Specialty Conference

New Advances in Surgical Oncology

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Remarkable advances have occurred in surgical oncology, including the development of doxorubicin hydrochloride (Adriamycin) for treating sarcoma, hyperthermia for treating deep-seated tumors by localized magnetic-loop induction and an in vitro assay for cloning human tumor cells. When preoperative administration of doxorubicin, given intraarterially, is followed by irradiation and en bloc resection, it is now possible to preserve the extremities of many patients who have skeletal or soft tissue sarcoma previously treated by amputation. For patients who have advanced cancer in visceral organs, deeply penetrating hyperthermia alone or in combination with irradiation or chemotherapy may destroy active tumor and extend survival. At present, the potential effectiveness of various chemotherapeutic drugs can be tested on clones of a patient's tumor stem cells in vitro, a procedure that may allow us to select the most active drug or combination of drugs for an individual patient.

DONALD L. MORTON, MD:* *We have chosen the following three studies for discussion: extremity preservation, hyperthermia and the clonogenic assay.*

The concept of nonamputative surgical treatment for primary malignant tumors of bone and soft tissues is certainly not new. Initial attempts to resect malignant tumors arising in these sites were largely abandoned in the early 1900s because of the high local recurrence rate.[1] Amputation was then adopted as the only procedure that offered a chance for local disease control.[2]

Early results from radiotherapy trials were also disappointing; these tumors thus gained the reputation of being "radioresistant."[3,4] Interest was rekindled, however, when Suit and associates[5] and Lindberg and colleagues[6] used Fletcher's concepts to show that sarcomas were not radioresistant but were too large and bulky to be treated by irradiation alone. Irradiation could be effective if residual microscopic disease could be eliminated.[6,7]

The discovery of doxorubicin hydrochloride (Adriamycin) in 1973 further modified treatment for both skeletal and soft tissue sarcoma. This antitumor antibiotic was a highly effective chemotherapeutic agent that could induce an overall objective response rate of more than 50% in patients with metastatic disease.[8] Subsequently doxorubicin was administered as an adjuvant after definitive surgical amputation for malignant skeletal and soft tissue sarcoma to eliminate microscopic residual disease in the chest. Several studies indicated that doxorubicin as a single agent, or in combination with other drugs, was highly effective against micrometastatic disease.[9-17]

Hyperthermia is our second subject for discussion. The use of heat in cancer treatment dates back to the ancients, with application of red-hot irons by Ramajama (200 BC), Hippocrates (400 BC) and Galen (200 AD). In 1898 Westermark[18] placed hot-water-circulating cisterns into patients who had advanced carcinoma

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of the uterus and found palliative shedding of some tumors. In 1927 Coley introduced "toxin" therapy for cancer, but stated that responses were associated with temperatures of 39°C to 40°C for several days, suggesting that the febrile reaction might have been the tumoricidal agent.[19] Simultaneously, Keating-Hart and Doyen introduced electrocoagulation of tumors, which is still in use today. Warren in 1932 was one of the first to apply heat from infrared and high-frequency currents to tumors and found remissions of some malignant lesions.[20] With the subsequent development and popularity of x-irradiation therapy, hyperthermia research was all but abandoned until modern times, when the selective thermosensitivity of tumor cells was more fully appreciated.

Last, we shall discuss the clonogenic assay and its current and future uses. Cancer chemotherapy has always been an empiric science. Drugs for an individual patient have been selected on the basis of past statistical response of a particular tumor type. The development of a test predictive of tumor sensitivity to anticancer agents analogous to the bacterial culture for sensitivity has been, understandably, a high priority of cancer research.[21] Early assays, however, did not show a consistent correlation between in vitro activity and clinical response of individual patients. In theory, these early approaches were limited by their failure to identify a critical subpopulation of stem or clonogenic cells.[22] It is now assumed that this unique population of cells has the capability of sustained replication that represents the biologic behavior of a tumor. Based on this principle, Hamburger and Salmon[23] pioneered an in vitro assay in which human tumor stem cells are cloned in agar. This clonogenic assay technology is increasingly used by oncologists as an aid for selecting chemotherapy, by biologists for studying biologic properties of tumor cells and by pharmacologists for evaluating the efficacy of newly developed antineoplastic agents. Recent modifications in technique suggest enhanced growth capacity of tumors and improved predictability of clinical response.

Limb Preservation Procedures for Skeletal and Soft Tissue Sarcomas

FREDERICK R. EILBER, MD:* In 1975 we began a treatment series based on the principles described.[24,25] The protocol called for doxorubicin to be given before radiation therapy and surgical excision. The sequence of therapeutic intervention was based on the premise that treatment of micrometastatic lesions at the periphery of a tumor, given preoperatively (when the blood supply was intact), would enable us to do a local surgical procedure.

From August 1975 until August 1981, a consecutive series of 83 patients with malignant skeletal sarcoma and 100 patients with soft tissue sarcoma was treated. All patients received identical preoperative treatment with intraarterially administered doxorubicin.[26] After percutaneous placement of an intraarterial catheter by the Seldinger technique, catheter location was confirmed by arteriogram. The catheter tip was placed in a high-flow vessel (the common femoral, common iliac or axillary artery). Cutaneous distribution of fluorescein dye was used to monitor proper placement under a Woods lamp daily to confirm the distribution of the continuous drug infusion. Doxorubicin at 30 mg per day was infused continuously through this line during a 24-hour period for each of three consecutive days (for a total of 90 mg). The catheter was then removed and the patients received radiation therapy the following day. A total dose of 3,500 rads was delivered through posteroanterior and anteroposterior parallel opposed ports at a dose fraction of 350 rads per day for at least ten treatments. The entire extremity was treated except for a strip of skin opposite the biopsy site. One to two weeks after preoperative therapy, patients had en bloc resection of all gross tumor. Tumor-free margins were confirmed during the operative procedure by frozen sections.

In patients with skeletal tumors, the affected bone was removed 10 cm proximal to disease as shown by radiographic scan and computed tomography. No major nerves or blood vessels were sacrificed. Operations were done through uninvolved tissue planes. Enneking and co-workers[27] defined these procedures as wide local resections.

Postoperative adjuvant chemotherapy of doxorubicin, 45 mg per sq m delivered for a period of two consecutive days, then, two weeks later, high-dose methotrexate of 200 mg per kg of body weight, was administered to patients with osteosarcoma. This cycle was repeated until a total dose of 450 mg per sq m of doxorubicin was reached. Methotrexate administration was continued once a month for a total treatment time of a year.[25]

Of 75 patients with grade III soft tissue tumors, 35 were treated with chemotherapy (doxorubicin and high-dose methotrexate) and 40 received no adjuvant chemotherapy.

All types of soft tissue sarcoma were classified and staged by the clinical pathologic staging system described by the American Joint Committee on Staging and End Results.[28] One patient had clinical pathologic stage I tumor, 20 had clinical pathologic stage II, 75 had clinical pathologic stage III and four patients were classified as having clinical pathologic stage IV. Clinically, 78 patients had primary disease because they received no previous treatment, 18 had locally recurrent disease after earlier surgical procedures and four patients presented with both intact primary and metastatic disease. The histologic types of primary tumor represented in these patients included 57 with osteosarcoma, 11 with chondrosarcoma, 2 with Ewing's sarcoma, 6 with giant cell tumor and 7 with miscellaneous types of sarcoma that included malignant fibrous histiocytoma

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(3), synovial cell sarcoma (1), leiomyosarcoma (1) and metastatic hypernephroma (1).

After excision of primary skeletal tumors, diseased bone was replaced with a freeze-dried cadaver allograft (National Naval Medical Center, Bethesda, Md) in 23 patients; with a metallic endoprosthesis in 41 patients, and with an intramedullary rod and sliding bone graft in four patients.[29] Of the 15 patients who had no bony replacement, 5 had tumor in the pubis, fibula and radius, and 10 patients had resection of all or a portion of the ilium.[30] Patients were followed at monthly intervals by routine physical examination and roentgenogram of the chest and at three-month intervals by whole lung tomograms.

In this consecutive series of 183 patients, 2 of 83 patients with skeletal sarcoma and 3 of 100 patients with soft tissue sarcoma had disease recurrences (2.7%). Median follow-up time for these patients is 32 months.

Complications of Preoperative Therapy

In 2 of the 183 patients who had preoperative infusions of doxorubicin, arterial thrombosis developed for which they subsequently needed an operative procedure. In one patient, embolectomy (removal of the thrombus) was done and the patient had no further difficulty. In the other patient, complications from the thrombus required amputation of the extremity. No other direct complications related to the infusion of doxorubicin or radiation therapy occurred.

In 13 of the 100 patients with soft tissue tumors, wound slough developed postoperatively, but none required operative intervention. In five patients fracture of adjacent long bones developed and four had lymphedema; the complications that occurred in the rest are as listed in Table 1. All of the fractures of adjacent bone (from which periosteum had been removed at the time of the operative procedure) were in the thigh of patients over 45 years of age. All fractures healed after intramedullary rod fixation. Examination of biopsy specimens at the time of operation confirmed the absence of tumor in the fracture site.

Complications from treatment of skeletal sarcoma largely revolve around the type of bony replacement. The complication rate from allografts was extremely high. Of the original 23 patients who had allografts placed, 6 patients (who had no allograft complications) died 3 to 12 months after their operation for metastatic disease. Of the remaining patients at risk for more than a year, only four have had no complication with the allograft. In all, 13 patients had a serious allograft complication (such as fracture, loosening or resorption and infection) that required additional surgical repair. Four of these patients, or 17%, required amputation, six were treated by allograft excision and replacement with metallic endoprosthesis and in three their allografts were revised. Therefore, of the 23 patients with allografts, 57% required subsequent operative revision. In 41 patients an endoprosthesis was placed and 3 subsequently required operative revision of their prosthesis.

TABLE 1.—*Complications of Treated Patients*

Complications	*Number of Patients N=24*
Wound slough	13
Fracture of adjacent bone*	5
Lymphedema	4
Fibrosis	1
Pyarthrosis	1
Femoral artery thrombosis	1
Pulmonary embolus	1

*Fractures: 4 in femur, 1 in tibia=5 of 53 at risk (9%).

TABLE 2.—*Tumor Size in Clinical-Pathologic Stage III Patients Related to Survival*

Tumor Size	*Patients*				
	Studied Number	*Alive Number*	*Percent*	*Follow-up Period Months (range)*	*median*
0-5 cm	18	16	89	(10-54)	33
5-10 cm	34	23	67	(3-84)	31
10-15 cm	23	15	65	(3-72)	25
TOTAL	75	54	72		

Seven of the remaining patients died in less than a year; 29, or 70%, of the total group have been observed for more than 14 months and have not required further surgical intervention.

The overall disease-free survival rate for patients with malignant skeletal sarcoma was compared with a series of patients seen at University of California, Los Angeles (UCLA), who were treated by amputation and the identical adjuvant treatment. There was no statistical difference between the overall survival for patients treated by amputation or by limb salvage. Furthermore, in 2 of the 24 patients who had amputation, local recurrences developed; thus the local recurrence rate was the same for patients treated by amputation or the limb preservation protocol.

The overall survival rate by clinical pathologic stage for patients who had soft tissue sarcoma was 68%. No age group seemed to be at higher risk; this finding, however, may be a statistical phenomenon because patients 10 to 20 years of age have only a 15% survival rate. In Table 2 the size of tumor and overall survival for stage III patients are compared and the size was directly related to overall survival rate.

The purpose of this study was to determine whether or not preoperative therapy with intraarterially given doxorubicin followed by irradiation would be of value for patients with malignant skeletal and soft tissue sarcoma and whether or not these modalities would reduce the incidence of local recurrence after nonamputative operation. It is not yet possible to determine which of the pretreatment methods, intraarterial administration of doxorubicin, radiation therapy or the surgical procedure itself, is responsible for the low recurrence rate or, conversely, the high percentage of primary tumor control in patients who have malignant skeletal and soft tissue sarcoma. The procedure is feasi-

ble, the complication rate is low and the overall ability to save a functional extremity is very high.

Like many studies, this one raises more questions than answers. Is administration of doxorubicin intra-arterially preferable to the intravenous route? As for irradiation, it is not clear whether rapid fractionation has any advantage over standard fractionation and whether the total dose of 3,500 rads is adequate or is excessive. To answer some of these questions, we have started a prospective trial to compare an additional series of patients who have malignant skeletal and soft tissue sarcoma treated by an identical protocol, except for a reduced radiation dose of one half, or 1,750 rads.

Although the exact reason for the improved local control rate is not known because of the multidisciplinary aspect of this trial, it is unlikely that the improvement is related to more adequate surgical procedures. We believe that this local disease control is the result of the multidisciplinary adjuvant therapy that destroys microscopic disease at the periphery of the tumor.

Hyperthermia for Cancer Therapy

F. KRISTIAN STORM, MD:* At temperatures between 41°C and 45°C (106°F to 113°F) cancer cells may be slightly more sensitive to heat than their normal-cell counterparts. In vitro and in vivo tumor models have shown irreversible damage and complete regression of various tumors, whereas normal cells were killed at temperatures at least one degree higher, or more than twice the duration of heating.[31-34]

Heat causes progressive necrosis in tumor cells, but not in stromal or vascular cells within tumors or in normal surrounding tissues.[35] Autolytic disintegration of heat-damaged cells is followed by a pronounced increase in connective tissue stroma and scar formation.[36] This process occurs in tissue cultures of tumor-derived and tumor-producing cells but not in normal and non-tumor-producing cells. When a cell subline derived from a non-tumor-producing line acquires high tumor-producing capacity, it also acquires greater thermosensitivity. Thus, malignant potential, both in vivo and in vitro, is accompanied by decreased thermotolerance.[37,38]

Mechanism of Thermal Kill

Hyperthermia alters DNA and RNA synthesis and depresses cellular enzymatic systems required for cell metabolism and division. Its major mode of action may be to increase plasma and lysosome membrane permeability, causing internal destruction of the cancer cell (Figure 1).

Selective Tumor Heating

Most studies so far have dealt with moderate hyperthermia of 42°C to 43°C alone or combined with x-ir-radiation or chemotherapy, based on the evidence of selective thermal sensitivity of tumor cells. Lethal temperature-exposure time relations have been established for many cell lines. Several investigators, however, have found that at temperatures approaching 45°C a linear kill takes place due to progressive and irreversible protein denaturation. At such high temperatures the differential susceptibility between malignant and normal cells decreases, and host tolerance becomes the prime consideration.[31,34,37] Therapeutic hyperthermia in this higher temperature range was not thought to be feasible until it was realized that, because of abnormal vascularity and poor blood flow, some solid tumors might act as a heat reservoir. When Shibata and

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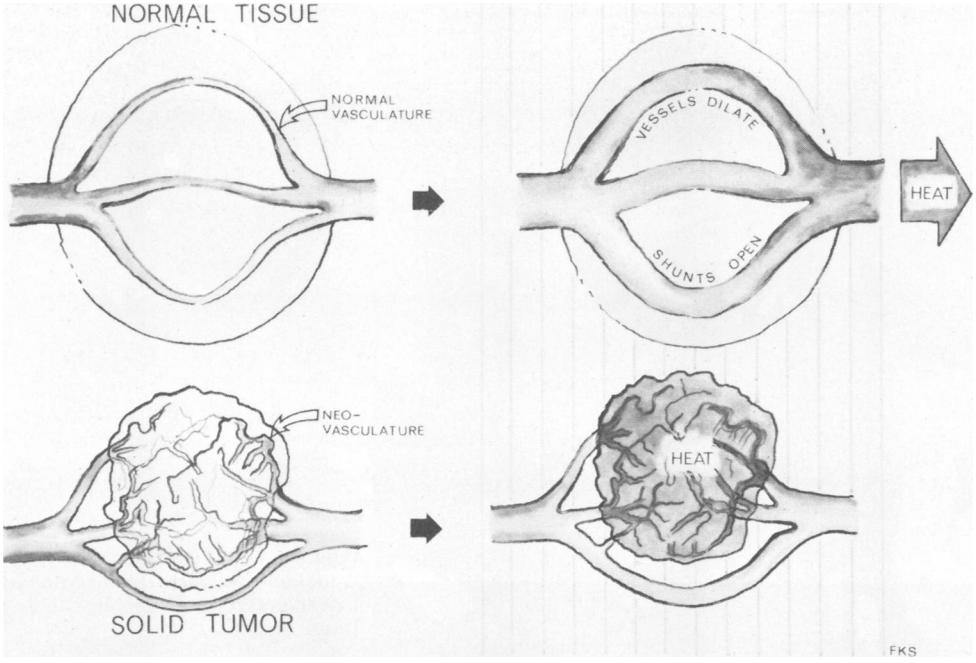

Figure 1.—Postulated mechanism of selective solid tumor heating by radio frequency. Normal tissue blood flow is augmented by heat, not present in solid tumors. Tumors act as relative heat reservoir. (From Storm et al[42]; reprinted by permission of *Cancer Research.*)

MacLean[39] evaluated cancers in humans, they found that the blood supply was poorer in all tumors studied.

Our evaluation of thermal tolerance on animal skin, extremities and viscera supported the safety of temperatures of less than 45°C. When normal animal muscle temperature reaches 43°C to 44°C, spontaneous cooling maintains the tissue well below its thermal tolerance limit. This phenomenon, also observed by others, implies that normal tissue does adapt to hyperthermia in a way that suggests augmented blood flow. When external radio-frequency hyperthermia was applied to normal canine viscera, no selective heating of any normal organ occurred.

Clinical Trials

Isolated limb perfusion. In 1967 Cavaliere and colleagues[34] did regional limb perfusions using prewarmed blood at 42.5°C to 43.5°C in 22 patients with large, recurrent or single metastatic lesions in the extremities. All evidence of gross tumor disappeared in ten patients, five had regressions, three failed to respond and four could not be evaluated. Even though the complication rate was high (six deaths and three immediate amputations), the treatment caused massive tumor necrosis.

Total body hyperthermia. In 1974 Pettigrew and associates[40] reported the cases of 38 patients with terminal cancer treated by total body hyperthermia (immersion in molten wax) at 41.8°C (107.2°F) for an average of four hours. An objective response, weight gain or relief from pain, as well as measured tumor regression or histologic evidence of necrosis, was seen in 18 of 38 patients, though four patients died of disseminated intravascular coagulation.

Larkin and co-workers[41] in 1976 reported their experience with total body hyperthermia applied by a water-circulating suit, maintaining 19 patients at 41.5°C to 42°C for from two to five hours, with an objective tumor response noted in 70%. Complications included one death, transient cardiac arrhythmias in 15%, superficial burns in 15% and transient respiratory distress in 11%, which was attributed to the seven to eight hours of anesthesia time required to raise and maintain body temperatures in these critically ill patients.

Localized hyperthermia. Electromagnetic waves are the most practical means of producing localized hyperthermia. All frequencies heat tissues in a similar way. Energy is transferred into tissue by field interaction; this causes ions in the tissue to oscillate or produce changes in the magnetic orientation of molecules that are locally converted into heat. Because the energy of a shortwave or microwave quantum is only about 10^{-5} eV, it cannot produce ionization or excitation. The biologic effects of these waves are primarily the result of heat production. But the absorption and penetration capabilities of electromagnetic waves depend on tissue composition and interfaces (that is, skin, muscle, fat, bone). Moreover, their depth of penetration is often limited. Incident energy absorption is a function of tissue resistance, so that surface tissues (skin, subcutaneous tissue, body wall musculature) preferentially absorb heat in amounts 10 to 150 times greater than internal organs. Therefore, if superficial tissues must be penetrated to heat deeper tissue, a high and potentially dangerous degree of surface energy deposition would be needed to produce deep heat effectively. In the past, satisfactory heating has been limited to depths of 2 to 3 cm with commercially available apparatus. To overcome this limitation, several investigators designed special equipment to function in the range of 915-MHz and 2,450-MHz microwave bands. Even with surface cooling, however, documented temperatures of 42°C to 44°C have been possible only at 2- to 3-cm

Figure 2.—Circular magnetic-loop induction applicator (20-inch transthoracic and transabdominal electrode shown).

depth, with the thermal gradient continuously decreasing as depth increases.

In 1977 Storm and associates[42,43] introduced magnetic-loop induction hyperthermia for safe thermal treatment of visceral organs (Figure 2). This fundamentally new approach provided potentially therapeutic levels of hyperthermia while sparing surface tissues. With this device (Magnetrode, Henry Medical Electronics, Los Angeles), investigations of the efficacy of localized internal hyperthermia in humans have provided virtually all the available information for response in deep-seated cancers.[44]

Clinical Investigations

Tumor-heating capacity. Of 89 tumors evaluated in skin, subcutaneous tissue or muscle, intra-abdominal viscera, intrathoracic viscera or bone, temperatures at 42°C or more were possible in 69 (78%) tumors, 45°C or more in 32 (36%) and 50°C or more in 22 (25%), while normal tissues remained within a physiologic temperature range of less than 45°C.[43] Of the 89 tumors, 53 were greater than 5 cm in *least* dimension and 36 were at or less than 5 cm. Effective heating at 42°C or higher occurred more frequently in larger tumors (89%) than in smaller tumors (61%).[43]

These reports suggest that potentially effective hyperthermia can be achieved in most superficial and visceral solid human tumors regardless of histopathologic type, though it is most effective for large tumors. Some tumors cannot be safely heated at 42°C or higher and seem to retain their ability to regulate blood flow and dissipate heat. The cooler normal tissue-tumor interface observed in most evaluable cases also suggests that the blood flow at the tumor periphery may have some bearing on the ability to heat tumors effectively.

Histopathologic and gross morphologic effects of heat. In 1971 in Denmark, Overgaard and co-workers[36] treated mouse mammary carcinoma at temperatures of 41.5°C to 43.5°C with localized radio-frequency hyperthermia and found distinct histologic changes in tumor cells but not in stromal or vascular cells within the tumor, or in adjacent normal tissues. They found rapid autolytic disintegration of heat-damaged tumor cells, followed by a pronounced increase in connective stroma associated with progressive scar formation.

In our experience,[44] superficial tumors generally sloughed off, whereas most visceral tumors did not change significantly in size after a transient increase in size during treatment. Serial biopsy specimens showed early coagulation necrosis, edema and vascular thrombosis, then slow replacement of fibrous tissue during many weeks. The vascular thrombosis occurring during hyperthermia slows or prevents the usual mechanisms of resorption. Therefore, a biopsy of the tumor, or careful assessment of tumor-doubling time—that is, stabilization of a previously progressive tumor—is necessary for a determination of the effects of high-temperature on internal tumor therapy. This finding indicates that tumor stabilization may be an important criterion of response to thermal therapy.

Dose response to thermal therapy. To evaluate the thermal death (cell death caused by heat) times of human cancer, we compared the net amount of tumor necrosis in 44 cases of advanced malignancy after electrode-induced hyperthermia at various temperatures and fractionated exposure times.[45] We found that one treatment at 50°C or higher for 17 to 45 minutes resulted in 20% to 100% tumor necrosis, whereas lower temperatures had no apparent effect. Two or three weekly treatments at 45°C to 50°C for 30 to 72 minutes total treatment time produced 70% to 100% necrosis, whereas 40°C to 45°C produced nearly equivalent necrosis but required more than twice the time. Five weekly and ten daily treatments for a total of 135 to 600 minutes produced some tumor necrosis at 40°C to 45°C; for similar amounts of treatment time, however, temperatures above 45°C were the most tumoricidal. Total tumor necrosis by the criterion used (that is, total absence of cell nuclei) was rarely possible even at high temperatures. Although only minimal follow-up was possible in these patients with far advanced cancer. these findings suggested that human tumors might be less responsive to thermal therapy than predicted for by models or induced tumors in animals.

The results of these studies to evaluate hyperthermia as a single agent suggested that higher temperatures, longer treatments and multiple treatments were the most effective. Optimal dose to time regimens and treatment fractionation schedules remain to be determined.

Thermoradiotherapy. Hyperthermia has been combined with radiation therapy in the hope of producing a synergistic and augmented response. Hypoxic cells are more radioresistant than aerobic cells.[46] Gerweck and co-workers[47] have concluded that hypoxic cells may be at least as sensitive to hyperthermia as aerobic cells. Ben-Hur and associates[48] have suggested that the primary effect of hyperthermia is to inhibit cellular recovery from sublethal radiation damage.

When tumor cells were exposed to hyperthermia followed by 600-rad irradiation, the result was a three-log increase in cells killed as compared with their survival at 37°C to 43°C. Clinical doses for local and regional treatment with the combined treatment may lie in the range of 200 to 600 rad per fraction. Recent investigations by Overgaard[49] indicate that hyperthermia is best used three to four hours after irradiation, at 48- to 72-hour intervals.

Thermochemotherapy. The combination of hyperthermia and chemotherapy also has been investigated, because heat is thought to alter the permeability of tumor cell membranes and enhance uptake of chemotherapeutic agents.

In 1970 Giovanella and colleagues[50] found a four-log kill in leukemia cells at 42°C in 3 hours. With the addition of dihydroxybutylaldehyde, a 100-fold kill enhancement was observed with no increase in toxicity. DL-glyceraldehyde, melphalan, sodium oxyamate and actinomycin D (dactinomycin) were also active in com-

bination with heat. In vitro data also suggest benefits from hyperthermia combined with doxorubicin.[51]

In 1977 Goss and Parsons[52] reported on the survival of four human fibroblast strains and seven melanoma cell lines after administering various concentrations of melphalan only and in combination with heat at 42°C for four hours. They found that combined treatment was not only synergistic but increased the differential between fibroblast and melanoma lines.

When Stehlin and associates[53] treated locally recurrent and in-transit melanoma of the extremities using hyperthermic limb perfusion, they found an increased response from 35% to 80% by adding heat (42°C) to melphalan perfusion.

Although few investigations are being done with humans, our recent studies[54] of combined thermochemotherapy for metastatic melanoma in liver are encouraging. Moreover, combined therapy has shown potential benefit and safety of application in animal brain tumors.[55] Clinical trials for refractory human brain malignant lesions are under way at UCLA.

Surgical adjuvant therapy. At temperatures over 45°C, tumors display extensive vascular thrombosis.[42] Thrombosis may not be associated with the number or size of the vessels per se, but rather with the inability of these vessels to augment blood flow in the presence of heat.[43] In selected patients (for example, those who have locally advanced sarcoma) we[56,57] administered high-temperature hyperthermia preoperatively; in some instances, the subsequent resection was facilitated by the avascular nature of the tumor.

Clinical Use of the Clonogenic Assay

DAVID H. KERN, PhD:* The double-layer agar system, called the "human tumor stem cell assay" by its developers, Hamburger and Salmon,[23] was first applied to mutiple myeloma and ovarian carcinoma. Subsequently, a variety of tumor types have been cultured in the clonogenic assay. Using modifications of this original process, the UCLA Division of Surgical Oncology has processed more than 1,000 tumors, of which 62% produced at least 30 colonies in soft agar culture. The major tumor types from which colonies were successfully grown included the following: cancer of the breast, 100 of 140 processed (71%); colon, 102 of 170 (60%); stomach, 29 of 52 (56%); lung, 65 of 85 (76%); ovary, 43 of 57 (75%); soft tissue and skeletal sarcoma, 72 of 120 (60%), and melanoma, 122 of 152 (80%).

To assess the ability of the clonogenic assay to predict clinical responses of patients with solid tumors, Mann and colleagues[58] instituted a prospective, correlative trial. Tumor specimens were obtained from patients with primary and metastatic tumors. Tumor cell suspensions were tested with intravenous formulations of standard chemotherapeutic agents that included doxorubicin, bleomycin sulfate, carmustine, dacarbazine, fluorouracil, methotrexate, mitomycin, melphalan, cisplatin and vinblastine sulfate. Because the alkylating agent cyclophosphamide (Cytoxan) is inactive in vitro, melphalan was used as the standard alkylating agent for in vitro testing; its effects were assumed to be similar to those of cyclophosphamide. Each drug was tested at a dose comparable with the highest concentration pharmacologically achievable in patient serum (Table 3).

TABLE 3.—*Clonogenic Assay: Drugs Tested at Standard Chemotherapeutic Formulations*

Drug	Concentration μg/ml
Doxorubicin hydrochloride	0.4
Bleomycin sulfate	2.0
Carmustine	2.0
Dacarbazine	10.0
Fluorouracil	10.0
Methotrexate	4.0
Mitomycin	3.0
Melphalan	1.0
Cisplatin	2.0
Vinblastine sulfate	5.0

Colony growth was assessed three times per week during the course of the investigation using an inverted microscope at × 100 and × 200. Small clusters of cells could be observed within the first week, and colonies (more than 30 cells) were usually apparent in 10 to 14 days. A maximum number of colonies per culture well was reached within three to four weeks, at which time their numbers were recorded and the means and standard deviations for each of the triplicate counts were calculated. Assays were not considered to be evaluable for determination of drug effect unless an average of at least 30 tumor colonies for control plates was observed. To verify the neoplastic validity of the clones, slides of the entire upper agar layer were prepared and stained with hematoxylin-eosin stain and compared with the original histologic findings in the tumor. Special stains, such as periodic acid-Schiff base and silver stains for melanin, were used when appropriate. Mean and standard deviations were calculated for controls and for each individual drug exposure. Antitumor activity of each drug was calculated as the percentage of inhibited colony growth of cells exposed to the drug relative to the number of colonies in the control dishes. In vitro sensitivity to a chemotherapeutic agent was defined as greater than 75% tumor colony inhibition (at the concentration shown in Table 3); less than 75% inhibition of tumor colonies was defined as in vitro resistance.

Responses of patients treated with chemotherapy were classified according to universally accepted clinical criteria. Complete response was defined as disappearance of all clinically apparent disease for longer than a month. Partial response was defined as at least a 50% reduction in the size of all measurable disease for at least a month. Patients whose disease became stable, but who had no objective evidence of tumor regression, were considered nonresponders. Patients received chemotherapy based on the best clinical judgment, independent of the assay result. In vitro assay

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TABLE 4.—*Correlations Between Chemosensitivity as Determined by the Clonogenic Assay and Observed Clinical Responses**

	In Vitro/In Vivo Sensitivity (total correlations = 84)			
	Group 1 (S/S)	*Group 2 (S/R)*	*Group 3 (R/S)*	*Group 4 (R/R)*
Number of patients	21	4	5	54

S = sensitive, R = resistant

*Patients were treated at the UCLA Division of Surgical Oncology and nearby community hospitals.

results were not used to select chemotherapy, but results were later given to the physicians. Responses to chemotherapy were assessed by a patient's oncologist and were later evaluated by independent reviewers. The results of the chemosensitivity testing were compared with the patients' clinical responses to the drug in a blind fashion.

Of 400 patients evaluated to date, 84 had objectively measurable disease and received at least one course of chemotherapy. The correlations between in vitro sensitivity and clinical response are summarized in Table 4.

The clonogenic assay predicted sensitivity for 25 tumors of varying histology; 21 of these 25 patients had evidence of clinical response. For 59 tumors resistant to the drug tested, 54 of these patients showed no response to chemotherapy. Thus, the clonogenic assay was 84% accurate for prediction of sensitivity (21 of 25), and 92% accurate for prediction of resistance (54 of 59). Similar results were obtained by other investigators for selected tumor types.[59,60]

Although good correlations have been found between in vitro chemosensitivity results and clinical responses, several major problems need to be resolved—that is, technical problems and interpretation of results. Technical problems are principally in the areas of tissue disaggregation, drug delivery, culture conditions and colony counting. Scirrhous primary carcinoma of the breast or colon is particularly difficult to dissociate, as is fibrous sarcoma. Although mechanical methods are adequate for most tumor types,[23,59,60] enzymatic dissociation techniques may provide greater cell yield and higher tumor cell viability.[58] Concentrations of anticancer drugs used in the assay vary among laboratories, as do times of exposure of tumor cells to drugs. Nevertheless, comparable results have been obtained from several laboratories, indicating that careful analyses of in vitro data may provide realistic criteria for defining in vitro sensitivity.[58,60]

The commonest tumor types can be cloned by the soft agar technique. It is reasonable, however, to assume that different tumors may have different growth requirements. Culture media and media supplements, such as animal sera, hormones and antibiotics, may need to be specifically tailored for each tumor type. Quantitation of colonies at the completion of the assay is often awkward, time-consuming and somewhat subjective when microscopic counting is used. At UCLA more than 500 assays have been counted using an Omnicon FAS II (Feature Analysis System, Bausch & Lomb, Inc, Rochester, NY). Agreement of machine counts with visual observations has been satisfactory, and the reproducibility of machine counts is excellent.

Even if all technical difficulties of the clonogenic assay are adequately dealt with, the interpretation of results is not a simple task. Perhaps now is the time to ask the question, "How can cancer patients benefit from the clonogenic assay?" Two groups of patients who will not benefit from in vitro chemosensitivity testing are those whose tumors fail to grow in soft agar culture and those whose tumors are most sensitive in vitro to first-line drugs that may be an oncologist's initial choice. There is a third group of patients whose tumors are resistant to all drugs tested. These patients, however, should benefit by being spared the needless side effects of ineffective drugs. Growth rates of tumors in the assay may be increased by improvements in tissue processing and culture conditions. The second group of patients is likely to respond to standard chemotherapy. Moreover, the potential usefulness of the clonogenic assay seems to be limitless.

The clinical response rates of most tumors to chemotherapy show that many tumors are not sensitive to standard or first-line drugs. The clonogenic assay may help identify those patients whose tumors are sensitive to drugs not normally used for a particular tumor type. The assay may be of particular benefit to those patients who had initial responses to drug therapy, but whose disease later recurred or progressed. The clonogenic assay may assist oncologists to select alternative antineoplastic agents for patients who do not respond with their initial courses of therapy. A major application of the clonogenic assay will likely be the rapid screening of new anticancer drugs, so that active drugs can move more rapidly into clinical trials. The availability of the clonogenic assay may influence surgical management of patients.[61] Finally, the selection of patients for treatment with new forms of therapy, such as hyperthermia or thermochemotherapy, may be aided by clonogenic assay results.[62]

The clonogenic assay seems to be applicable to a variety of tumor types. Accurate predictions of chemosensitivity have been obtained for patients with multiple myeloma, sarcoma, melanoma and cancer of breast, ovary, lung, stomach and colon. This assay may have great value for selecting the most efficacious therapy for each patient who has cancer and for alleviating otherwise needless toxic reactions from drugs destined to be ineffective. Large randomized prospective studies will be necessary to determine the overall impact of this predictive test on the treatment of solid tumors. Based on initial encouraging results, these studies are clearly warranted.

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