K-ras gene, to generate a recombinant, replication-defective adenoviral vector expressing the ribozyme rAd/anti-K-ras Rz. The catalytically active anti-K-ras Rz has been shown to be at least 2-fold more potent in decreasing cellular K-ras mRNA levels, and inhibiting cell proliferation and colony formation in soft agar than a catalytically inactive control ribozyme. It also increases the ratio of wild-type to mutated K-ras mRNA in human pancreatic adenocarcinoma CFPAC-1 cells. These results suggest that both catalytic cleavage and antisense effects contribute to the ribozyme's activity (Giannini CD, etal, Nucleic Acids Res, 1 Jul 1999;27(13):2737-44).

When rAd/anti-K-ras Rz was used to transfect cultured Capan-1 human pancreatic cells, a 22% suppression in mutant K-ras gene expression was observed, together with cell-growth inhibition. Maximum growth suppression was observed at 3 days postinfection, along with characteristic morphologic changes of apoptosis such as nuclear condensation and oligonucleosomal DNA fragmentation, as well as suppression of bcl-2 oncoprotein. These changes were not demonstrated in control virus-infected cells. In a murine model system, rAd/anti-K-ras Rz treatment caused efficient reversion of the malignant phenotype in Capan-1 human pancreatic tumor xenografts with K-ras gene mutation (Tsuchida T, etal, Cancer Gene Ther, Mar 2000;7(3):373-83, and Kijima H, etal, ICGTC00, Abs. PD-13:S4). Originally, this work was being conducted in collaboration with Berlex Biosciences (Richmond, CA) that has since terminated its involvement.

GROWTH FACTOR SUPPRESSION

Transforming growth factors (TGF)

Transforming growth factors (TGF) are peptides that can affect the growth and phenotype of cultured cells, bringing about in untransformed fibroblastic cells phenotypic properties that resemble those of malignant cells. Two types of TGF have been well characterized, type α (TGF-α) and type β (TGF-β), distinguished both chemically by their unique amino acid sequences, and biologically by their different actions on cells. TGF-β is produced by a variety of normal and malignant cells, and although its major physiologic role appears to be the stimulation of mesenchymal matrix formation, it is capable of transforming the morphology of certain cell lines via distinct cell surface receptors (type I and type II) possessing serine/threonine kinase activity within their cytoplasmic domains (Datto M and Wang XF, Cytokine Growth Factor Rev, Mar-Jun 2000;11(1-2):37-48).

In the process of malignant progression, TGF-β appears to play two different and opposite roles. As a tumor suppressor, picomolar concentrations of TGF-β have been shown to completely inhibit the growth of most tumor cells during the early stages of carcinogenesis. However, contrary to earlier notions that TGF-β may be downregulated in cancer cells to promote their growth, at some point during the development and progression of malignant neoplasms, bioactive TGF-β is significantly increased in the tumor microenvironment. In fact, the most aggressive forms of certain cancers (i.e., colon carcinoma and glioblastoma multiforme) were actually shown to be autocrine and/or paracrine growth-stimulated by TGF-β (Kim SJ, etal, Cytokine Growth Factor Rev, Mar-Jun 2000;11(1-2):159-68).

TGF-β has also been shown to promote immunosuppression and tumor immune escape by induction of growth arrest and apoptosis in immune cells, by downregulation of MHC II antigen expression resulting in interference with the generation of tumor-specific cytotoxic T lymphocytes (CTL), and by changes in the cytokine release profiles of immune and tumor cells (Haufel T, etal, Anticancer Res, Jan-Feb 1999;19(1A):105-11, Stander M, etal, Cell Tissue Res, May 1999;296(2):221-7, De Visser KE and Kast WM, Leukemia, Aug 1999;13(8):1188-99, and Shah AH, etal, ICGTC00, Abs. PD-92:S28). Furthermore, TGF-β may facilitate tumor angiogenesis by overcoming the extracellular matrix cell adhesion requirement for surface expression of α5β1 integrin, a necessary effect for the induction of anchorage-independent growth (Dalton SL, etal, J Biol Chem, 15 Oct 1999;274(42):30139-45).

Based on observations of high-level expression of TGF-β in developed AK7 malignant mesothelioma in murine models, and of the induction of rapid tumor growth in mice subjected to andenovirus-mediated transfection of tumor cells with an active form of TGF-β, scientists at INSERM (Villejuif, France), in collaboration with Brown University (Providence, RI), have used an adenovirus coding for decorin (Ad-decorin), a natural inhibitor of TGF-β, to modulate the effects of this inflammatory cytokine both *in vitro* and *in vivo* (Valeyrie L, etal, ICGTC00, Abs. P-23:S7). *In vitro*, tumor cells transduced with Ad-decorin exhibited reduced proliferation, massive apoptosis, and cell cycle arrest, the latter effect independent of p21(waf1/cip1), the classic decorin pathway. Injection of mice with a tumorigenic dose of AK7 cells, infected *ex vivo* with Ad-decorin, further confirmed that decorin could inhibit the growth of mesothelioma. Injection of Ad-decorin into pre-established AK7 tumors also significantly inhibited tumor growth. These effects were, however, transient *in vivo*, probably as a consequence of a favorable balance of TGF-β far away from the virus injection site.

ANTIANGIOGENIC GENE THERAPY

Angiogenesis, the development of new blood vessels, plays a fundamental role in many normal physiological processes as well as in various pathologic conditions, including solid tumor growth and metastasis. Antiangiogenic strategies target the tumor microvascula-

ture, seeking to eliminate or restrict blood flow to tumor tissues, causing the disruption of oxygen and nutrient delivery as well as the accumulation of waste metabolites. Tumor cells cannot survive extended severe ischemia, and without adequate vascularization, tumors cannot grow larger than a few millimeters in size and are incapable of metastasizing to other sites. According to nm|OK, there are currently over 115 different constructs in development, exploiting a variety of antiangiogenic/antivascular mechanisms with at least 28 having completed or being currently evaluated in over 100 clinical trials in nearly all cancers as monotherapy or in combination with chemotherapy and/or multimodality therapy.

In vivo Delivery and Expression of Endostatin

In vivo delivery and expression of antiangiogenic genes has been suggested as an attractive alternative to the chronic, systemic administration of recombinant antiangiogenic proteins to cancer patients, avoiding the difficulties associated with obtaining sufficient quantity and quality of the protein(s) for optimal therapeutic benefit as well as the problem of often short circulating half-lives of therapeutic proteins (Feldman AL, etal, Cancer Res, 15 Mar 2000;60(6):1503-6, Sauter BV, etal, Proc Natl Acad Sci USA, 25 Apr 2000;97(9):4802-7, and Chen CT, etal, Hum Gene Ther, 20 Sep 2000;11(14):1983-96).

Editor's note: *In 2001, a court ruling in the USA found that Dynepo, a gene-activated endogenous erythropoietin (EPO) approach in development by a collaboration between Transkaryotic Therapies (TKT; Cambridge, MA) and Aventis, infringed on Amgen's recombinant EPO (Epogen). The notion that a gene-activated endogenous production of a protein by the human host may infringe on a patent involving a man-made recombinant version of the same protein, has far reaching implications and it may be necessary that it is tested in the courts again.*

Endostatin, a 20 kDa, carboxy-terminal fragment of collagen XVIII, is one of several endogenous inhibitors of angiogenesis. Endostatin is a specific inhibitor of endothelial cell proliferation and migration, with potent angiogenic activity as well as tumor growth inhibition (Blezinger P, etal, Nat Biotechnol, Apr 1999;17(4):343-8, Dhanabal M, etal, Biochem Biophys Res Commun, 10 May 1999;258(2):345-52, and Cirri L, etal, Int J Biol Markers, Oct-Dec 1999;14(4):263-7). Endostatin is undergoing clinical testing as a recombinant antitumor agent (Ryan CJ and Wilding G, Drugs Aging, Oct 2000;17(4):249-55). Endostatin, and its sister protein, angiostatin, were originally discovered by Dr. Judah Folkman's group at Children's Hospital at Harvard Medical School (Boston, MA) and licensed exclusively to EntreMed (Rockville, MD) for further development. In phase I clinical trials, intravenous treatment with both of these proteins was well tolerated. In addition, in August 1999, EntreMed announced a research collaboration with Cell Genesys (Foster City, CA) to evaluate a gene therapy approach via which angiostatin and endostatin genes will be combined with Cell Genesys' adenoviral and adeno-associated viral (AAV) gene delivery systems. The two companies will consider a potential business relationship based on the results of the studies performed under this collaboration.

Schering-Plough Research Institute (Kenilworth, NJ) has conducted preclinical studies with a Canji (San Diego, CA)-developed recombinant, E1-deleted, replication-deficient Ad5 vector carrying the secreted form of mouse endostatin (rAd-MEndo). The antitumor activity of rAd-MEndo (1×10^{10} viral particles) was tested in the syngeneic MidT2-1 mammary tumor model in FVB mice and in the MDA-MB-231 human breast tumor model in SCID mice (Nielsen LL, etal, ICGTC00, Abs. O-11:S4). Two intratumoral doses of rAd-MEndo halted further growth of MidT2-1 tumors but had minimal impact on MDA-MB-231 growth. By comparison, an initial IV administration of rAd-MEndo also retarded the growth of MidT2-1 tumors; however, the FVB mice rapidly developed neutralizing antibodies against the adenoviral vector, rendering subsequent IV doses only minimally effective. In her presentation, Loretta L. Nielsen, PhD, of Schering-Plough noted that this work was not being conducted for eventual commercialization but was rather performed as a proof-of-concept study.

At the University of Bergen in Norway, Tracy-Ann Read, PhD, and her colleagues, encapsulated HEK-293 cells transfected with the gene for human endostatin in immunoprotective microcapsules constructed from guluronic-enriched, ultra-pure sodium alginate (Read TA, etal, Int J Dev Neurosci, Aug-Oct 1999;17(5-6):653-63, and Read T-A, etal, ICGTC00, Abs. O-10:S3). The resulting microspheres, which ranged from 300 μm to 500 μm in diameter, typically secreted 2-2.5 μg/ml of endostatin at 3 weeks post-encapsulation. When the encapsulated endogenous endostatin was administered locally to implanted BT4C gliomas in BDIX rats, it caused a significant reduction in tumor size compared to control animals; the lifespan of treated rats was 70% longer than controls. Treated tumors exhibited large central necrosis and multiple areas of hypoxia as well as apoptosis. Local and circulatory distribution of the transgene protein did not appear to affect normal chamber microcirculation. However, tumor associated microcirculation was severely affected, with tumors exhibiting a 60% reduction in the density and 50% reduction in the diameter of microvessels.

Scientists at the University of Southern California (USC;Los Angeles, CA) are studying lentiviral vectors for the delivery of transgenes for endostatin as well as thrombospondin-1 and angiostatin, to the tumor microvascular for stable, long-term antiangiogenic gene therapy (Tai C-K, etal, ICGTC00, Abs. P-50:S15). Like endostatin, angiostatin, a 38 kDa internal fragment of plasminogen, and thrombospondin-1, a disulfide-linked structural glycoprotein subunit, are endogenous substances that have been shown to modulate cell attachment, migration, and proliferation. In comparing lentiviral vectors to adenoviral vec-

tors encoding the same transgenes, the researchers found the latter to be capable of mediating gene delivery of antiangiogenic factors directly to endothelial cells of the human lung microvasculature and human umbilical vein, but that high titers were required to achieve effective cell transduction. In addition, adenoviral vectors were incapable of infecting tumor cells that had lost expression of the coxsackie-adenovirus receptor (CAR), while lentiviral vectors readily infected such tumor cells.

Tumor Suppressor Activity and Inhibition of Angiogenesis

In addition to inducing apoptosis of tumor cells, both the mda-7 and VHL tumor suppressor genes have demonstrated antiangiogenic activity. The mda-7 protein, released from cells treated with Introgen's INGN 241, has been observed to inhibit tumor cell angiogenesis *in vitro* as well as in animal models (Mhashilkar AM, etal, *ibid*). The antiangiogenic properties of VHL were suggested by the finding that mutations in the VHL gene can result in the constitutive stabilization of transcription factors hypoxia-inducible factors 1α and 2α, which bind to specific enhancer elements of the vascular endothelial growth factor (VEGF) gene and stimulate angiogenesis (Harris AL, Oncologist 2000;5 Suppl 1:32-6).

In fact, when Dr. Seth of the Human Gene Therapy Institute and his associates subcutaneously injected 786-0 renal cancer cells expressing defective VHL into nude mice, a significant induction of angiogenesis was observed (Seth P, etal, *ibid*). These investigators then exposed human umbilical vein endothelial cells (HUVEC) to an adenoviral vector expressing wild-type VHL (AdVHL), and found that the vector not only induced G1/S arrest but caused complete inhibition of capillary-like network blood vessel formation.

SUICIDE GENE-BASED APPROACHES

Suicide gene strategies are typically applied either to virus-based vector systems (self-deleting vectors) to avoid problems that might be encountered with conventional viral vectors, such as recombination with helper viruses or transcriptional repression of transduced genes, or to facilitate an *in situ* prodrug approach to gene therapy of malignant disease.

Gene-directed Enzyme Prodrug Therapy (GDEPT)

Suicide gene-based prodrug approaches represent essentially two-step, tumor-targeted chemotherapy, in which an exogenous gene is delivered to tumor cells, that when expressed, is capable of converting a nontoxic prodrug into a cytotoxic species that can kill the cells. Alternatively, suicide genes can be used to enhance the long-term safety of transplanted cells by permitting their ablation should they develop unacceptable characteristics in the form of malignancy, hyperfunction, or graft-versus-host disease (GvHD). The most frequently used suicide genes code for either herpes simplex virus thymidine kinase (HSVtk), which renders transfected cells suscepti-ble to ganciclovir (GCV), or *E. coli* cytosine deaminase, which converts 5-fluorocytosine into 5-fluorouracil (5-FU). Because these systems rely on the enzyme-mediated activation of a prodrug after the gene encoding for the enzyme has been targeted to the malignant cell, they are referred to as gene-directed enzyme prodrug therapy (GDEPT).

Investigators Olga Greco and Gabi U. Dachs of the Gray Laboratory Cancer Research Trust (CRT) at Mount Vernon Hospital (Northwood, Middlesex, UK) have laid out certain criteria for the selection of an appropriate enzyme/prodrug combination (Greco O and Dachs GU, J Cell Physiol, Apr 2001;187(1):22-36). Ideally, the enzyme should be monomeric, of low molecular weight, and have no requirement for glycosylation; this will allow for ease of handling and potential protein modification. The enzyme should also exhibit strong catalytic activity under physiological conditions, and fast prodrug activation even at low concentrations of the substrate, without dependence on further catalysis by other enzymes. Expression of the enzyme in itself should not lead to cytotoxic effects, and the reaction pathway should be different from any endogenous enzyme, to avoid cytotoxic activation of the prodrug in normal tissues. The ideal prodrug should be chemically stable under physiological conditions, freely diffusible throughout the targeted tumor, and capable of being converted intracellularly to a metabolite that is at least 100-fold more toxic than the prodrug and that is not cell cycle-specific in its mechanism of action. The active drug should also be readily diffusible, with a half-life long enough to allow it to exert a cytotoxic bystander effect on surrounding untransfected cells, but short enough to ensure that any drug escaping into the circulation will be inactive. Several GDEPT systems are being investigated, most of which do not meet the all of these conditions. Nonetheless, results in tissue culture and animal models have been encouraging, and some of these systems have advanced into phase I/II clinical testing.

HSVtk/ganciclovir is perhaps the most well studied enzyme/prodrug combination in GDEPT for cancer. Ganciclovir is a nucleoside analog that, along with its derivatives, is widely used in the treatment of HSV infections. GCV is readily converted to the monophosphate form by thymidine kinase from HSV-1, and subsequent catalysis by cellular enzymes leads to several toxic metabolites, the most active being the triphosphates. GCV-triphosphate competes with deoxyguanosine triphosphate for incorporation into elongating DNA during cell division, resulting in inhibition of DNA polymerase and formation of single strand breaks. GDEPT systems using HSVtk/GCV are limited to target cells that are actively dividing, as GCV-triphosphate is an S phase-specific cytotoxin.

Although HSVtk suicide gene transduction can sensitize cancer cells to chemotherapy, it is difficult to deliver the gene to all areas of established cancers, an issue compli-

cated by the fact that the HSVtk/GCV system is not membrane permeable, and relies on cell-to-cell contact for diffusion into surrounding cells. This problem has been overcome to some degree by using adenoviral vectors to achieve *in situ* locoregional tumor gene delivery. However, application of this approach to systemic disease is limited by ectopic expression of the transgene within the liver, resulting in fatal toxicity.

At the University of Alabama at Birmingham researchers have explored a targeting strategy in gastrointestinal cancer in which the promoter region of the cyclooxygenase-2 (cox-2) gene is used to control the expression of HSVtk in an adenoviral vector (Yamamoto M, etal, ICGTC00, Abs. O-65:S19). Under physiologic conditions, the cox-2 gene is virtually undetectable in most tissues, including the liver, but is expressed in a majority of gastric and colon cancers. Compared to a CMV promoter, the cox-2 promoter exhibits very little activity in the liver, but is highly active in cox-2-positive subcutaneous tumors. Adenoviral vectors expressing HSVtk under the control of the cox-2 promoter exhibit cytocidal effects specific to cox-2-positive cells. When administered to mice, the cox-2 promoter construct successfully mitigated the *in vivo* hepatotoxicity seen with GCV and an adenoviral vector expressing HSVtk under the control of a CMV promoter.

In another approach to conferring selective cytotoxicity to GCV in human carcinoma cells, scientists at the Chiba Cancer Center Research Institute and Chiba University School of Medicine, together with investigators at the Nagoya University School of Medicine, and Kagoshima University, all in Japan, have used a cis-acting transcriptional control sequence in the midkine (MK) gene to promote expression of the HSVtk gene (Tomizawa M, etal, ICGTC00, Abs. PD-69:S21). Midkine is a heparin-binding growth factor with mitogenic activity for fibroblasts and neuroectoderm cells. Although the biological function of this protein during tumorigenesis is unclear, expression of the MK gene is elevated in various types of human cancers, such as lung, breast, and gastrointestinal tumors as well as neuroblastoma. In normal human adult tissues, MK expression is restricted to lung alveoli, the kidney, and mucosal tissues of the gastrointestinal tract. These features suggest that the MK promoter may prove useful for the selective transcription of the HSVtk gene in tumor cells.

Frederick L. Moolten, MD, discussed work conducted at the Edith Nourse Rogers Memorial Veterans Hospital (Bedford, MA) involving the preemptive introduction of suicide genes into tissues at risk for cancer, to impart drug sensitivity as a clonal property to cancers arising from sensitized cells (Moolten FL, etal, ICGTC00, Abs. O-60:S18). In testing this approach, a retroviral vector was used to transduce the HSVtk gene into the TM4 line of preneoplastic murine mammary epithelial cells. While control tumors were insensitive to GCV treatment, tumors that arose from the transduced cells were susceptible to GCV-mediated growth inhibition, and durable regressions were induced in 7/20 mice (Moolten FL, etal, Hum Gene Ther, 20 Jun 1996;7(10):1197-204).

Donor lymphocyte infusions (DLI) in allogenic BMT have been shown to induce remissions in some patients with relapsed hematologic malignancies. However, DLI is complicated by the development of GvHD, attributable to donor lymphocytes attacking normal tissue in the recipient (Link CJ Jr, etal, Cancer Treat Res 1999;101:369-75). To abrogate the effects of GvHD, should it occur, Tatiana Seregina, PhD, and associates at the Human Gene Therapy Research Institute, in collaboration with Northwestern University School of Medicine (Chicago, IL) and Medical College of Wisconsin (Milwaukee, WI), have used a retroviral vector to transfect donor lymphocytes *ex vivo* with the gene for HSVtk, thereby rendering the cells susceptible to selective destruction *in situ* with GCV (Seregina T, etal, ICGTC00, Abs. O-54:S16).

Patients with various hematologic malignancies, including chronic myelogenous leukemia (CML), acute myelogenous leukemia (AML), Hodgkin's disease (HD), non-Hodgkin's lymphoma (NHL), and cutaneous T-cell lymphoma (CTCL), were entered into a phase I/II study designed to assess the safety and potential effectiveness of the infusion of HSVtk-transduced donor lymphocytes in allogenic BMT. Eight patients have been treated with at least one infusion of transduced lymphocytes having, at a minimum, 85% HSVtk-positive cells. After infusion, detectable HSVtk-transduced lymphocytes in the peripheral blood ranged from 0% to 3.8%. Upon second relapse, in a patient with CTCL refractory to chemotherapy, a transfusion of 4.5 billion HSVtk-transduced lymphocytes from the original marrow donor, resulted in a resolution of the CTCL lesions over 6 to 8 weeks, but chronic extensive cutaneous GvHD developed several months later. Although refractory to conventional therapy, GvHD resolved following GCV infusion. However, transgene expression became undetectable in both blood and skin after GCV treatment, and CTCL recurred several months later, suggesting that further cycles of HSVtk-transduced DLI may be required.

Cytosine deaminase/5-fluorocytosine (CD/5-FC) is another enzyme/prodrug system which, like HSVtk/GCV, is based on the production of a toxic nucleotide analog. An enzyme found in certain bacteria and fungi but not in mammalian cells, CD catalyses the hydrolytic deamination of cytosine to uracil, and is thereby capable of converting the nontoxic prodrug 5-FC to 5-FU which in turn is transformed by cellular enzymes to potent pyrimidine antimetabolites. Unlike the HSVtk/GCV system, 5-FU enjoys nonfacilitated diffusion into and out of cells, rendering a strong bystander effect to the CD/5-FC system that does not require cell-to-cell contact. On the other hand, although not cell cycle phase-specific, 5-FU exhibits both proliferation-dependent and proliferation-independent actions, and *in vitro* studies have shown 5-FU and CD/5-FC to have reduced cytotoxic effects in hypoxic tumor

cells, a common feature of solid tumors that may limit the therapeutic effectiveness of this system (Greco O, unpublished data).

In a suicide gene therapy approach in prostate cancer, researchers at St. James' Hospital (Dublin, Ireland) and St. Luke's Hospital (Dublin, Ireland) constructed a eukaryotic expression plasmid in which fragments of the PSA promoter from human genomic DNA are subcloned into the CD gene (Foley R, etal, ICGTC00, Abs. PD-72:S21). One of these fragments extends 700 bp upstream of the transcription start site, while a second fragment spans a 1.6 kb upstream region; both contribute to the upregulation of PSA transcription by androgens. Liposomal formulations of PSA-CD plasmid have achieved transfection efficiencies of 20%-30% in LNCaP, DU145, and PC-3 human prostate cancer cell lines, which combined with the bystander effect characteristic of the CD/5-FC system, may allow CD expression to exert an activated prodrug effect in a significant proportion of malignant prostate cells.

Svend O. Freytag of the Henry Ford Health System (Detroit, MI), described work being conducted there and at the Harvard Gene Therapy Initiative (Cambridge, MA) on the development of a trimodal approach to adenovirus-mediated suicide gene therapy in managing local recurrence of prostate cancer following definitive radiation therapy (Freytag SO, etal, ICGTC00, Abs. O-59:S18). Taking advantage of the cytolytic properties of replication-competent adenovirus, an Ad5 vector has been constructed in which a tumor-specific transcriptional promoter is used to confer conditional virus replication competency upon prostate cancer cells. The lytic activity of the virus is enhanced by using it to selectively deliver a CD/HSVtk fusion gene to the tumor cells and invoking the CD/5-FC and HSVtk/GCV enzyme/prodrug systems.

Results of preclinical testing indicated that intraprostatic administration of the lytic, replication-competent Ad5-CD/HSVtk virus to humans, concomitant with double suicide gene therapy, would be associated with acceptable toxicities and not result in vertical transmission of viral-encoded genes through the germ line (Paielli DL, etal, Mol Ther, Mar 2000;1(3):263-74). This vector system was subsequently approved for phase I clinical testing, in which an escalating dose of the vector (10^{10} to 10^{12} virus particles), administered intraprostatically using transrectal ultrasound guidance, is followed two days later by the daily infusion of 5-FC (150 mg/kg) and GCV (10 mg/kg), for seven days. Among 12 patients treated with 10^{10} virus particles, 6 were evaluable for response. In a first cohort of three patients, with the exception of one case of transient Grade 3 hypermagnesemia, all toxicities were minimal and self-limiting. In terms of results, one patient exhibited a 51% decrease in serum PSA by day 55 that was sustained for over 6 months, and two patients demonstrated transient stabilization of PSA levels. In a second cohort of three patients, two demonstrated decreases in serum PSA levels of 40% to 46%, while one did not respond.

Scientists at the University of North Carolina (Chapel Hill, NC) have engineered a double suicide gene vector for episomal gene therapy of B-cell lymphoma, using human EBV as a gene delivery system (Wang J and Vos JMH, ICGTC00, Abs. PD-67:S20). The B lymphotropic nature of EBV is well established through its association with most B-cell lymphomas in immunocompromised patients and its ability to transform human B cells to B lymphoblastoid cells *in vitro*. Researchers have constructed a nononcogenic, helper-dependent miniEBV vector carrying only the minimal *cis* elements required for episomal replication, viral amplification, and packaging; this vector is capable of accommodating exogenous cDNAs of up to 180 kb in size (Banerjee S, etal, Nat Med, Dec 1995;1(12):1303-8, and Sun TQ, etal, Dec 1996;3(12):1081-8). Genes encoding HSVtk and CD have been cloned into the miniEBV vector, and placed under control of the CMV immediate early promoter/enhancer and the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), to maximize coexpression of these genes. It is hoped that the transfer of the HSVtk and CD genes into B-cell-derived lymphomas will render the transduced as well as nontransduced (bystander effect) cells susceptible to synergistic GCV/5-FC-mediated killing.

Researchers at the University of Yonsei College of Medicine (Seoul Korea) and University of Ulsan College of Medicine (Seoul, Korea), together with the University of Pittsburgh Medical School (Pittsburgh, PA), have engineered a double suicide gene vector as a potential therapy system for brain tumors, by incorporating a fusion gene for HSVtk and CD in a recombinant, replication-defective adenovirus (Kim E, etal, ICGTC00, Abs. P-101:29). *In vitro*, additive cytotoxicity was evident in transduced C6 glioma cells in the presence of GCV and 5-FC. In female Wistar rats, stereotactically injected with transduced C6 glioma cells into the right caudate-putamen, treatment with GCV and 5-FC resulted in significantly smaller tumor volumes (14.7 ± 1.8 mm^3) than did treatment with GCV or 5-FC alone (57.4 ± 7.1 mm^3), or tumor volumes in control rats (157 ± 8.8 mm^3).

Cytochrome P450/cyclophosphamide (CYP/CP) represents a system in which the oxazaphosphorine prodrug cyclophosphamide (CP) is activated by liver cytochrome P450 (CYP) metabolism via a 4-hydroxylation reaction; a 4-hydroxy intermediate breaks down to form the bifunctional alkylating toxin phosphoramide mustard, which leads to DNA crosslinks, G2-M arrest and apoptosis in a cell cycle-independent fashion (Chen L and Waxman DJ, Cancer Res, 1 Feb 1995;55(3):581-9, and Chen L, etal, Cancer Res, 15 Mar 1996;56(6):1331-40). An isomeric analog of CP, isophosphamide (IP), may be activated in a similar fashion. Although CYP is present in some human cancers, including colon, breast, lung, liver, kidney, and prostate carcinomas, the enzyme is also present in the normal liver. However, local conversion of CP appears to be superior to its activation in the liver, probably because of

the short half-life (3.3 minutes) of 4-hydroxy-CP in human plasma, and the genetic engineering of tumor cells to overexpress CYP leads to selective sensitization to oxazaphosphorines, thereby reducing CP (or IP) side effects in critical host tissues. While the CP metabolite phosphoramide mustard does not diffuse efficiently across cell membranes, the CYP/CP system nonetheless exhibits a bystander effect, probably attributed to the diffusible nature of the 4-hydroxy-CP precursor (Greco O and Dachs GU, *ibid*).

The flavoenzyme NADPH-P450 reductase (RED), widely expressed in many cell types, including tumor cells, is a rate-limiting component of CYP-dependent intratumoral CP activation. Cotransduction of RED with CYP has been found to substantially enhance intratumoral prodrug activation, causing a 50- to 100-fold increase in tumor cell kill *in vivo* over that provided by hepatic drug activation alone (Chen L, etal, Cancer Res, 1 Nov 1997;57(21):4830-7, and Waxman DJ, etal, Drug Metab Rev, May 1999;31(2):503-22). To further enhance CYP/RED/CP gene therapy, Youssef Jounaidi, PhD, and David J. Waxman, PhD, of Boston University (Boston, MA), used an antiangiogenic schedule of CP administration (Jounaidi Y and Waxman DJ, ICGTC00, Abs. P-110:45). In this approach, CP is administered on a 6 day repeating schedule. When 9L gliosarcoma cells transduced with CYP as well as RED, and grown subcutaneously in SCID mice, were challenged with CP (140 mg/kg) every 6 days, significant tumor regression was observed in both early-stage tumors (1.5%-3% of body weight) with 6/8 tumors eradicated, and large, late-stage tumors (10%-15% of body weight) with ≥95% regression in 12/16 tumors, and tumor eradication in 2 cases. Little or no overt drug-related toxicity was observed, and CP administration using the same schedule was far less effective in inducing the regression of nontransduced tumors. Although CP resistance developed in the transduced cells, this was primarily caused by loss of expression of the CYP and/or RED gene, rather than intrinsic cellular resistance to activated CP.

Horseradish peroxidase/indole-3-acetic acid (HRP/IAA) is an enzyme/prodrug system being developed by the Gray Laboratory CRT, that is capable of causing lipid peroxidation, strand breaks and adducts in supercoiled plasmid DNA, and the abrogation of colony-forming ability in cultured mammalian cells (Folkes LK, etal, Int J Radiat Oncol Biol Phys, 1 Nov 1998;42(4):917-20, and Folkes LK, etal, Biochem Pharmacol, 15 Feb 1999;57(4):375-82). Nontoxic in isolation, at neutral pH, the plant hormone IAA is oxidized by the plant enzyme HRP to a radical cation, which undergoes scission of the exocyclic carbon-carbon bond to yield a carbon-centered skatolyl radical. Under normoxic conditions, the skatolyl radical rapidly forms a peroxyl radical, which then decays to several products, including indole-3-carbinol, oxindole-3-carbinol and 3-methylene-2-oxindole (MOI). Decarboxylation of the radical cation can also take place under anoxic conditions, in which case the carbon-centered radical preferentially reacts with hydrogen donors (Candeias LP, etal, Biochemistry, 9 Jan 1996;35(1):102-8, and Candeias LP, etal, Biochemistry, 10 Jun 1997;36(23):7081-5).

Although the activated drug resulting from HRP/IAA metabolism has not yet been unequivocally identified, the effect of HRP/IAA is thought to be attributed in part to the formation of MOI, which may conjugate with the sulphydryl regions of histone DNA or RNA and react with protein thiols (Folkes LK and Wardman P, Biochem Pharmacol, 15 Jan 2001;61(2):129-36). Although endogenous peroxidases can be present in leukocytes and phagocytes as well as tumor cells, nonspecific activation of IAA in normal tissues is considered unlikely, as HRP is significantly more efficient at converting IAA into a cytotoxin. The activated drug is long-lived and apparently does not require cell-to-cell contact for a bystander effect to occur, relying instead on diffusion of the active metabolite across cell membranes (Greco O, etal, Cancer Gene Ther, Nov 2000;7(11):1414-20).

In her presentation, Ms. Olga Greco of the Gray Laboratory CRT reported that four different cell lines of human origin (T24, bladder cancer; FaDU, nasopharyngeal squamous carcinoma; MCF-7, mammary cancer; and HMEC-1, human dermal microvascular endothelium), transiently transfected with a plasmid vector containing HRP cDNA, exhibited selective sensitization to IAA (Greco O, etal, ICGTC00, Abs. O-63:S19). Significant cytotoxicity was observed under both normoxic and anoxic conditions, with up to three-log cell kill induced at nontoxic doses of IAA; cytotoxicity was increased in T4 cells with the HRP/IAA system compared to the HSVtk/GCV system. Several IAA analogs were tested in combination with HRP, and in transfected T4 cells, 1-methyl-IAA demonstrated 100-fold more effective cell kill than parental IAA under both normoxic and anoxic conditions, whereas 5-br-IAA exhibited up to three-log cell kill, but only under anoxic conditions. These results suggest that the HRP/IAA system may provide a novel approach to GDEPT, with the potential to kill hypoxic tumor cells.

Carboxylesterase/irinotecan (CE/CPT-11) is a system in which the camptothecin-derived prodrug irinotecan (CPT-11) available commercially as Campto from Aventis Pharmaceuticals, or Camptosar from Pharmacia, is converted to the active metabolite SN-38 by carboxylesterase (CE). SN-38 inhibits topoisomerase I, causes DNA damage, and induces apoptosis. In its active form, the drug has been shown to be up to 1000-fold more active than CPT-11 *in vitro*. In its usual application, CPT-11 is activated by endogenous CE, found mainly in the microsomes of hepatocytes; however, CPT-11 metabolism by human CE is relatively inefficient *in vivo*, and high doses of CPT-11 are often associated with Grade 3/4 delayed diarrhea, leukopenia, and neutropenia.

In an effort to improve the conversion of CPT-11 to its active metabolite, Mary K. Danks, PhD, and her associates at St. Jude Children's Research Hospital (Memphis, TN), have developed a CE/CPT-11 system for GDEPT based on rabbit liver CE. The rabbit and human CE exhibit approximately 86% homology, and are 100% identical in the active site amino acids, but rabbit CE is some 100- to 1000-fold more efficient than human CE at converting CPT-11 to SN-38 *in vitro*, and is 12- to 55-fold more efficient in sensitizing transfected cells to CPT-11 (Danks MK, etal, Cancer Res, 1 Jan 1998;58(1):20-2, Potter PM, etal, Cancer Res, 15 Jun 1998;58(12):2646-51, and Morton CL, etal, Cancer Res, 1 Aug 2000;60(15):4206-10).

In *in vitro* transfection studies, use of a replication-deficient adenoviral vector encoding rabbit CE cDNA under the control of an RSV promoter (Ad.RSV.CE), resulted in very high levels of intracellular CE activity in a panel of human tumor cells following transduction, causing significant sensitization to CPT-11 (Wierdl M, etal, ICGTC00, Abs. PD-73:S22). Adenovirally transduced tumor cells demonstrated reductions in IC_{50} values for CPT-11 ranging from 11- to 127-fold and, in comparison with an adenovirus expressing a secreted form of rabbit CE, a bystander effect was achieved that resulted in a 4- to 19-fold reduction in IC_{50} values. In *in vivo* studies, immune-deprived mice bearing Rh30 rhabdomyosarcoma xenografts were treated with CPT-11. As expected, xenografts transduced with rabbit CE were more sensitive to CPT-11 than were control xenografts, or xenografts expressing the human enzyme. Although each of the three types of xenografts regressed with treatment, following cessation of CPT-11 administration recurrent tumors were detected in 7/7 bearing control Rh30 xenografts as well as in 2/7 mice bearing human CE-expressing Rh30 xenografts, whereas no tumors recurred in mice bearing xenografts that had been transduced with rabbit CE (Danks MK, etal, Clin Cancer Res, Apr 1999;5(4):917-24).

In evaluating the rabbit CE/CPT-11 system for purging neuroblastoma (NB) cells from hematopoietic cells prior to autologous stem cell rescue, mixtures of 90% CD34+ cells and 10% human NB cells were first exposed *ex vivo* to Ad.RSV.CE and then treated with CPT-11. In unpurged samples, NB cells were readily detected, while no viable NB cells were detected in purged samples. Mice administered unpurged cell mixtures died from disseminated NB, whereas all mice administered purged cells exhibited no subsequent tumor development (Wagner LM, etal, ICGTC00, Abs. O-61:S18). According to Dr. Danks, St. Jude researchers have initiated a clinical protocol to establish the efficacy of adenovirus-mediated rabbit CE transfection in CPT-11 *ex vivo* purging of patient bone marrow samples for autologous stem cell transplantation.

Tumor-specific promoters, such as those regulating PSA or CEA, may confer target selectivity to enzyme/prodrug systems and have been used to achieve tumor-selective expression of transduced genes. Targeting of prodrug activating enzymes to specific tumor types can also be accomplished by using promoters that are activated by tumor-overexpressed transcription factors. Researchers at St. Jude are using this technique to enhance the tumor-selective toxicity of the rabbit CE/CPT-11 system (Pawlik CA, etal, Mol Ther, May 2000;1(5 Pt 1):457-63).

Many neuroblastomas overexpress the transcription factor n-MYC, and several other tumor types, including brain, breast, and colon carcinomas, overexpress c-MYC; this overexpression is commonly associated with poor prognosis. The ornithine decarboxylase (ODC) promoter can be used to regulate expression of rabbit CE in MYC-expressing cells. IMR32 human NB cells stably transfected with a plasmid containing an ODC promoter/CE cassette were approximately 10-fold more sensitive to CPT-11 than cells transfected with a CMV promoter/CE cassette.

To further enhance the potency of the ODC promoter in regulating tumor-selective rabbit CE transgene expression, additional MYC-responsive binding sites were placed 5'-, 3'-, or both to the ODC promoter (Krull EJ, etal, ICGTC00, Abs. O-62:S18). Although the modified ODC promoter was found to be similar to the CMV promoter in regulating the expression of the neomycin resistance gene *in situ* in n-MYC as well as c-MYC expressing tumor cell lines, stable cell lines could not be established in which the modified ODC promoter regulated CE expression because of the cytotoxicity of CE overexpression.

Methioninase/selenomethionine (METase/SeMET) is an enzyme/prodrug system being developed by AntiCancer (San Diego, CA), with collaborative research being conducted at the University of California at San Diego, and Yokohama City University, and University of Tokyo in Japan. Methionine metabolism and transmethylation are central to the growth and differentiation of all known cells, and in all tumor cell types tested, an elevated requirement for methionine compared to normal cells has been demonstrated (Hoffman RM, Hum Cell, Mar 1997;10(1):69-80). Recombinant METase has been under investigation by AntiCancer as a potential anticancer agent to selectively target the methionine dependence of tumor cells (Tan Y, etal, Anticancer Res, Sep-Oct 1997;17(5B):3857-60, and Miki K, etal, Cancer Res, 15 May 2000;60(10):2696-702).

In the GDEPT approach, however, METase gene transfer is used to convert the physiological compound SeMET to cytotoxic methylselenol (Miki K, etal, ICGTC00, Abs. O-64:S19). To achieve this goal, the L-methionine alpha-deamino-gamma-mercaptomethane-lyase (methioninase, METase) gene from *Pseudomonas putida* is cloned from *E. coli* (Tan Y, etal, Protein Expr Purif, Mar 1997;9(2):233-45), and ligated into a recombinant adenovirus vector under the control of a CMV-5 or CEA promoter (rAd-METase).

According to AntiCancer's Robert M. Hoffman, PhD, the IC_{50} of SeMET in cancer cells transduced with METase is up to 1000-fold less than in nontransduced cancer cells or those transduced with a control adenovirus. The aden-

oviral-mediated METase/SeMET system was active against various cancer cell types, including head and neck, pancreatic, ovarian, and lung cancer cell lines. A strong bystander effect was observed attributed to release of methylselenol from METase-transduced cells. SeMET treatment of cultures containing only 3% METase-transduced cells resulted in the death of over 80% of the nontransduced cells. High levels of superoxide were also produced in SeMET-treated METase-transduced cells.

Beta-glucuronidase/HMR 1826 is an enzyme/prodrug system in which the human enzyme beta-glucuronidase (BG) converts an inactivated, hydrophilic, glucuronidated derivative of doxorubicin to the lipophilic, cell-permeable, cytotoxic anthracycline form (Papot S, etal, Bioorg Med Chem Lett, 22 Sep 1998;8(18):2545-8, and Desbene S, etal, Anticancer Drug Des, Apr 1999;14(2):93-106). The BG enzyme is present at high levels in many tumors, suggesting that use of a nontoxic, glucuronide prodrug such as HMR 1826 might facilitate more selective delivery of chemotherapy to tumors, avoiding or reducing dose-limiting organ toxicities (Murdter TE, etal, Cancer Res, 15 Jun 1997;57(12):2440-5, and Sperker B, etal, Naunyn Schiedebergs Arch Pharmacol, Aug 2000;362(2):110-5).

In regards to cardiotoxicity, HMR 1826 demonstrated an excellent *in vivo* tolerability profile (Bosslet K, etal, Cancer Res, 15 Mar 1998;58(6):1195-201), being about 100-fold less cardiotoxic than doxorubicin (Platel D, etal, Br J Cancer, Sep 1999;81(1):24-7). Also, because doxorubicin is membrane-permeable, BG/HMR 1826-based GDEPT exhibits a pronounced bystander effect in nontransduced cells when a secreted or transmembrane form of the normally lysosomal human BG is used to establish an extracellular cytotoxic effector system. When tested *in vitro*, this approach was shown to be clearly superior to conventional chemotherapy with doxorubicin (Weyel D, etal, Gene Ther, Feb 2000;7(3):224-31, and Brusselbach S, etal, ASGT00, Abs. 437). HMR 1826 was originally synthesized in the laboratory of Dr. Claude Monneret at Institut Curie (Paris, France), in collaboration with Dr. K. Bosslet's laboratory at Hoechst's Behring Institute (Marburg, Germany), and ended up with Aventis after various mergers. Although the compound was slated for clinical trials in the past, at this point, it appears that Aventis is not actively pursuing development of this agent.

As an alternative approach to achieving local intensification of the bystander effect with the BG/HMR 1826 system, scientists at M. D. Anderson Cancer Center are attempting to modulate the intracellular retention of lysosomal BG following transduction, by combining adenoviral vector-mediated transfer of lysosomal BG (AdBG) with transduction of the proapoptotic bax gene (AdBax). The expectation is that bax transduction will lead to BG release from dying cells and engender a locally progressive bystander effect as prodrug is cleaved (Mohuiddin IT, etal, ICGTC00, Abs. PD-68:S20). No appreciable cellular killing was observed in an AdBG-transduced Fisher rat fibrosarcoma cell line (RFS) treated with HMR 1826; cell killing was demonstrated in cells transduced with both AdBG and AdBax, but this was significantly less than that which occurred in AdBG/AdBax-transduced cells treated with HMR 1826 (55.5% and 75.9%, respectively, at a AdBG:AdBax ratio of 1:1).

Self-Deleting Vectors

Retroviral vectors are particularly efficient mediators of gene transfer. However, their use *in vivo* has raised concerns that viral sequences, such as the RNA primer binding site for transcription, or the dimerization and encapsidation signals that are not required for expression of the transferred gene, may recombine with endogenous and/or exogenous retroviruses to produce new, unpredictable retroviral forms. These sequences are also targets for transcriptional repressors that can inhibit transduced gene expression. To avoid these problems, scientists at the University of Frankfurt Medical School (Frankfurt am Main, Germany) have developed a retroviral vector system that self-deletes from the genome of an infected cell upon integration (Russ AP, etal, J Virol, Aug 1996;70(8):4927-32, and Grez M, etal, Stem Cells 1998;16 Suppl 1:235-43).

This system exploits the duplication of terminal control regions U5 and U3 to generate long terminal repeats (LTR), as part of the natural retrovirus life cycle, and the ability of P1 phage site-specific recombinase (Cre) to excise any sequences positioned between two loxP target sequences from the mammalian genome. In a typical vector, the gene of interest, flanked by loxP target sequences, is cloned into the U3 region of the retrovirus (typically Maloney murine leukemia virus). A separate cassette expressing the Cre-recombinase gene is inserted between the LTR into the body of the virus. This places the Cre-expressing vector sequence between loxP sites in the integrated provirus, enabling Cre to excise most viral and nonviral sequences not required for gene expression from the provirus.

Taking advantage of the fact that p53 is mutationally inactivated in more than 50% of human cancers, and is functionally deficient in many other tumors because of mutations in interacting genes, Harold von Melchner, MD, of the University of Frankfurt and his colleagues, together with researchers at Asta-Medica (Frankfurt am Main, Germany), the European Molecular Biology Laboratory (Heidelberg, Germany), and MainGen Biotechnologie (Frankfurt am Main, Germany), produced a self-deleting retrovirus vector based on the Cre/loxP site-specific recombination system, for which deletion from the genome of an infected cells is dependent on transcriptional activation by p53 (Andreu T, etal, ICGTC00, Abs. O-32:S9). Self-deletion of this vector occurs only in cells expressing functional p53, whereas cells lacking functional p53 retain the virus and can be selectively killed by a cytotoxic protein encoded by a gene

expressed by the provirus. This vector system has entered preclinical testing using the gene coding for GCV.

OTHER DIRECT CYTOTOXICITY APPROACHES

Transfer of Genes Encoding Viral Fusogenic Membrane Glycoproteins

Richard G. Vile, MD, PhD, and associates at the Mayo Clinic (Rochester, MN) directly kill tumor cells through the transfer of genes encoding viral fusogenic membrane glycoproteins or FMG (Bateman A, etal, Cancer Res, 15 Mar 2000;60(6):1492-7, Diaz RM, etal, Gene Ther, Oct 2000;7(19):1656-63, and Bateman A, etal, ICGTC00, Abs. O-82:S24). FMG kill cells by fusing them into large multinucleated syncytia, which die by sequestration of cell nuclei and subsequent nuclear fusion by a mechanism that is nonapoptotic. *In vitro* transfection studies have shown gibbon ape leukemia virus (GALV) FMG, or measles virus F and H FMG, to be significantly better than either HSVtk/GCV or CD/5-FC suicide gene systems in terms of direct cytotoxicity. FMG also exhibit bystander killing on the order of one to two logs greater than these conventional enzyme/prodrug systems.

Intratumoral transduction using a GALV FMG-expressing VSV-G pseudotyped lentiviral vector is capable of eradicating small, established human tumor xenografts growing subcutaneously in nude mice. Although the direct and bystander killing effects of the FMG transgene are major contributors to the success of this approach, immunologic activation may also be a factor. FMG can act as potent antigens in their own right, and tumor-associated FMG expression in immunocompetent animals generates specific antitumor immune responses. In addition, syncytial formation is accompanied by the induction of several immunostimulatory genes, including heat-shock proteins, which contribute to the recruitment, loading and activation of antigen-presenting cells. To augment these effects, vectors were constructed coexpressing genes for FMG as well as immunostimulatory molecules such as GM-CSF. To lend tumor specificity to the fusion event, transcriptional control systems have been developed using either tissue-specific promoters, Cre/loxP excision, and/or inducible promoters that allow for tight regulation of FMG expression in target cells.

Tumor-specific Activation of dsRNA-dependent PKR

Scientists at Hebrew University (Jerusalem, Israel) are attempting to use tumor-specific activation of double stranded RNA (dsRNA)-dependent protein kinase R (PKR) to directly kill tumor cells (Shir A and Levitzki A, ICGTC00, Abs. P-102:30). dsRNA-dependent PKR is an IFN-induced, growth inhibitory protein located at 2p21-22, that inhibits translation initiation through the phosphorylation of the a subunit of the initiation factor eIF-2 (eIF-2α) and also controls activation of several transcription factors such as NF-kappaB or p53 (Basu S, etal, Cancer Res, 1 Mar 1997;57(5):943-7, Jagus R, etal, Int J Biochem Cell Biol, Jan 1999;31(1):123-38, and Gil J, etal, Apoptosis, Apr 2000;5(2):107-14).

Because many tumor cells express mutated genes containing deletions or chromosomal translocations producing unique sequences, the researchers suggest that its should be possible to use antisense RNA, complementary to fragments flanking the deletion or translocation, to produce a dsRNA molecule of sufficient length to activate PKR. In a test of this hypothesis, expression of a 39-nucleotide long antisense RNA complementary to the unique exon 1 to 8 junction of the U87MGDEGFR cell line, which expresses a truncated form of EGFr, was found to cause selective death of cells harboring the truncated EGFr, and to have no effect on cells expressing wild type EGFr.

Attenuated, Replication-competent Adenoviruses

At Calydon (Sunnyvale, CA), the oncolytic properties of attenuated, replication-competent adenoviruses are being exploited in gene therapy for prostate cancer. In CN706, a minimal promoter enhancer construct (PSE), located 5' to the PSA gene, is used to drive expression of the E1A gene of E3-deleted Ad5 adenovirus, resulting in the regulated replication of CN706 in cells that express PSA (Henderson DR, ICGTC00, Abs. P-29:35). Intratumoral injection of CN706 has demonstrated complete eradication of pre-existing human prostate cancer xenografts in preclinical animal studies (Rodriguez R, etal, Cancer Res, 1 Jul 1997;157(13):2559-63).

A phase I/II dose-escalation study, conducted in collaboration with researchers at the Brady Urological Institute of the Johns Hopkins Hospital Oncology Center (Baltimore, MD), closed to patient enrollment in April 2000 and was completed in October 2000. In this trial, CN706 was administered intraprostatically by a transrectal ultrasound-guided transperineal brachytherapy technique at doses ranging from 10^{11}-10^{13} viral particles, to 20 men with biopsy-proven prostate cancer, which recurred following radiation therapy. Treatment was well tolerated; drug-related adverse events included transient Grade 2 fever, responsive to antipyretics, and transient Grade 1 liver transaminase elevation. Of 11 patients in the top two dose groups, 9 (80%) demonstrated greater than 35% decreases in PSA from baseline, and 5 exhibited PR lasting at least 4 weeks; in 3 of these patients, PR persisted for at least 9 months. In the highest dose group, 3 of 5 (60%) men exhibited a PR. PSA doubling time was prolonged in 18 of 20 patients, particularly in the upper two dose groups (9 of 11 patients not yet showing biochemical progression). Viral replication was confirmed by electron microscope analysis of post-treatment biopsy samples, and 60% of day-22 biopsies demonstrated a significant reduction in PSA staining. According to Calydon's Daniel R. Henderson, PhD, CN706 is under consideration for phase II clinical trials in the treatment of newly diagnosed prostate cancer.

Calydon is developing a second generation version of CN706, CV787, currently in multicenter, open-label phase

I/II clinical trials for locally recurrent (intratumoral administration) as well as end-stage metastatic (IV administration) prostate cancer (Yu D-C, etal, ICGTC00, Abs. O-29:S9). CV787 contains the entire Ad5 E3 region, and incorporates a prostate-specific rat probasin promoter driving the E1A gene, and the PSE promoter driving the E1B gene. CV787 replicates in PSA-positive cells as well as PSA-negative cells, but in the latter, its cytolytic efficiency is some 10^4-fold lower. When administered IV into the tail vein of nu/nu mice carrying subcutaneous LNCaP human prostate cancer xenografts, a single injection of CV787 eliminated tumors sized at 300 mm^3 within 4 weeks (Yu D-C, etal, Cancer Res, 1 Sep 1999;59(17):4200-3).

POTENTIATION OF CONVENTIONAL THERAPY

Gene-based therapies for cancer also include strategies for potentiating the anticancer response to more established therapeutic approaches, through gene-mediated increase in both the sensitivity of tumors to chemotherapy or radiation therapy and the protection of normal tissues.

Chemotherapy Sensitization

Based on preclinical studies that have shown p53 gene replacement to result in synergistic cancer cell kill when combined with DNA damaging agents, such as cisplatin or doxorubicin (Dell J, etal, AACR98, Abs. 3774:555, and Gurnani M, etal, Cancer Chemother Pharmacol 1999; 44(2):143-51), SCH58500, under development by Canji, has been combined with chemotherapy for the treatment of ovarian cancer and nsclc. In a phase II clinical trial, heavily pretreated patients with recurrent ovarian cancer were treated with either single-dose (SD) or multidose (MD) regimens of SCH58500 combined with platinum-based chemotherapy (Buller RE, etal, ICGTC00, Abs. O-3:S1). Twelve patients were treated with a single intraperitoneal (IP) injection of SCH58500 at doses ranging from 7.5 x 10^{10} to 7.5 x 10^{12} viral particles, followed by administration of carboplatin, while 19 patients were treated with multidose IP treatment with SCH58500 at 7.5 x 10^{12} to 7.5 x 10^{13} particles for 5 days per cycle; carboplatin was added to the 2nd and 3rd cycles. Comparisons between SD and MD subjects revealed no significant differences in treatment response on the basis of age or interval from primary diagnosis to first SCH58500 administration. However, while SD patients had been treated with more prior cycles of chemotherapy, all died of their disease, whereas only 10 MD patients had died at the time of this presentation. MST among SD subjects was 5.0 months, compared to 12.0 months for the MD group. Phase II/III studies of SCH58500 combined with platinum-based chemotherapy, and/or paclitaxel, have been initiated in patients with newly diagnosed Stage III epithelial ovarian cancer.

Previous phase I studies have suggested the benefit of p53 gene replacement in nsclc (Kauczor HU, etal, Eur Radiol 1999;9(2):292-6). To evaluate the potential enhanced efficacy of p53 gene therapy in patients undergoing first-line chemotherapy for advanced nsclc, 25 patients with Stage IIIb or Stage IV nsclc were enrolled in an open-label, multicenter phase II clinical trial, and treated with either carboplatin plus paclitaxel (regimen A) or cisplatin plus vinorelbine (regimen B), in combination with the bronchoscopic or CT-guided percutaneous intratumoral injection of 7.5 x 10^{12} particles of SCH58500 (Schuler M, etal, ICGTC00, *ibid*). Although transgene expression was confirmed in tumor samples from 68% of the patients, and subgroup analysis revealed increased mean local tumor regressions in patients enrolled in treatment regimen B, no significant difference was observed in the objective response rate of lesions treated with chemotherapy alone (44%), or with SCH58500 and chemotherapy (48%). There was also no survival difference between the two chemotherapy regimens, with MST of the cohort being 10.5 months.

Oncolytic adenoviruses selectively targeting p53-deficient cancer cells have also been considered as a neoadjuvant approch to chemotherapy. In preclinical studies, the cytolytic effect of Onyx's ONYX-015 has been shown to be increased by a factor of 10 when combined with a low-dose cisplatin and paclitaxel regimen (You L, etal, ASCO99, Abs.1768:458a). Clinical evidence for the potential synergy of ONYX-015 with chemotherapy has been obtained from studies in head and neck as well as colorectal cancer (Kirn D, *ibid*). There were substantial objective responses in a phase II trial, conducted at M.D. Anderson Cancer Center, of intratumorally injected ONYX-015 combined with systemically administered cisplatin and 5-FU in patients with recurrent squamous cell cancer of the head and neck (Kirn DH, etal, ASCO99, Abs. 1505:389a, and Khuri FR, etal, *ibid*).

Among 26 evaluable patients, there were 6 CR (22%), 10 PR (40%), and disease stabilized in 8 (30%), and progressed in 2 (8%). The overall response rate (ORR) of 62% and CR rate of 22% compare favorably with past multicenter studies using similar doses and regimens of cisplatin and 5-FU. At 6 months, none of the responding tumors had progressed, whereas all noninjected tumors treated with chemotherapy alone had progressed. Toxicities were acceptable, and similar to those expected with chemotherapy alone. In June 2000 Onyx, in collaboration with Pfizer, initiated a randomized, multicenter phase III clinical trial of ONYX-015 in the treatment of recurrent head and neck cancer to compare intratumoral injection of ONYX-015 combined with cisplatin and 5-FU against standard chemotherapy alone, in approximately 300 patients at about 40 centers in the USA and Europe.

Tony Reid, MD, PhD, of Stanford University (Palo Alto, CA), reported results from a multicenter phase I/II trial, in which patients with liver-predominant metastases from malignancies of the gastrointestinal tract (primarily colorectal carcinoma) are being treated with 5-FU and leucovorin (LCV) in combination with hepatic artery infusion of ONYX-015. No DLT has been observed, although dose-related Grade 1/3 fevers and rigors have occurred in most patients. Among 13 evaluable patients, objective evidence

of intrahepatic activity has been obtained at ONYX-015 doses of ≥3 x 10^{10} pfu, with 2 PR, and 1 MR; disease stabilized in 3 patients and progressed in none. Five of the six responding patients had previously failed one or more chemotherapy regimens, including 5-FU/LCV; two patients demonstrated extrahepatic tumor progression while maintaining intrahepatic tumor regression (Reid T, etal, ASCO00, Abs. 953:246a).

In an ongoing phase I clinical trial patients with refractory cancer containing a p53 gene mutation are being treated with ONYX-015 in combination with carboplatin and paclitaxel (Nemunaitis J, etal, *ibid*). In this trial, ONYX-015 is administered once a week by IV push in 3-week cycles, with viral doses escalating from 2 x 10^{10} to 2 x 10^{13} particles; carboplatin and paclitaxel are added after 2 cycles or 1 week. In 42 cycles (126 infusions), Grade 2 fever and chills occurred in all patients within 8 hours following each dose in cycle 1 or cycle 2; all patients administered 2 x 10^{12} or more viral particles developed transient transaminitis. Three of 4 evaluable patients treated with ≥2 x 10^{12} particles exhibited rising viral genome concentrations at 48 hours compared to 6 hours after the first ONYX-015 infusion, suggesting ongoing viral replication. Among 10 treated patients, there was one MR, while disease stabilized in 6 and progressed in 3.

Another oncolytic adenovirus variant that has been evaluated for potential synergistic activity when combined with standard chemotherapy is Calydon's CV787 (Yu D-C, etal, ICGTC00, *ibid*). *In vitro*, the addition of paclitaxel and/or docetaxel to CV787 resulted in greater than additive cytotoxicity toward the human prostate cancer cell line LNCaP, regardless of the timing of administration. *In vivo*, a single IV dose of 10^{8} CV787 particles, combined with docetaxel, eliminated large, pre-existing PSA+ LNCaP xenografts in murine models, without evidence of a synergistic increase in toxicity.

Enhanced expression of the human multidrug resistance protein MRP2, an ATPase-transporter encoded by the cMOAT gene, has been associated with resistance of tumor cells to platinum-containing chemotherapeutics, such as cisplatin (Kavallaris M, etal, AACR96, Abs. 2130:313, and Kartenbeck J, etal, AACR98, Abs. 1148:168). At Humboldt University (Berlin, Germany), scientists have designed hammerhead ribozymes having high catalytic cleavage activity toward the GUC sites in codon 704 and codon 708 of the open reading frame of cMOAT-specific mRNA. These ribozymes, that efficiently cleave their substrates at physiological pH and temperature, were used to construct CMV promoter-driven ribozyme expression vectors (Lage H, etal, ICGTC00, Abs. PD-14:S5). When the cisplatin-resistant human ovarian carcinoma cell line A2780RCIS was transfected using these constructs, the anti-cMOAT ribozyme-transfected clones demonstrated a 35% to 50% reduction in the level of cisplatin resistance. The ribozyme-expressing clones also exhibited increased sensitivity to carboplatin, daunoblastin, and etoposide, but not to vinca alkaloids.

As noted earlier, caspase 3 is a key mediator of apoptotic pathways. Cells lacking in caspase 3 exhibit resistance to drug-induced DNA fragmentation. Researchers at Humboldt University and University of Muenster in Germany, constructed a retroviral vector containing the cDNA for caspase 3 (C3-LSXN), which was used to impart sensitivity to drug-induced apoptosis in cancer cells with innate and acquired drug resistance (Daniel PT, etal, ICGTC00, Abs. PD-22:S6).

The breast carcinoma cell line MCF-7, which is devoid of caspase 3, is insensitive to epirubicin, etoposide, and paclitaxel, but when transduced with C3-LSXN, these same drugs led to the breakdown of nuclear DNA. The breast cancer cell line variant MT1/ADR, which expresses only low levels of caspase 3, also exhibits defective apoptosis induction upon drug exposure, but here again, MT1/ADR clones stably transfected with caspase 3, after retroviral transduction, demonstrate heightened sensitivity to these cytotoxic drugs, with DNA fragmentation nearly reaching the levels induced in the original drug-sensitive MT1 cell line. Interestingly, this effect was not expected, as the drug resistance of MT1/ADR cells is attributed to a defect at the level of the mitochondrial apoptosome. Since activation of the mitochondrial apoptosome is the dominant signaling pathway for drug-induced apoptosis, and caspase 3 activation is presumed to occur downstream of caspase 9 activation, caspase 3 transfection should, in theory have no impact on drug resistance. However, the results of this study suggest that the vector-mediated induction of caspase 3 overexpression can bypass this pathway, and that caspase 3 activation may occur independently of mitochondrial signaling defects that may be caused by loss of Bax or overexpression of Bcl-2.

In earlier research conducted by Transgene (Strasbourg, France) using replication-defective adenovirus vectors incorporating a CMV promoter to deliver IFN-γ cDNA *in situ* for antitumor gene therapy, sustained expression of IFN-γ within the tumor was observed when the expression vector (Ad-pCMV-IFNγ) was injected into established B16F0 tumors growing in syngenic mice (Slos P, etal, *ibid*). However, while IFN-γ expression resulted in a significant increase in survival compared to control groups, complete tumor rejection was not achieved. To enhance antitumor activity, intratumoral injections of Ad-pCMV-IFNγ were combined with systemic administration of cisplatin, resulting in a greater decrease in tumor volume than obtained with either agent alone. Survival also increased in mice treated with the combination compared to mice treated with Ad-pCMV-IFNγ alone. However, tumor regression, observed in 10% to 40% of the mice treated with Ad-pCMV-IFNγ and cisplatin, was still transient, with tumors reappearing 2 to 3 weeks after cessation of therapy. Transgene scientists are continuing to study the combination of Ad-pCMV-IFNγ and cisplatin as well as Ad-pCMV-IFNγ combined with 5-FU, in the treatment of renal cell carcinoma and colon cancer models.

The E2F family of transcription factors has been implicated in the regulation of genes whose products are involved in cell proliferation, particularly those involved in progression through G1 and into the S-phase of the mammalian cell cycle. An important role for E2F in tumorigenesis is suggested by the finding that in most human neoplasias, genetic or epigenetic alterations occur that ultimately result in the deregulation of E2F-dependent transcription (Johnson DG and Schneider-Broussard R, Front Biosci, 27 Apr 1998;3:d447-8, and Watanabe G, etal, Mol Cell Biol, Jun 1998;18(6):3212-22). Although E2F-1, the first member of the E2F family identified, can stimulate cellular proliferation, it also has the properties of a tumor suppressor. Overexpression of E2F-1 in some cancer cell lines has been shown to induce premature S-phase entry and G2 arrest, followed by apoptosis (Rabbani F, etal, JNCI, 19 May 1999;91(10):874-9, Dong YB, etal, Cancer, 15 Nov 1999;86(10):2021-33, and Amanullah A, etal, Blood, 15 Jul 2000;96(2):475-82), coinciding with the ability of E2F-1 to induce accumulation of p53 protein (Kowalik TF, etal, Cell Growth Differ, Feb 1998;9(2):113-8).

Under normal proliferative conditions, the p53 tumor suppressor gene is regulated by the murine mdm2 protein through a negative feedback mechanism; mdm2 protein inhibits p53 function by binding to its transcriptional transactivation domain, and by promoting the ubiquitination and proteasome-dependent degradation of p53, possibly by acting as a ubiquitin ligase (Lu W, etal, Oncogene, 13 Jan 2000;19(2):232-40, and Zhang R and Wang H, Curr Pharm Des, Mar 2000;6(4):393-416). A domain of E2F-1 (amino acids 390 to 406) exhibits striking similarity to the mdm2 binding domain of p53, and it is the interaction of mdm2 with p53 through this domain that is required for mdm2-dependent degradation of p53 (Blattner C, etal, Mol Cell Biol, May 1999;19(5):3704-13). It has been shown that E2F-1 can bind to and coprecipitate with mdm2, inhibiting mdm2-mediated p53 degradation, and resulting in increased p53 protein expression (Itoshima T, etal, Clin Cancer Res, Jul 2000;6(7):2851-9). Disruption of the mdm2-p53 complex in cells that overexpress mdm2 is sufficient to trigger p53-mediated cell death (Wasylyk C, etal, Oncogene, 18 Mar 1999;18(11):1921-34).

Scientists at the James Graham Brown Cancer Center of the University of Louisville (Louisville, KY) have constructed an Ad5 adenovirus vector carrying the E2F-1 gene under the control of a CMV promoter (Ad5CMV-E2F-1). Adenovirus-mediated transfer of the E2F-1 gene into mdm2-overexpressing tumor cell lines has been observed to result in significant growth inhibition and rapid loss of cell viability (Yang HL, etal, Clin Cancer Res, Aug 1999;5(8):2242-50, and Dong YB, etal, Cancer, *ibid*). Because E2F-1 overexpression has been associated with sensitivity to S phase-specific topoisomerase II poisons (Nip J, etal, Mol Cell Biol, Mar 1997;17(3):1049-56, and Hofland K, etal, Clin Cancer Res, Apr 2000;6(4):1488-97), researchers have investigated the effect of combined treatment with adenovirus-mediated E2F-1 gene transfer and topoisomerase II inhibitors on the growth of human melanoma (Dong YB, etal, ICGTC00, Abs. P-98:26) and osteosarcoma (Yang HL, etal, ICGTC00, Abs. P-103:31) cells.

Infection with Ad5CMV-E2F-1 alone produced less than 3% apoptosis in the human melanoma cell lines SK-MEL-28 and SK-MEL-2, whereas combined treatment with Ad5CMV-E2F-1 and low-dose etoposide or doxorubicin significantly sensitized cells to apoptosis. No enhancement of drug sensitivity was observed with cisplatin or 5-FU, and the protein synthesis inhibitor cycloheximide demonstrated a cytotoxicity-protective effect against E2F-1/topoisomerase II inhibitor-induced apoptosis, suggesting that new protein synthesis is required for this process. A slight enhancement of sensitivity to roscovitine, a cyclin-dependent kinase (CDK) inhibitor, was observed in combination with E2F-1 overexpression. The CDK family of protein kinases regulate cell cycle progression in proliferating eukaryotic cells. Inhibition of the activity of these proteins has been associated with disruption of S phase progression and subsequent apoptosis in certain cancer cell lines (Atienza C Jr, etal, Int J Mol Med, Jul 2000;6(1):55-63).

Significantly increased sensitivity to topoisomerase II inhibitors was also observed in human p53-positive sarcoma cells (OsACL and U2OS) transduced with Ad5CMV-E2F-1; the cooperative effect of E2F-1 and topoisomerase II inhibitors was less significant in p53-null SAOS-2 cells. *In vivo*, adenovirus-mediated E2F-1 overexpression enhanced considerably tumor cell killing by etoposide or doxorubicin in nude mice bearing human sarcoma OsACL or U2OS xenografts, achieving decreases in tumor size of 85% (etoposide) and 95% (doxorubicin) relative to controls.

Radiation Therapy Sensitization

According to Dr. Roth of M.D. Anderson Cancer Center, Introgen's RPR/INGN 201 is being evaluated as a radiosensitizer in the treatment of nsclc, based on previous animal studies that demonstrated that adenovirus-mediated transfer of the p53 gene induces radiation sensitization in previously radiation-resistant tumors (Roth JA, etal, Semin Radiat Oncol, Oct 2000;10(4):333-42, and Colletier PJ, etal, Int J Radiat Oncol Biol Phys, 1 Dec 2000;48(5):1507-12). A small phase II clinical study of INGN 201 in combination with radiation therapy (RT) for the treatment of patients with localized nsclc who were not candidates for surgery or chemoradiation, had been initiated at UTMDACC under the direction of Stephen Swisher, MD (Swisher S, etal, ASCO00, Abs. 1807:461a, and Roth JA, etal, ICGTC00, *ibid*). Seventeen patients have been entered in this trial and treated with 3 intratumoral injections of RPR/INGN 201 on days 1, 18 and 32, in combination with a 5-week course of RT (60 Gy). RPR/INGN 201 doses were escalated from 3×10^{11} to 3×10^{12} viral particles and injected directly into the primary tumor by bronchoscopy or computed tomographic (CT) guidance. The

most common side effects have been arrhythmia and nausea; no Grade 3 or 4 toxicities have been observed. Of the 17 patients entered into the trial, 10 demonstrated ≥50% reduction in tumor size, and 12 patients have shown no active cancer cells at the treated site, as confirmed by biopsies obtained three months after completion of treatment, compared to a local tumor control rate of <20% in patients treated with RT alone. Five patients were still alive at 5 to over 22 months post-treatment.

Researchers at the Baylor College of Medicine and the Harvard Gene Therapy Initiative initiated a phase I/II clinical study of HSVtk/VCV gene therapy in combination with RT for the treatment of patients with locally recurrent and stage D1 prostate cancer following prior definitive RT. High-risk and stage D1 patients also underwent neoadjuvant androgen ablation (Chikara M, etal, ICGTC00, Abs. O-58:S17). This approach is based on preclinical studies demonstrating prostate tumor growth suppression and prolongation of survival in animals treated with HSVtk/GCV gene therapy (Shalev M, etal, World J Urol, Apr 2000;18(2):125-9), and on results from phase I clinical trials demonstrating minimal toxicity and anticancer activity (assessed by decreases in serum PSA levels) in patients with recurrent prostate cancer treated with intraprostatic injections of a replication-deficient adenovirus containing the HSVtk gene (AdV-HSVtk), followed by either IV GCV or oral valaciclovir (VCV) as enzymatic substrates (Herman JR, etal, Hum Gene Ther, 1 May 1999;10(7):1239-49, and Shalev M, etal, J Urol, Jun 2000;163(6):1747-50).

According to Estuardo Aguilar-Cordova, PhD, of the Harvard Gene Therapy Initiative, 36 patients out of an anticipated total enrollment of 150 have been treated with 4 ultrasound-guided, intraprostatic injections of AdV-HSVtk followed by oral administration of VCV. Toxicities have included transient Grade 3 ALT (n=1) and transient Grade 2 fever, fatigue, and thrombocytopenia (n=8). Although there appears to be a trend to decreased serum PSA levels with this treatment regimen, long-term data is not yet available. One goal of HSVtk/VCV gene therapy is to reduce the external RT load and minimize patient exposure to RT.

Scientists at Gray Laboratory CRT are developing radiation-responsive vectors for cancer gene therapy. These vectors use specific transcriptional control (CArG) elements within the radiation-inducible early growth response 1 (Egr1) gene promoter to drive expression of exogenous therapeutic genes (Marples S, etal, Gene Ther, Mar 2000;7(6):511-7). As reported by Simon D. Scott, PhD, clinically relevant doses of ionizing radiation (1-3 Gy) increased expression of a green fluorescent protein (GFP) reporter gene by 2- to 5-fold over vector expression without radiation activation of CArG elements. When used to drive the HSVtk/GCV suicide gene system, an approximately 10% enhancement in tumor cell killing was observed following a single 2 Gy radiation dose (Scott SD, etal, ICGTC00, Abs. O-66:S20).

In order to provide long-term, constitutive gene expression and more enhanced tumor cell killing, even after the withdrawal of the radiation stimulus, scientists designed a dual-vector, radiation-triggered molecular switching scheme based on promoter elements from the Egr-1 gene and the P1 bacteriophage site-specific Cre/loxP recombination system (Scott SD, etal, Gene Ther, Jul 2000;7(13):1121-5). Using this system, a single, minimally toxic dose of radiation induces Cre-mediated excision of a loxP-flanked stop cassette in a silenced expression vector, resulting in a 40-fold enhancement in GFP production. When applied to the HSVtk/GCV system, a 30% increase in tumor cell killing was observed at 2 Gy. Also, further enhancement in cell killing efficiency was achieved using a single vector system.

Based on studies in animal models showing that low doses of TNF-α can augment the lethal effects of radiation against certain tumor types, including human colon tumor xenografts (Gridley DS, etal, Anticancer Res, May-Jun 1994;14(3A):1107-12), human lung adenocarcinoma xenografts (Gridley DS, etal, Oncol Res 1996;8(12):485-95), and glioma xenografts (Gridley DS, etal, Oncol Res 1997;9(5):217-27), Daila S. Gridley, PhD, and her colleagues at Loma Linda University (Loma Linda, CA) have constructed a plasmid-based human TNF-α expression vector, pGL1-TNFα, complexed with a cationic polyamine for the treatment of glioblastomas in combination with RT (Li J, etal, Oncol Res 1998;10(7):379-87, Baher AG, etal, Anticancer Res, Jul-Aug 1999;19(4B):2917-24, Gridley DS, etal, Anticancer Res, Nov-Dec 2000;20(6B):4195-203, and Gridley DS, ICGTC00, Abs. O-80:S24).

The pGL1 locus is involved in hereditary head and neck paragangliomas (Baysal BE, etal, Hum Genet, Mar 199;104(3):219-25), and is used to impart tumor selectivity. In preclinical studies, athymic nude mice were implanted subcutaneously with C6 glioma cells. Subsequently, established tumors were injected with pGL1-TNFα (at doses of 15 μgm, 150 μgm, and 450 μgm), and the mice were irradiated 16-18 hours later, with each modality administered three times over 8-9 days. Expression of TNF-α was transient and dose-dependent, with the highest levels detected at 18-hours post-injection. The highest levels of NK cells were observed after administration of the 15 μgm and 150 μgm doses of pGL1-TNFα.

Although the administration of pGL1-TNFα alone did not slow tumor progression, and radiation alone demonstrated only a modest effect on tumor growth, when combined with RT, pGL1-TNFα significantly enhanced the antitumor effect of both gamma and proton radiation. Administration of pGL1-TNFα together with proton radiation resulted in tumor volumes that were 23% smaller than those following pGL1-TNFα and gamma-ray treatment; pGL1-TNFα combined with proton radiation reduced mean tumor volumes by 51% and 43% compared to controls at pGL1-TNFα doses of 15 μgm and 150 μgm, respectively. Body weights as well as blood and spleen cell analy-

ses did not reveal treatment-related toxicity, although high basal proliferation of blood leukocytes and increased B cell levels in the spleen were associated with combined pGL1-TNFα and RT (gamma and proton).

In other work combining cytokine gene transfer with RT, scientists at Japan's Cancer Institute (Tokyo, Japan) and the National Research Institute for Radiotherapy and Radiohygiene (Budapest, Hungary) have studied the adjuvant effect of autologous, cytokine-producing cancer cell vaccines and local RT in experimental murine gliomas (Safrany G, etal, ICGTC00, Abs. P-114:52). In this work, cells extracted from brain tumors established by intracranial transplantation of murine G1261 glioma cells, were transduced *ex vivo* with adenoviral vectors encoding various murine cytokines, including IL-2, IL-4, IL-12, or GM-CSF, and then inactivated by irradiation before autologous transplantation. Administration of the transduced tumor cells induced activation of G1261-specific cytotoxic T lymphocytes and, depending on the cytokine level produced by the cells, resulted in CRs in 20% to 40% of the glioma-bearing mice. However, when combined with local RT, survival rates increased significantly, with CR achieved in 70% to 100% of tumor-bearing mice.

Active transport of iodide across the basal membrane and into the thyroid and other tissues, such as salivary glands, gastric mucosa, and lactating mammary gland, is mediated by human sodium iodide symporter, hNIS, an intrinsic transmembrane protein (De La Vieja A, etal, Physiol Rev, Jul 2000;80(3):1083-105). At the University of Ulsan researchers have constructed a recombinant adenoviral vector incorporating the cDNA for the hNIS gene (Ad-hNIS) as a mechanism for enhancing exogenous hNIS-mediated radioiodide concentrating activity in the thyroid and other tissues for effective radioiodide treatment of patients with thyroid and nonthyroid cancers (Chang H, etal, ICGTC00, Abs. P-94:S28). This approach may be particularly useful for metastasized thyroid cancer, in which loss of iodide uptake because of diminished expression of hNIS is frequently observed (Smit JW, etal, Thyroid, Nov 2000;10(11):939-43). Infection rates approaching 100% have been achieved with this vector in thyroid and breast cancer cells *in vitro*, and vector-mediated hNIS overexpression in these cells has elicited over 5-fold increases in the I^{125} uptake, suggesting the potential utility of this method in improving the efficiency of targeted radioiodide therapy.

Combined Chemo/Radiotherapy Sensitization

Ionizing radiation and certain chemotherapeutics cause double-stranded DNA breaks, which if unrepaired, can be lethal to cancer cells. Pangene (Mountain View, CA) has targeted the regulation of DNA repair mechanisms as a means of increasing the antitumor efficiency of DNA-damaging chemotherapeutics and RT, thereby promoting better long-term survival while reducing the side effects that stem from high dose RT or drug treatment. One regulatory target under consideration is human Rad51 recombinase (Bates A, etal, ICGTC00, Abs. P-24:S7), a highly conserved eukaryotic homolog of the prokaryotic *E. coli* replication protein RecA, a heterotrimeric single-stranded DNA binding protein that has been shown to stimulate homologous pairing and DNA strand exchange (Li MJ, etal, PNAS USA, 17 Sep 1996;93(19):10222-7, Gupta RC, etal, PNAS USA, 21 Jan 1997;94(2):463-8, and Golub EI, etal, Nucleic Acids Res, 1 Dec 1998;26(23):5388-93).

Rad51 protein, first identified and characterized by researchers at Yale University (New Haven, CT), catalyzes the recombinational repair of DNA damage (Haaf T, etal, J Cell Biol, 11 Jan 1999;144(1):11-20). Rad51 binds several major human tumor suppressor proteins, including p53, which appear to regulate the function of Rad51 protein, possibly by maintaining it in an inactive state (Thompson LH and Schild D, Biochimie, Jan-Feb 1999;81(1-2):87-105, Amaudeau C, etal, J Mol Biol, 25 Jun 1999;289(5):1231-8, Dasika GK, etal, Oncogene, 20 Dec 1999;18(55):7883-99, and Susse S, etal, Oncogene, 14 Sep 2000;19(39):4500-12). Working in collaboration with the Yale University School of Medicine, Pangene has found that Rad51 apparently binds p53 at multiple contact sites. DNA strand exchange assays are being used to identify peptides or other small molecules that bind to the p53 contact site(s) on Rad51 and regulate Rad51 activity.

Protection of Critical Host Tissues

Transfer of the multidrug resistance gene, MDR-1, into normal hematopoietic stem cells has been suggested as an approach to alleviate dose-limiting leukocytopenia arising from post-transplantation chemotherapy, and may serve as a selectable marker for the transfer of other genes into the bone marrow, restoring gene expression *in vivo* (Licht T, etal, Gene Ther, Feb 2000;7(4):348-58, and Hafkemeyer P, etal, Hum Gene Ther, 1 Mar 2000;11(4):555-65). However, clinical trials have so far failed to demonstrate the applicability of this approach, probably because of the low transduction efficiencies achieved in hematopoietic stem and progenitor cells with the retroviral vectors being used, resulting in insufficient expression of MDR-1 cDNA in the target cells (Schilz AJ, etal, ASCO00, Abs. 1881:479a).

To facilitate MDR-1 transduction as a chemoprotection/selectable marker strategy, scientists at the Hebrew University Medical School and National Institutes of Health (NIH; Bethesda, MD) have cloned the human MDR-1 gene into an SV40 pseudoviral vector containing the SV40 origin of replication (ori) and encapsidation signal (ses), to produce an SV40/MDR-1 pseudovirion (Rund D, etal, Hum Gene Ther, 20 Mar 1998;9(5):649-57, and Kimchi-Sarfaty C, etal, ICGTC00, Abs. PD-49:S14). Expression of the 170 kDa P-glycoprotein (Pgp) MDR1 gene product on the surface of transduced cells was demonstrated in murine MEL cells and human HeLa cells infected with SV40/MDR-1, using MRK16, a monoclonal antibody (MAb) specific for Pgp. Functional Pgp activity was demonstrated by rhodamine-123 dye exclusion assay and fluorescence-activat-

ed cell sorter (FACS) analysis. Highly efficient gene transfer and expression was observed in all murine and human cell types tested, including primary human bone marrow cells, with over 95% of cells becoming MDR-1+ when multiplicities of infection were used. In addition, MDR-1 transduction using the pseudovirion vector was transient, with no significant levels of gene expression detectable 20 days after infection, making this approach ideal as a bone marrow chemoprotection strategy.

CELL MARKING STUDIES

The transfer of selectable gene markers, such as , the neomycin resistance (NeoR) gene obtained from *E. coli*, or the gene for green fluorescent protein (GFP), enable researchers to identify transduced cells even though they may represent only a small fraction of the total number of cells present. Gene-based cell marking is more effective than conventional radioisotope labeling in determining the distribution and survival of cells in the circulation as well as in establishing clinical correlations between cell subpopulations localized to a particular target site.

In an application of gene marking for determining transgene delivery efficiency, investigators at AntiCancer and the Kitasato University School of Medicine (Sagamihara, Japan) have used GFP gene transduction to assess genetic modification of hair follicles (Saito N, etal, ICGTC00, Abs. PD-48:S14). In their approach, mouse anagen skin fragments in histoculture are treated with collagenase to make them accessible to adenovirus-mediated GFP gene transfer. Transduced fragments are transplanted onto nude mice, and the visualization of GFP in the hair follicles allows the efficiency of the alteration of the growing hair shaft to be assessed. GFP was visualized in as many as 75% of hair follicles, including large numbers of growing hair shafts.

In clinical studies being conducted at Baylor College of Medicine, the NeoR gene is inserted *ex vivo* into EBV-specific cytotoxic T lymphocytes (CTL) to serve as a cell marker in the administration of autologous CTL as immunotherapy in patients with relapsed EBV-positive Hodgkin's disease (Roskrow MA, etal, Hum Gene Ther, 20 May 1998;9(8):1237-50, and Aguilar LK, etal, ICGTC00, Abs. O-55:S16). Gene marking revealed localization to tumor sites as well as persistence of transferred CTL in the peripheral blood of patients for up to 9 months following transfusion. In one patient with erosion of tumor through the left bronchus, who died 2 months after CTL infusion, gene-marked CTL were found within part of the tumor but not at the site of bronchial erosion. Gene-marked CTL were found localized to a malignant pleural effusion in one patient 3 weeks after lymphocyte infusion.

Typically, implantation of human bladder cancer cells into murine bladders by transurethral installation to study the biology and effects of intravesical gene therapy, has not been very efficient, or has had limited reproducibility. At Hokkaido University and M. D. Anderson Cancer Center, scientists have developed a modified intravesical technique that has allowed human bladder cancer cell lines to be efficiently and reproducibly implanted and grown initially as superficial bladder tumors in athymic mice (Watanabe T, etal, Cancer Gene Ther, Dec 2000;7(12):1575-1580).

To allow for better evaluation of the efficacy of gene transfer and to optimize treatment schedules for gene therapy regimens researchers from M. D. Anderson Cancer Center and AntiCancer have developed human bladder cancer cell clones transduced with the marker gene for GFP (Rosser CJ, etal, ICGTC00, Abs. PD-105:37). Use of transduced cells has allowed both primary bladder tumors and metastatic lesions to be visualized and quantitated *in situ* in live (using an intravesical probe) as well as sacrificed mice. Information derived from preclinical studies of GFP-expressing human bladder cancer xenografts in murine models will be used to develop a phase I clinical trial of gene therapy in patients with superficial bladder cancer.

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