Cell and Environment Interactions in Tumor Microregions: The Multicell Spheroid Model

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Abnormal vascularization of malignant tumors is associated with the development of microregions of heterogeneous cells and environments. Experimental models such as multicell spheroids and a variety of new techniques are being used to determine the characteristics of these microregions and to study the interactions of the cells and microenvironments. The special cellular microecology of tumors influences responsiveness to therapeutic agents and has implications for future directions in cancer research.

BY THE TIME A TUMOR HAS GROWN TO A DETECTABLE SIZE the cancer cells and their local environments have often become heterogeneous. Subpopulations of cells may have developed with a variety of growth and functional properties as well as diverse responses to therapeutic modalities. Malignant progression, traditionally considered to be due to genetic changes and the instability of the transformed phenotype, may also be influenced by the abnormal microenvironments that can develop even at very early stages of tumor growth.

Many microenvironmental changes occur as a result of inefficient vascular function within a tumor. Small (<1 mm) tumor nodules as well as microregions of larger tumors can be considered as microecological niches in which there may be major gradients of critical metabolites such as oxygen (O_2), glucose, lactate, and H^+ ions, and probably of other nutrients, hormones, and growth factors (Fig. 1) (*1*). Because of the selective pressure of the heterogeneous environments and the instability of the malignant genome, new and diverse cell phenotypes emerge that show altered responses to therapeutic agents. However, it may be possible to exploit these abnormal environments in order to enhance sensitivity to certain therapies or to develop new diagnostic and therapeutic strategies. In this article, I discuss the use of the multicell spheroid model in the study of tumor microregions and the application of the results to tumors in vivo.

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Vascular Pathophysiology

Angiogenesis generally occurs in tumors that have reached 1 to 2 mm in diameter (Fig. 2, A and B), and details of the process and its regulation are gradually being elucidated (*2*). The endothelial cells of the blood vessels degrade basement membrane (extracellular matrix), migrate, proliferate, and produce new basement membrane. The endothelial cells in tumors have a much higher mitotic activity than endothelial cells in normal tissues (*2*). Several agents that stimulate endothelial cell migration and proliferation in vitro and in vivo have been identified (*2*). Morphometric analyses of xenografted human melanomas implanted at the same site indicate that angiogenic stimulation by tumors of similar histopathology can differ substantially (*3*). Furthermore, the same tumor cell line implanted in different tissues shows a variety of oxygenation states, indicating a significant influence of the tissue environment on the efficiency of the vascular supply that develops (*4*). There is evidence that macrophages found in tumors also stimulate angiogenesis (*2*).

Deficiency of vascularization may be evident as areas of necrosis in metastatic tumors of 0.1 to 1.0 cm in well-vascularized tissues such as lung (Fig. 2, B and C). Vascular density varies in both human and xenograft tumors (*5*). Such abnormal patterns of tumor blood vessel growth and variable blood flow can account for deficiencies in oxygenation and nutrient supply in some tumor microregions.

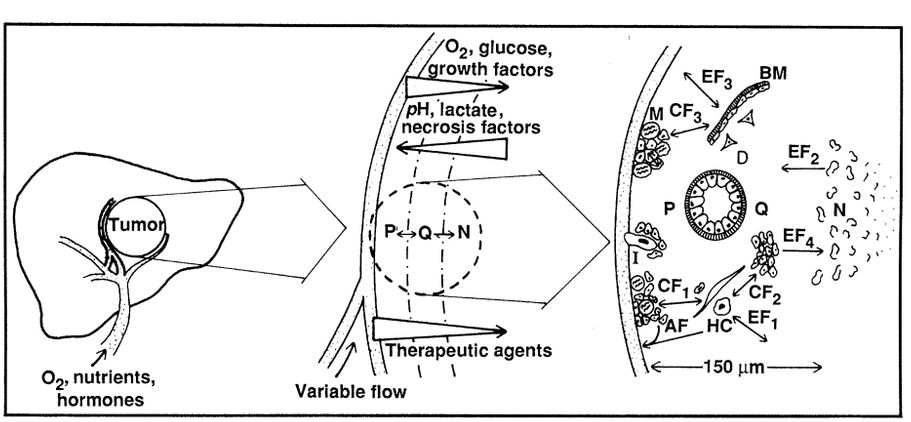

Fig. 1. Diagram of tumor microregion showing some of the factors that contribute to the development of heterogeneous microenvironments and cells. Abbreviations: P, proliferating; Q, quiescent; N, necrotic; D, differentiation; I, invading; M, mitotic; HC, host cells (lymphocytes, macrophages, and fibroblasts); EF, environmental factors; CF, cell factors; AF, angiogenic factors; and BM, extracellular biomatrix.

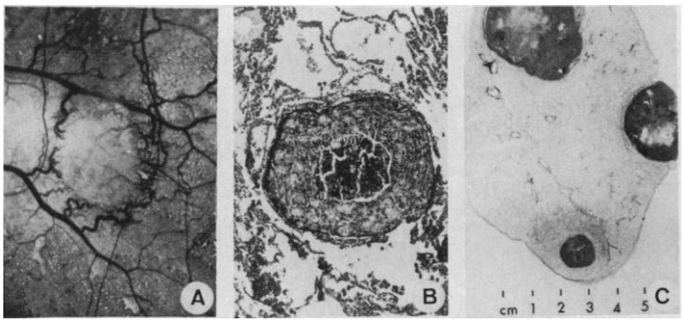

Fig. 2. (**A**) Early angiogenesis in a small tumor (approximately 1 mm) growing in a hamster cheek pouch "window" preparation (*78*). (**B**) Human adenocarcinoma of colon micrometastasis (1.5 mm) in lung. Central necrosis is evident at this small size even in this well-vascularized site. (**C**) Human epidermoid carcinoma metastases in lung. Significant necrosis can be seen.

Oxygen tension (Po_2) and *p*H have been measured in rodent and human tumors (*6, 7*). These data are characterized by a large degree of inter- and intratumor heterogeneity. Generally, the frequency distributions of Po_2 values are shifted toward hypoxia, with at least 50% of the measurements often being less than 10 mmHg. The *p*H may range from 5.8 to 7.6, and most often acidic median values (*p*H 6.8 to 7.0) are observed. Direct evidence for vascular inefficiency in tumors of the human head and neck as well as of the rectum has been obtained by using a cryospectrophotometric technique to measure oxyhemoglobin saturation in tumor microvessels (*8*). These data suggest that the intratumor distributions of important substrates such as glucose or other nutrients as well as growth factors or hormones may also be deficient and heterogeneous.

Tumor Cell Heterogeneity

Tumor growth is characterized by a phase of exponential cell proliferation followed by a phase of declining growth rate associated with an increase in nonproliferating (quiescent) cells and necrotic cells. Proliferating cells are usually located within a few cell layers of functional blood vessels. Quiescent and necrotic cells are located at progressively greater radial distances from the vessels. The radii measured between the vessels and necrotic areas in a variety of rodent and human tumors range from approximately 50 to 250 μm.

In addition to the quiescent cells that presumably develop as a result of O_2, nutrient, or growth factor deprivation, another class of quiescent cell phenotype, the differentiated quiescent cell, may be present. Although differentiation is genetically based, the process is also influenced by the cellular environment, particularly the three-dimensional relation of cells to each other and to extracellular matrix. Various chemical mediator signals and cell receptors controlling growth and differentiation are known. Some of these natural signaling factors, also known as biological response modifiers, have the capacity to induce proliferation or quiescence, and some quiescent cells may at a later stage of tumor progression act as stem cells to cause regrowth of the tumor.

The fraction of stem cells in human tumors is variable, but usually small (<1%). Stem cells are the targets for therapy, and it is from these cells that resistant variants can emerge. Cloned stem cell lines from the same tumor or from different tumors of the same histological type express a range of sensitivities to drugs and to radiation (*9*) but are generally considered as relatively stable phenotypes. Similarly, relatively stable but highly metastatic subpopulations of cells have been cloned from tumors (*10*). However, phenotype stability may depend on the stability of the selective pressures of the environment. A dynamic heterogeneity model incorporating forward and backward rates of phenotypic variance has been applied to explain the behavior of growing tumor cell populations with different metastatic propensities and drug resistances (*11*).

This cellular heterogeneity of tumors is also reflected by variations in the expression of cellular antigens, which can complicate the use of antibodies conjugated with cytotoxic agents (*12*), and by variations in specific chromosomal abnormalities (*13*). Multiple clones with differences in DNA content have been found in specimens of some human malignancies (*14*). The products of one or more oncogenes may be overexpressed in some tumor cells (*15*), and such overexpression may be correlated with malignant progression in several types of human cancer (*16*). The expression of certain growth factors and growth factor receptors is critical to cellular interactions in a growing tumor (*17*). The responsiveness to some growth factors in vitro can depend on whether the cells are anchorage-independent or grown as monolayers on glass or plastic (*18*) or attached to extracellular matrix (*19*). In a progressing malignancy, normal structural associations (cell-cell and cell-matrix) are disrupted, thereby creating an environment where the normal processing of these intercellular signals may be altered (Fig. 1).

The Multicell Spheroid Model

One approach to studying the biology of tumor microregions is to culture cancer cells in the form of three-dimensional multicell spheroids that simulate micrometastases or intervascular microregions of larger tumors (*1, 20–28*) (Fig. 3). This tumor model is intermediate in complexity between standard two-dimensional monolayer cultures in vitro and tumors in vivo. The spheroidal geometry and spatial resolution possible with various microtechniques facilitate studies of the relation of tumorlike microenvironments to the development of specific cellular subpopulations (*23–25*). Methods have been developed for optimal growth and utilization of spheroids of a variety of different histological types of rodent and human tumors (*20–28*).

Spheroids grown from established tumor cell lines or, less frequently, directly from primary tumor specimens, show growth kinetics similar to those of tumors in vivo. As growth progresses, the number of cells that are proliferating decreases, and the proportion of nonproliferating (quiescent) cells increases. When the cells become deprived of O_2, glucose, and other substrates, and when toxic metabolic waste products accumulate, there are steep gradients in these metabolites, and cell death and necrosis will occur in the centers of the spheroids. The distance from the periphery of the spheroid at which necrosis occurs may vary from 50 to 300 μm, depending on the cell types and their substrate consumption rates, the cell packing densities, and the concentrations of substrates in the growth media. For most types of human tumor cells grown under optimal nutrient and oxygen conditions the thickness of the viable rims of cells surrounding the necrotic centers of spheroids ranges from 100 to 220 μm with cell packing densities producing extracellular volumes of 35 to 55%, generally similar to the values for tumors in vivo (Fig. 3, B, C, and D).

It is over these very small distances, approximately 10 to 20 cell diameters (simulating the radial distance from small blood vessels in tumors), that significant differences in cell microenvironments may develop. Generally, most of the proliferating cells in spheroids are located in the outer three to five cell layers (75 μm). The quiescent cells are located more centrally and include a significant proportion of cells that are reproductively viable when removed from these environments (*23–25, 29*). These cells can be recruited to repopulate the proliferating compartment.

Subpopulations of cells from peripheral or central regions of spheroids can be isolated in order to study their biological properties and responses to therapeutic agents. The methods used for such isolation include selective dissociation with low concentrations of proteolytic enzymes (*30*), centrifugal elutriation (*23–25, 29*), and fluorescence-activated cell sorting based on gradients of intracellular fluorescent probes for viable cells (*31*) from the peripheries to the centers of spheroids.

Spheroids have been used to study the relative importance for tumor cell growth of the supply of O_2 and glucose (*20–25, 32–34*). Direct measurements of the Po_2 within spheroids were performed with microelectrodes (Fig. 4A). Gradient profiles of Po_2 across the viable rims and necrotic centers of many different types of cancer cell spheroids have been obtained (*21, 22, 32*). These gradients can often be very steep, in agreement with theoretical calculations based on known O_2 concentrations, diffusion constants, and consumption rates. However, some cells may adapt to changes in O_2 supply by modifying consumption rates and consequently decreasing the steepness of the O_2 gradients. The relative concentrations of O_2 and glucose may affect pathways of energy metabolism and thereby change oxygenation and may also influence the fraction of quiescent cells and viable rim thickness. Glucose concentration affects the development of central necrosis even when O_2 levels are significant. Although initial spheroid growth rates are similar in different O_2 and glucose environments, the spheroid size at which growth saturation occurs depends on O_2 and glucose concentrations and is associated with the onset of necrosis. Necrotic or prenecrotic materials can produce growth inhibitory feedback effects on the proliferating population of cells. These data have been incorporated into a theoretical model of growth regulation in relation to supply of critical metabolites (*34*).

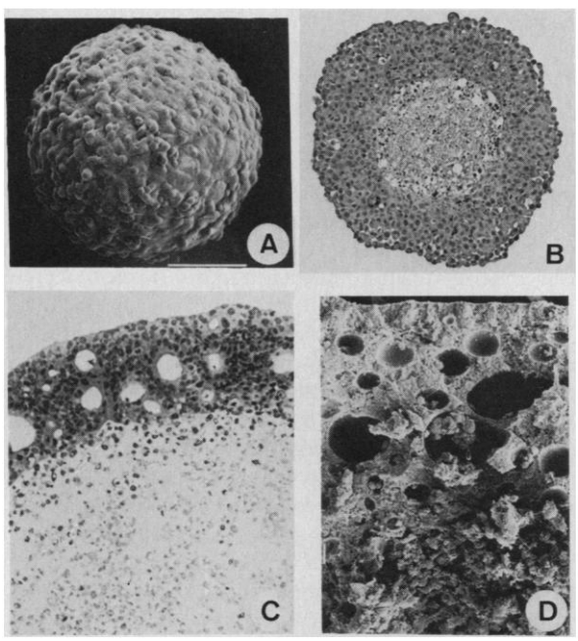

Fig. 3. (**A**) Scanning electron micrograph of human cervical squamous cell carcinoma (CaSki) spheroid (approximately 300 μm in diameter) containing 3900 cells (bar, 100 μm). (**B**) Histologic section through the center of CaSki spheroid similar to that shown in (A). Viable rim of cells of approximately 120 μm surrounds the necrotic center. (**C**) Viable rim of spheroid of CO112 human colon adenocarcinoma demonstrating differentiation of pseudoglandular structures. (**D**) Scanning electron micrograph of viable rim (top) and necrotic center (bottom) of CO112 spheroid. The pseudoglandular structures are predominantly in the more quiescent deeper regions of the viable rim. The spheroid was dried under critical point conditions, cleaved, and then coated with gold (bar, 10 μm) (*79*).

An unexpectedly large decrease (factor of 3 to 4) in both O_2 and glucose consumption occurred during the growth of intact spheroids (*33*). It has been hypothesized that a significant part of this decreased metabolism results from the increased fraction of quiescent cells and cooperative cellular biochemical interactions that modulate energy metabolism during spheroid growth.

Another biochemical event associated with hypoxia and glucose deprivation is the increased rate of synthesis of a specific class of oxygen-regulated proteins (ORPs) (*35*). The synthesis of these ORPs, most of which are present at low concentrations constitutively, begins after about 1 hour of hypoxia and increases for periods of up to about 12 hours of hypoxia. The kinetics and extent of induction of the five major ORPs (33, 80, 100, 150, and 260 kD) that have been identified vary among the rodent and human normal and malignant cell lines that have been studied. The proteins are synthesized at increased rates in the cell layers surrounding the necrotic centers of spheroids.

The ORPs are induced in the presence of normal glucose concentrations. However, low glucose (in well-oxygenated environments) can induce synthesis of several of these proteins. Combined stress of hypoxia and low glucose concentrations shortens the exposure time required for induction but not the synthesis rates. The induction of these proteins does not appear to vary at different phases of the cell cycle. The possible significance of these proteins for determining sensitivity to therapy or as diagnostic markers has yet to be determined.

Differentiation in Spheroids

The growth of cells in three-dimensional aggregates has been widely used for studies of regulation of embryological development (*36*). Evidence for enhanced differentiation has been obtained in studies of characteristics of malignant cell subpopulations when grown as spheroids. Spheroids of human colon adenocarcinoma differentiate to develop pseudoglandular structures that possess the features of tumors in vivo (Fig. 3, C and D) (*27*). These spheroids express large amounts of carcinoembryonic antigen in association with these structures, eight times more than when cultured as two-dimensional monolayers. Studies of these and less differentiated human colon carcinoma spheroids with microelectrodes showed considerable differences in oxygenation (*27*).

Differentiation as determined by morphological, biochemical, and immunological criteria has also been demonstrated in spheroids of other malignant and normal cell lines. Of special interest are the responses to hormones and growth factors that have been observed in spheroids of different cell types from tissues such as thyroid, liver, and pituitary (*37*). Functional differentiation can be maintained for many weeks. The sensitivity to epidermal growth factor is modulated markedly when human squamous carcinoma cells are grown in close cell-cell contact even as small spheroids (groups of five to ten cells). In spheroids, reactions with some antibodies to differentiation antigens of fetal tissues are qualitatively and quantitatively different than in monolayers, and the reactions change as spheroids grow. An important environmental factor involved in stimulating differentiation within some types of spheroids is probably the production of extracellular biomatrix in close association with the cells during growth (*38*).

Cell-Cell Contact in Spheroids

Spheroids are held together by surface membrane microprojections, extracellular matrix, and a variety of cell-cell junctions (desmo-

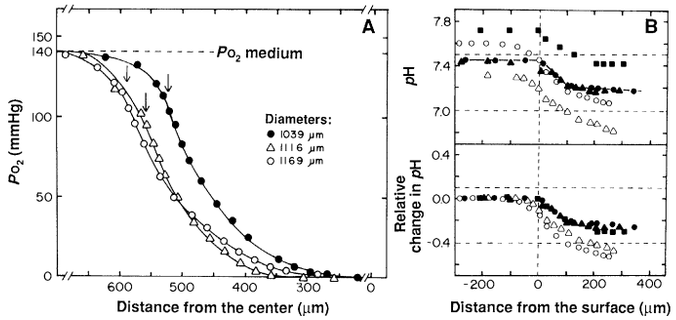

Fig. 4. (**A**) Microelectrode profiles of Po_2 in HT29 human colon spheroids (in Dulbecco's minimum essential medium and 10% fetal bovine serum), demonstrating steep gradients across the viable rim and low values in the central regions. (**B**) Gradients of *p*H in human U-118 MG glioma spheroids (650 μm) under different buffer and *p*H conditions [symbols: ●, initial conditions with a buffer capacity of 11.3 m*M* per *p*H unit and a medium *p*H of about 7.4; the buffer capacity was changed to 4 m*M* per *p*H unit with a medium *p*H of 7.3 (△) and then 7.6 (○). The buffer capacity was then shifted back to 11.3 m*M* per *p*H unit with a medium *p*H of 7.4 (▲). The medium *p*H under this last buffer condition was finally changed to 7.7 (■)]. [Reprinted from (*65*) with permission, copyright Cancer Research, Inc.]

somes, tight junctions, junctional complexes, and gap junctions). In some spheroids specialized junctions are rare, but when they are present, the most common junction is the desmosome. Gap junctions are of special interest because they are thought to play a regulatory role in embryogenesis. Although it is possible that loss of coupling between cells is a critical element in the uncontrolled proliferation in cancer (*39*), this field of research is controversial. Abnormalities of cell-cell communication are probably involved in some but not all cancers. Whether through gap junctions or by other structures, intercellular permeability may not be constant between cells at different stages of spheroid growth (*40*).

There is a positive correlation between lines of cancer cells that are highly electrically coupled and their resistance to ionizing radiation as small spheroids (*41*). This "contact effect" was first demonstrated in small spheroids of Chinese hamster V79 lung cells after 1 or 2 days of growth (5 to 15 cells) (Fig. 5A) (*42*) and was subsequently shown for other cytotoxic agents (*43, 44*) such as heat, ultrasound, and the drug Adriamycin (ADR). This increased resistance usually takes the form of an increased threshold shoulder on the dose-response survival curves of cells from dissociated spheroids after exposure of the intact spheroids to the cytotoxic agents. Resistance to radiation in V79 cells is not lost until approximately the duration of one cell cycle after dissociation of the spheroids, indicating that direct cell-cell communication at the time of irradiation is not critical for the contact effect. A history of growth in close cell-cell contact appears to be the most important factor for most cell lines that express this phenomenon. Thus, although there appears to be a correlation between the ability to express this contact effect and cell-cell electrical coupling, the evidence does not conclusively support a direct relation. Other factors appear to be involved in the mechanism of this altered sensitivity.

Another covariant with cell-cell contact is altered cell shape when the cells are grown in small spheroids rather than monolayer cultures. There is evidence that the altered cell shape, in association with the development of intercellular membrane contacts and junctions, may stimulate mechanochemical transductions from cell membrane through cytoskeleton and nuclear matrix to the chromatin, thereby affecting DNA packaging and DNA-enzyme interactions (*45*). This concept, along with a specific hypothesis of altered DNA loop sizes in cells in small spheroids compared with cells in monolayer cultures, has been proposed to explain the decreased DNA damage in irradiated spheroids (*46*).

This increased resistance to radiation attributable directly or indirectly to cell-cell interactions has now been demonstrated in other rodent and human tumor xenograft models in vivo (*41, 47*). In experiments with human melanoma spheroids a contact effect was found in one of five established cell lines at early passage and two of four primary cultures from fresh surgical specimens (*48*). It is important to determine the frequency and magnitude of this effect among and within different histological classifications of human tumors since radiation therapy is usually given in many small dose fractions in which the sensitivity of the threshold shoulder region of the cell survival curve will have a major influence on the outcome of the total course of therapy. Currently this is of special interest because of the suggestion that the low dose response of cells in culture, the so-called intrinsic sensitivity to radiation, may predict the general clinical responsiveness of different classifications of tumors (*9*). Refinement of such potentially predictive assays may be possible with the use of small spheroids to assess cell-cell contact effects and large spheroids to determine influences of the development of heterogeneous cell subpopulations and microenvironments during growth.

Microregions and Resistance to Therapy

Radiation-resistant hypoxic cells have been demonstrated in most rodent tumors, and there is evidence for their existence in human tumors (*49*). Decreased radiation sensitivity begins to occur at a Po_2 of less than 10 mmHg; the equivalent partial pressure of O_2 that produces one-half the maximum sensitivity is approximately 3 to 5 mmHg. Such levels (and even lower) have been measured in many rodent and human tumors. Different rates and extents of reoxygenation, and therefore enhanced sensitization, may occur during multifraction radiation therapy. The relation of reoxygenation to changes induced in tumor vascularization, inhibition of tumor cell O_2 consumption, possible migration of hypoxic cells, or other characteristics of tumor microregions is not clear.

More detailed information on the interrelation of some of these factors in response to therapeutic agents has been obtained with spheroids. This model is particularly appropriate for simulating chronically hypoxic and quiescent cells as they may occur in a dynamic equilibrium between proliferating and necrotic compartments in vivo. A radiation-resistant hypoxic fraction is present in spheroids of both rodent (*24, 50*) and human origin (Fig. 5C) (*51*). Tumors that contain radiation-resistant hypoxic cells when grown as spheroids also usually show a resistant hypoxic fraction of cells when grown in mice. In one study relative differences in radiation responsiveness of xenografts of five human melanoma cell lines were predicted from studies of the sensitivity of corresponding spheroids (*52*). When spheroids are subjected to fractionated doses of radiation, simulating radiotherapy, reoxygenation occurs that, because of the absence of a vascular network, demonstrates the importance of decreased cellular respiration (*53*). Other research has shown that agents that inhibit O_2 consumption by the outer proliferating compartment of spheroids can reoxygenate the more central compartment and thereby increase radiation sensitivity (*53*).

Major differences in sensitivity to drugs attributable to effects on accessibility and uptake as well as local microenvironments have been demonstrated in tumor cells grown as spheroids (*44, 54–56*). One example of this is the resistance to ADR where, in several tumor spheroid systems, a gradient in drug distribution develops with the greatest concentrations in the outer cells (Fig. 6A) (*44, 55, 56*). A similar gradient occurs in vivo where the greatest concentrations of ADR are found in cells near blood vessels. As a result, at drug concentrations achievable in the serum in vivo, cells in

spheroids exhibit increased resistance (several logs) in the surviving fraction of clonogenic cells (Fig. 5D). Studies of penetration of other drugs have shown that some, such as 5-fluorouracil and vincristine, penetrate readily.

The ability of antibodies with attached cytotoxic agents to penetrate into tumor microregions may be poor because of the high molecular weights involved. Heterogeneous distributions of antigens and their accessibility even within volumes as small as 1 to 2 mm^3 are beginning to be regarded as critical for the therapeutic use of such agents. Recently, we showed that $F(ab')_2$ and Fab fragments of antibodies labeled with radioisotopes can penetrate about six to eight cell layers in spheroids of human colon adenocarcinoma compared with only one to three cell layers for intact antibody (*57*). Radiation dosimetry studies of antibody distributions in animal tumors, as well as theoretical modeling of expected doses for tumors of different geometries, including spheroids, have pointed out the advantages of using combinations of antibodies or antibody fragments and different isotopes with a range of energies and effective radiation distances in order to maximize the therapeutic potential of this approach.

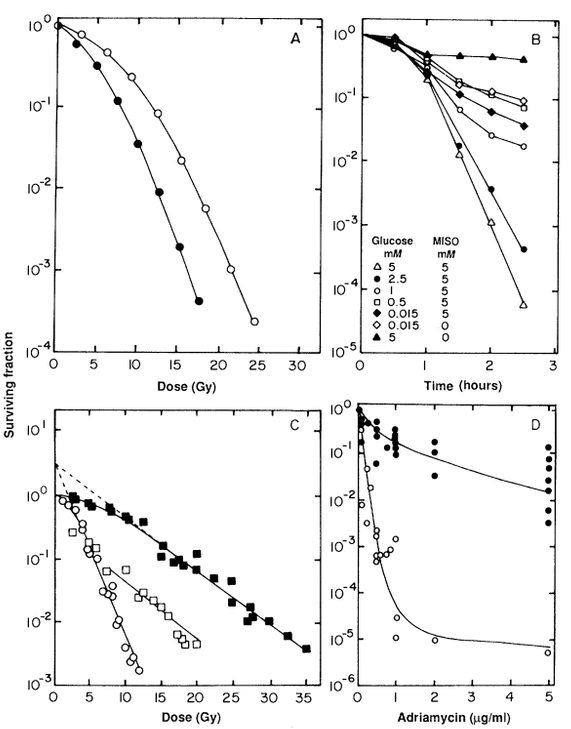

Fig. 5. (**A**) Radiation sensitivity of small spheroids (five to ten cells per spheroid) of Chinese hamster V79-171b lung cells. Symbols: ○, spheroids irradiated and immediately dissociated for colony formation assay of separated cells; ●, monolayer cultures irradiated in suspension and assayed for colony formation (*42*). (**B**) Surviving fraction of hypoxic EMT6/Ro mouse mammary tumor cells incubated in suspension with different concentrations of glucose and MISO. Changes in the redox state of the cells affect the cytotoxicity of this drug. [Reprinted from (*63*) with permission, copyright Macmillan Press] (**C**) Radiation response of multicell spheroids of WiDr human colon adenocarcinoma cells. Spheroids were irradiated in air at 4°C (○) to inhibit cellular O_2 consumption and reoxygenate the hypoxic, resistant cells present when spheroids are irradiated under normal growth conditions in air at 37°C (□). Spheroids irradiated in N_2 at 37°C (■) show a resistant survival curve that parallels the curve for irradiation in air at 37°C, also indicating the presence of a fraction (about 8%) of hypoxic, radiation-resistant cells. Spheroid diameter, 1239 ± 32 μm. All colony formation assays were performed on the cells after completely dissociating the spheroids immediately after irradiation. [Reprinted from (*51*) with permission, copyright Academic Press] (**D**) Relative sensitivities of EMT6/Ro spheroids and monolayer cultures in exponential growth phase to different exposure doses of ADR for 1 hour. The cells were exposed to the drug as spheroids (●) (various sizes between 400 and 900 μm in diameter) or as monolayers (○) and were then dissociated with trypsin to produce single cell suspensions that were than assayed for colony formation. [Reprinted from (*55*) with permission, copyright Pergamon Journals]

Therapy Sensitization

A major area of research on radiation-resistant hypoxic cells has been the development and application of radiation-sensitizing drugs. The early demonstration that certain nitro-containing chemicals could penetrate to hypoxic zones within spheroids and tumors and enhance radiation sensitivity through fixation of reactive free-radical–induced damage (*58*) helped stimulate the search for other active agents. Analogs of nitroimidazoles with a range of electron affinities and lipid solubilities have been compared, and some effective hypoxic-cell sensitizers have been identified. Misonidazole (MISO), for example, has received extensive clinical evaluation, but its clinical use may be limited because of its neurotoxic side effects. The binding of MISO is much greater in hypoxic than in normoxic cells (Fig. 6B), and the extent of binding can be calibrated by means of autoradiography in spheroids equilibrated in different O_2 environments. Thus MISO can be used in experimental tumor systems to detect those tumors that contain resistant hypoxic cells at the initiation of therapy (*59*). Relatively noninvasive techniques, including positron emission tomography (PET), magnetic resonance spectroscopy (MRS), and magnetic resonance imaging (MRI) may be used to measure appropriately labeled agents that preferentially bind to hypoxic cells in vivo. Similar techniques may also be useful in other forms of ischemic disease.

The finding that certain nitroimidazoles also preferentially kill hypoxic quiescent cells in spheroids (*60*) led to a search for other drugs with similar reactivities. A number of nitroimidazoles and related nitro-containing compounds have been identified and compared for their chemotherapeutic activity with the use of monolayer cultures, spheroids, and tumor models in vivo. Drugs that are many times as effective as hypoxic cytotoxic agents have now been identified. Some combinations of drugs that are more effective against the proliferating cell subpopulations and drugs such as MISO that kill the hypoxic, quiescent cells show not only the expected additive effect but also a synergistic (chemosensitization) effect (*61*).

Since interactions of the O_2 and glucose supply affect spheroid oxygenation, the consequences for radiation response of different concentrations of glucose have been investigated (*24*). When mouse mammary tumor spheroids were grown in glucose concentrations greater (4.5×) than normal, the fraction of radiation-resistant hypoxic cells decreased and the cells were more sensitive. The change in the hypoxic fraction can be explained by decreases in O_2 consumption leading to higher concentrations of O_2 within the spheroid. The change in the inherent sensitivity in this cell subpopulation may reflect changes in intracellular levels of known modulators of radiation response, such as glutathione, or in the fraction of quiescent cells. It has been possible recently to isolate enriched populations of quiescent cells from spheroids and plateau-phase monolayer cultures and to measure directly their radiation sensitivity (*29*). These cells are more radiosensitive when they are isolated and then irradiated. However, many of these cells would be hypoxic in spheroids and tumors in vivo and may also efficiently repair radiation damage. Therefore, they may be more resistant than the normoxic proliferating cells, and their ability to survive and to be recruited into the proliferating compartment is an important consideration for assessing their contribution to the overall responsiveness of tumors.

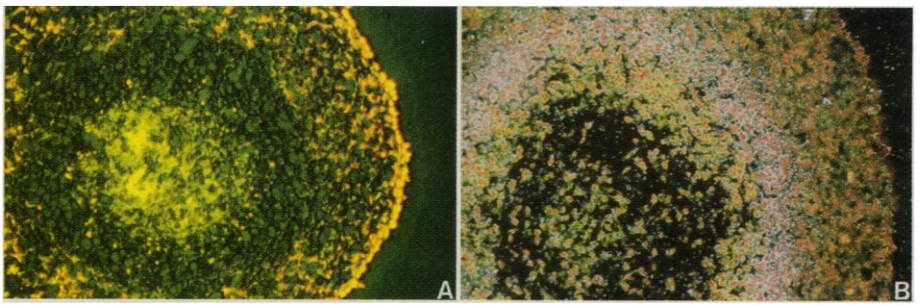

Fig. 6. (**A**) ADR gradient (yellow fluorescence) in viable rim of EMT6/Ro mouse mammary tumor spheroid. Necrotic center is at the left. Population of cells (green) with little uptake of the drug is shown. Rim thickness, approximately 200 μm (*55*). (**B**) Preferential activation and binding of radiolabeled MISO (white zone) in the hypoxic region of cells surrounding the necrotic center of EMT6/Ro mouse mammary tumor spheroids (*80*).

In addition to affecting the O_2 environment and cell viability and quiescence, the glucose supply also influences the intracellular redox state in ways that are important for determining the cellular response to cytotoxic agents. For example, the ability of MISO to bind and to kill hypoxic tumor cells is highly dependent on the concentration of glucose (Fig. 5B) (*62, 63*). As glucose is increased, activation and binding are increased so that the cells become sensitive. This is partly because the supply of pyridine nucleotide–reducing equivalents increases, mainly by way of the hexose monophosphate pathway. The effectiveness of other bioreductively activated drugs could be similarly altered. For many such drugs, critical interactions among environmental factors such as O_2, glucose, *p*H, and drug concentration would be expected to influence the sensitivity of different subpopulations of cells, even in tumor microregions less than 0.5 mm in diameter, as in spheroids.

The ability of low O_2 and glucose supply to induce specific proteins (ORPs) has stimulated research to assess the possible relation of these proteins to therapeutic sensitivity. A strong correlation has been established between the induction and decay kinetics of ORPs and the sensitivity of cells to ADR (*64*). After periods of exposure to hypoxic conditions the cells are more resistant to a range of concentrations of ADR administered for 1 hour under normoxic conditions. Reoxygenation of the cells after exposure to these hypoxic conditions causes the ADR resistance and the protein synthesis rates to return to normal. Longer periods of glucose deprivation, which induce most of these same proteins, also result in ADR resistance. Similar effects can be produced by the glucose analog glucosamine or by methods that alter intracellular calcium levels such as treatment with calcium ionophores or calcium chelating agents. The mechanism of this resistance is not known, although it may partly account for the greatly increased resistance of intact spheroids to ADR (Fig. 5D) (*44, 55, 56*).

The difference in *p*H between tumors and normal tissues may also be therapeutically exploitable. Spheroids have been used to measure gradients in *p*H that might be expected in tumors at distances of up to 200 μm (10 to 15 cell diameters) from vessels (*65*). The results obtained with microelectrodes show that gradients of 0.1 to 0.5 *p*H unit can occur depending on the metabolism of the particular cell type, the concentrations of O_2, glucose, and other substrates in the macroenvironment, and the convection and buffering properties of the surrounding fluid medium (Fig. 4B). Such *p*H differences enhance the sensitivity of tumor cells to hyperthermic therapy, and cells in the inner regions of the viable rims of spheroids are preferentially killed by hyperthermia (*66, 67*). Methods are being developed to increase the acid milieu in tumors as opposed to normal tissue and thereby obtain an even greater therapeutic advantage. These methods may also be applicable to treatment with drugs. A variety of mechanisms related to inherent differences in cell sensitivity to certain drugs caused by *p*H effects on metabolism, or related to effects of *p*H on drug structure and degradation products, or to uptake and distribution within cells, are being considered.

Cellular Immune Reactions in Spheroids

Among the host cells in a tumor that can play significant roles in tumor growth or response to treatment are cytotoxic T lymphocytes and macrophages. These cells, which may constitute a large percentage (up to 50%) of the total tumor cell population (*68*), may be directly cytotoxic and may modulate, that is help or suppress, cytotoxic or other immune functions. Many of these functions of host cells are mediated by different cytokines released into the tumor microenvironment (Fig. 1).

The reactions between host immune cells and tumor cells in environments simulating tumors in vivo have been studied by implanting spheroids into the peritoneal cavities of normal or immunologically sensitized or deficient mice and recovering the spheroids at different times (*69*). Kinetics of host cell infiltration, functional capacity of host cells recovered from spheroids, and destruction of the tumor cells by the host cells within the spheroids have been determined. In studies with syngeneic systems the primary cytotoxic cell was the T lymphocyte, and a factor produced by certain tumor cells was discovered that inhibits the generation of cytotoxic immunological reactions (*69, 70*). Spheroid-associated macrophages have been shown to produce tumor necrosis factor (*71*).

By using the spheroid model in vitro and in vivo, it is possible to separate direct effects of therapeutic manipulations on tumors from indirect effects mediated through the host. For example, mature immune effector cell cytotoxic activity is relatively radiation resistant as determined from studies of spheroids that were recovered from mice after infiltration with host cells and then irradiated (*72*). By comparison, irradiation of the host mice before spheroid implantation, early in the immune response to the tumor cells, produced a much greater impairment of cytotoxic immune activity. Treatment of spheroids with moderate hyperthermia before implantation into mice did not enhance their immunogenicity (*67*). Peritoneal implantation of spheroids has also been used to study the effects of drugs that require activation in vivo (*73*).

Implications and Future Research

A variety of technologies are becoming available for studying the characteristics of tumor microregions. These include cryobiological techniques used in conjunction with microspectrophotometry for quantitative analysis of oxyhemoglobin saturations in individual tumor vessels or the use of bioluminescence (Fig. 7B) for determining the distribution of metabolites and substrates such as adenosine triphosphate (ATP) and glucose in tumors and spheroids. These data can be evaluated in conjunction with other quantitative information obtained by using computer-interfaced microscopy imaging techniques to determine cellular heterogeneity with antibodies and immunocytochemistry methods, biochemical markers, and molecular probes for gene expression.

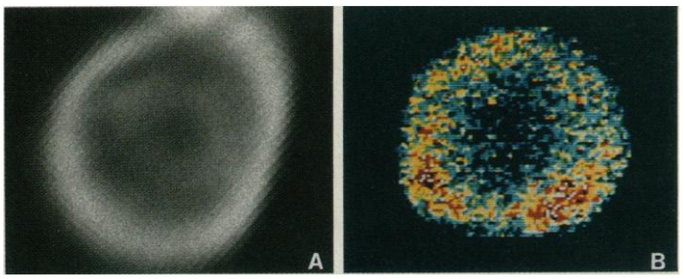

Fig. 7. (**A**) T_1-weighted (TR 450 msec, TE 16 msec) proton image of human HT29 colon carcinoma spheroid (2000 μm in diameter). Discrimination of the viable rim of the spheroid of approximately 200 μm thickness and other differences in the central necrotic region can be seen. Image obtained with a GE CSI 2 T spectrometer operating at 85.56 MHz for protons. Image slice thickness is 160 μm with a spatial resolution of 156 by 78 μm (*81*). (**B**) Bioluminescence image of ATP distribution in HT29 colon carcinoma spheroid (800 μm in diameter). The color coding indicates relative concentrations: red > orange > yellow > green > blue > black. ATP is present in greatest concentrations in the actively proliferating outer regions of the spheroid viable rim; it is lower in the inner region of the rim and lowest in the necrotic center (*82*).

Relatively noninvasive techniques such as MRI, MRS, and PET may provide insight into the metabolism and physiology of tumor tissue, although the resolution of these methods is inadequate to examine in detail the small microregional volumes described in this article. However, MRI of spheroids can currently distinguish necrotic centers from the layers of surrounding viable cells of about 160 μm in thickness (Fig. 7A); similar results have been obtained recently with ultrasound microscopy (*74*). Spectroscopy methods with the use of ^{31}P, for example, can provide information on metabolites related to phosphate-containing metabolic pathways to indicate energy status of tumors. Estimates of tumor *p*H can also be obtained from the shift in inorganic phosphate peaks. Studies with PET can provide details of glucose and O_2 metabolism in tissues, especially brain, and the methods are being refined for application in tumor tissue to study other metabolic pathways and specific receptors. Results from these methods can be interpreted relative to microregional heterogeneities measured in the same tumors with the use of cryospectrophotometry and bioluminescence.

The concept of dynamic and transient changes in genetic expression is emerging as an important area for investigation. Microenvironmental factors, including metabolic substrates, growth factors and hormones, and *p*H may influence genetic expression. Recently, hypoxia was shown to be an effective inducer of gene amplification, which can lead to the production of drug-resistant cells (*75*). Cell-cell interactions, directly or indirectly, may also be important modulators of malignant cell phenotype as indicated by changes of drug and radiation resistance in mixed-cell experiments (*76*) and by alterations in the potential of malignant cells to invade and mix with normal cells (*77*).

Eventually it may be possible to exploit and manipulate tumor environments to produce preferential effects of therapeutic agents on tumors. It may also be possible to treat tumors with combined therapy modalities that are effective against specifically characterized subpopulations and heterogeneous environments, and to control or stabilize the heterogeneity in favorable directions, for example, differentiation or expression of antigens or receptors that can then be treated with specific agents.

REFERENCES AND NOTES

1. R. M. Sutherland, *Cancer* **58**, 1668 (1986).
2. J. Folkman, *Cancer Res.* **46**, 467 (1986); J. L. Marx, *Science* **237**, 23 (1987); J. Folkman and M. Klagsbrun, *ibid.* **235**, 442 (1987).
3. E. K. Rofstad, *Cell Tissue Kinet.* **17**, 91 (1984).
4. P. Vaupel and W. Mueller-Klieser, *Adv. Exp. Med. Biol.* **200**, 633 (1986).
5. E. Siracka, J. Siracka, N. Pappova, L. Revesz, *Neoplasma* **29**, 183 (1982); M. L. Mlynek, D. Van Beunien, L. D. Leder, C. Streffer, *Br. J. Cancer* **52**, 945 (1985); O. V. Solesvik, E. K. Rofstad, T. Brustad, *ibid.* **46**, 557 (1982).
6. P. W. Vaupel, S. Frinak, H. I. Bicher, *Cancer Res.* **41**, 2008 (1981); R. A. Gatenby *et al.*, *Radiology* **156**, 211 (1985).
7. J. L. Wike-Hooley, J. Haveman, H. S. Reinhold, *Radiother. Oncol.* **2**, 343 (1984).
8. W. Mueller-Klieser, P. Vaupel, R. Manz, R. Schmidseder, *Int. J. Radiat. Oncol. Biol. Phys.* **7**, 1397 (1981); P. Wendling, R. Manz, G. Thews, P. Vaupel, *Adv. Exp. Med. Biol.* **180**, 293 (1985).
9. B. Fertil and E. P. Malaise, *Int. J. Radiat. Oncol. Biol. Phys.* **7**, 621 (1981); *ibid.* **11**, 1699 (1985); J. Deacon, M. J. Peckham, G. G. Steel, *Radiother. Oncol.* **2**, 317 (1984); R. R. Weichselbaum *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2684 (1986).
10. G. Poste and I. J. Fidler, *Nature (London)* **283**, 139 (1980).
11. V. Ling, A. F. Chambers, J. F. Harris, R. P. Hill, *Cancer Metastasis Rev.* **4**, 173 (1985); A. F. Chambers, J. F. Harris, V. Ling, R. P. Hill, *Invasion Metastasis* **4**, 225 (1984); C. Cillo, J. Dick, V. Ling, R. Hill, *Cancer Res.* **47**, 2604 (1987).
12. C. Cillo, J.-P. Mach, M. Schreyer, S. Carrel, *Int. J. Cancer* **34**, 11 (1984).
13. N. Wake, M. M. Hreshchyshyn, S. M. Priver, S. Matsui, A. A. Sandberg, *Cancer Res.* **40**, 4512 (1980); J. Whang-Peng *et al.*, *Science* **215**, 181 (1982).
14. F. Mauro, W. Gohde, J. Schumann, L. Teodori, M. Spano, *Int. J. Radiat. Biol.* **49**, 307 (1986).
15. T. A. Libermann *et al.*, *Nature (London)* **313**, 144 (1985); K. Saksela *et al.*, *Cancer Res.* **45**, 1823 (1985); Y. Shiloh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3761 (1985).
16. G. M. Brodeur, R. C. Seeger, M. Schwab, H. E. Varmus, J. M. Bishop, *Science* **224**, 1121 (1984); D. J. Slamon *et al.*, *ibid.* **235**, 177 (1987).
17. C.-H. Heldin and D. Westermark, *Cell* **37**, 9 (1984); F. Cuttitta *et al.*, *Nature (London)* **316**, 823 (1985); C. J. Sherr *et al.*, *Cell* **41**, 665 (1985); J. E. De Larco and G. J. Todara, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4001 (1978).
18. A. B. Roberts *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3494 (1980); S. Watanabe, E. Lazard, M. B. Sporn, *ibid.* **84**, 1258 (1987).
19. L. A. Liotta, *Cancer Res.* **46**, 1 (1986); G. F. Rieber, M. S. Rieber, C. Urbina, *Int. J. Cancer* **37**, 411 (1986).
20. R. M. Sutherland, J. A. McCredie, W. R. Inch, *J. Natl. Cancer Inst.* **46**, 113 (1971); R. M. Sutherland and R. E. Durand, *Curr. Top. Radiat. Res.* **11**, 87 (1976).
21. W. Mueller-Klieser, *J. Cancer Res. Clin. Oncol.* **113**, 101 (1987).
22. H. Acker, J. Carlsson, R. Durand, R. M. Sutherland, Eds., *Spheroids in Cancer Research: Methods and Perspectives* (Springer-Verlag, New York, 1984).
23. K. D. Bauer, P. Keng, R. M. Sutherland, *Cancer Res.* **42**, 72 (1982); J. P. Freyer and R. M. Sutherland, *ibid.*, p. 3513; W. Mueller-Klieser, J. P. Freyer, R. M. Sutherland, *Br. J. Cancer* **53**, 345 (1986).
24. C. Luk and R. Sutherland, *Int. J. Radiat. Oncol. Biol. Phys.* **13**, 885 (1987).
25. R. M. Sutherland, *ibid.* **12**, 611 (1986); J. Carlsson and H. Acker, *ibid.* **11**, 535 (1985); H. Acker, in (*22*), pp. 116–133; A. J. Franko, H. T. Freedman, C. J. Koch, in (*22*), pp. 162–167; C. K. N. Li, *Cancer* **50**, 2066 (1982); *ibid.*, p. 2074; I. F. Tannock and I. Kopelyan, *Cancer Res.* **46**, 3105 (1986); *Br. J. Cancer* **53**, 823 (1986).
26. J. M. Yuhas, A. E. Tarleton, K. B. Molzen, *Cancer Res.* **38**, 2486 (1978); E. Wibe *et al.*, *Int. J. Cancer* **34**, 21 (1984); O. Bruland, O. Fodstad, A. Pihl, *ibid.* **35**, 793 (1985).
27. R. M. Sutherland *et al.*, *Cancer Res.* **46**, 5320 (1986).
28. E. K. Rofstad, A. Wahl, T. Brustad, *Br. J. Radiol.* **59**, 1023 (1986).
29. C. Luk, P. C. Keng, R. M. Sutherland, *Br. J. Cancer* **54**, 25 (1986); C. E. Ng, P. C. Keng, R. M. Sutherland, *ibid.* **56**, 301 (1987).
30. J. P. Freyer and R. M. Sutherland, *Cancer Res.* **40**, 3956 (1980).
31. R. E. Durand, *J. Histochem. Cytochem.* **30**, 117 (1982).
32. H. Acker, G. Holterman, J. Carlsson, T. Nederman, in *Oxygen Transport to Tissue*, J. Matzka, Ed. (Plenum, New York, 1983), vol. 4, pp. 445–462; W. Mueller-Klieser, in (*22*), pp. 134–149; H. Acker, J. Carlsson, W. Mueller-Klieser, R. M. Sutherland, *Br. J. Cancer* **56**, 325 (1987).
33. J. P. Freyer and R. M. Sutherland, *J. Cell. Physiol.* **46**, 3504 (1986).
34. ———, *Cancer Res.* **46**, 3504 (1986).
35. J. J. Sciandra, J. R. Subjeck, C. S. Hughes, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4843 (1984); C. S. Heacock and R. M. Sutherland, *Int. J. Radiat. Oncol. Biol. Phys.* **12**, 1287 (1986).
36. J. Holtfreter, *J. Exp. Zool.* **95**, 171 (1944); A. Moscona, *Exp. Cell Res.* **3**, 535 (1952).
37. R. J. Mulcahy, W. A. Rosenkrans, D. P. Penney, R. A. Cooper, *In Vitro* **21**, 513 (1985); J. Ono, R. Takaki, H. Okano, M. Fukuma, *ibid.* **15**, 95 (1979); B. Vander Schueren, C. Denef, J. J. Cassiman, *Endocrinology* **110**, 513 (1982); J. Landry, D. Bernier, C. Ouelet, R. Goyette, N. Marcean, *J. Cell Biol.* **101**, 914 (1985).
38. T. Nederman, B. Norling, B. Glimelius, J. Carlsson, B. Brunck, *Cancer Res.* **44**, 3090 (1984).
39. W. R. Loewenstein, *Physiol. Rev.* **61**, 829 (1981).
40. D. F. Huelser and F. Brummer, *Biophys. Struct. Mech.* **9**, 83 (1982).
41. H. Dertinger and D. Huelser, *Radiat. Environ. Biophys.* **19**, 101 (1981); in (*22*), pp. 67–83.
42. R. E. Durand and R. M. Sutherland, *Exp. Cell Res.* **71**, 75 (1972).
43. J. C. Wigle and R. M. Sutherland, *J. Cell. Physiol.* **122**, 281 (1985); P. G. Sacks, M. W. Miller, R. M. Sutherland, *Radiat. Res.* **87**, 175 (1981).
44. R. E. Durand, *Cancer Res.* **41**, 3495 (1981).
45. J. Folkman and A. Moscona, *Nature (London)* **273**, 345 (1978); A. Ben-Ze'ev, S. R. Farmer, S. Penman, *Cell* **21**, 365 (1980).
46. P. L. Olive and R. E. Durand, *Radiat. Res.* **101**, 94 (1985); P. L. Olive, J. Hilton, R. E. Durand, *ibid.* **107**, 115 (1986).

47. R. P. Hill, R. Ng, B. F. Warren, R. S. Bush, *ibid.* 77, 182 (1979).
48. E. K. Rofstad, *Br. J. Cancer* **54**, 569 (1986).
49. R. S. Bush *et al.*, *ibid.* **37** (suppl. 3), 302 (1978); J. Overgaard, H. S. Hansen, K. Jorgensen, M. H. Hansen, *Int. J. Radiat. Oncol. Biol. Phys.* **12**, 515 (1986); S. Dische, M. I. Saunders, M. F. Warburton, *ibid.*, p. 1335.
50. A. J. Franko and C. J. Koch, *Radiat. Res.* **96**, 497 (1983).
51. C. M. L. West and R. M. Sutherland, *ibid.* **112**, 105 (1987).
52. E. K. Rofstad, A. Wahl, T. Brustad, *Int. J. Radiat. Oncol. Biol. Phys.* **12**, 975 (1986).
53. R. E. Durand and R. M. Sutherland, *ibid.* **1**, 1119 (1976); R. E. Durand and J. E. Biaglow, *Radiat. Res.* **69**, 359 (1977).
54. T. Hoshino, D. F. Deen, M. E. Williams, Y. Sano, *Cancer Res.* **41**, 4404 (1981); T. T. Kwok and P. R. Twentyman, *Br. J. Cancer* **51**, 211 (1985); R. E. Durand, *J. Natl. Cancer Inst.* 77, 247 (1986).
55. R. M. Sutherland, H. A. Eddy, B. Bareham, K. Reich, D. Vanantwerp, *Int. J. Radiat. Oncol. Biol. Phys.* **5**, 1225 (1979).
56. R. E. Durand and S. M. Brown, *ibid.* **8**, 603 (1982); C. Erlichman and D. Vidgen, *Cancer Res.* **44**, 5369 (1984).
57. R. M. Sutherland, F. Buchegger, M. Schreyer, J.-P. Mach, *Cancer Res.* **47**, 1627 (1987).
58. R. M. Sutherland and R. E. Durand, *Int. J. Radiat. Biol.* **22**, 613 (1972); R. M. Sutherland, *Pharmacol. Ther.* **8**, 105 (1980); G. Adams, *Br. Med. Bull.* **29**, 48 (1973); A. C. Begg, P. W. Sheldon, J. L. Foster, *Br. J. Radiol.* **47**, 399 (1974); J. M. Brown, *Radiat. Res.* **64**, 633 (1975); P. W. Sheldon, J. L. Foster, J. F. Fowler, *Br. J. Cancer* **30**, 560 (1974); J. D. Chapman, A. P. Reuvers, J. Borsa, J. S. Henderson, R. D. Migliore, *Cancer Chemother. Rep.* **58**, 559 (1974); A. M. Rauth and K. Kaufman, *Br. J. Radiol.* **48**, 209 (1975); J. F. Fowler, G. E. Adams, J. Denekamp, *Cancer Treat. Rev.* **3**, 227 (1976).
59. R. C. Urtasun, J. D. Chapman, J. A. Raleigh, A. J. Franko, C. J. Koch, *Int. J. Radiat. Oncol. Biol. Phys.* **12**, 1263 (1986); J. S. Rasey *et al.*, *Radiat. Res.* **102**, 76 (1985); J. A. Raleigh *et al.*, *Br. J. Cancer* **56**, 395 (1987).
60. R. M. Sutherland, *Cancer Res.* **34**, 3501 (1974).
61. D. W. Siemann, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 1029 (1982).
62. L. L. Ling, C. Streffer, R. M. Sutherland, *ibid.* **12**, 1231 (1986).
63. L. L. Ling and R. M. Sutherland, *Br. J. Cancer* **54**, 911 (1986).
64. J. J. Sciandra, G. Michel, R. M. Sutherland, 34th Annual Meeting of the Radiation Research Society, Las Vegas, NV, 12 April 1986 (abstr.), p. 125; in preparation; J. Shen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3278 (1987).
65. H. Acker, J. Carlsson, G. Holtermann, T. Nederman, T. Nylen, *Cancer Res.* **47**, 3504 (1987).
66. R. M. Sutherland, in *Proceedings of International Symposium on Cancer Therapy by Hyperthermia and Radiation* (Washington, DC, 28 to 30 April 1975), p. 99; ——— and R. Sridhar, in *Proceedings of 2nd International Symposium on Cancer Therapy by Hyperthermia and Radiation,* C. Streffer, Ed. (1978), p. 271.
67. J. Landry, E. Lord, R. M. Sutherland, *Cancer Res.* **42**, 93 (1982).
68. E. M. Lord and P. C. Keng, *J. Immunol. Methods* **68**, 147 (1984).
69. E. M. Lord, *Br. J. Cancer* **41**, 123 (1980); H. R. MacDonald and B. Sordat, *Contemp. Top. Immunobiol.* **10**, 317 (1980); K. M. Wilson and E. M. Lord, *Br. J. Cancer* **55**, 141 (1987).
70. L. J. daSilva, M. A. Taupier, S. Reynolds, L. Harwell, E. M. Lord, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 1625 (1984).
71. E. Lord, K. Wilson, G. Siegal, *J. Leukocyte Biol.* **42**, 588 (1987).
72. K. M. Wilson and E. M. Lord, *Cancer Immunol. Immunother.* **23**, 20 (1986).
73. J. M. Yuhas, A. E. Tarleton, J. G. Hartman, *Cancer Res.* **38**, 3595 (1978).
74. M. D. Sherar, M. B. Noss, F. S. Foster, *Nature (London)* **330**, 493 (1987).
75. G. C. Rice, C. Hoy, R. T. Schimke, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5978 (1986).
76. P. J. Tofilon, N. Buckley, D. F. Deen, *Science* **226**, 862 (1984); T. Sasaki, M. Yamamoto, T. Yamaguchi, S. Sugiyama, *Cancer Res.* **44**, 345 (1984).
77. A. Schleich, in *Chemotherapy of Cancer Dissemination and Metastasis*, S. Garattini and G. Franchi, Eds. (Raven, New York, 1973), pp. 51–58; M. M. Mareel, *Int. Rev. Exp. Pathol.* **22**, 65 (1980); G. A. Storme and M. M. Mareel, *Cell Biol. Int. Rep.* **8**, 147 (1984); T. Brauner and D. F. Huelser, Third International Conference on Spheroids in Cancer Research, Cambridge, England, 26 and 27 July 1987 (abstr).
78. H. Eddy and R. Sutherland, unpublished data.
79. Work conducted with D. Penney and N. Kutyreff of the Experimental Pathology/ Ultrasound Facility of the University of Rochester Cancer Center.
80. R. M. Sutherland *et al.*, unpublished data; A. J. Franko, J. D. Chapman, C. J. Koch, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 737 (1982).
81. Work conducted with T. Ceckler and R. L. Howell, University of Rochester.
82. Image obtained by using equipment at the Max Planck Institute for Neurological Research, Cologne, West Germany; provided by W. Mueller-Klieser from a method adapted for spheroids in collaboration with W. Paschen. See W. Paschen, I. Niebuhr, K.-A. Hossman, *J. Neurochem.* **36**, 513 (1981).
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