EDITORIALS

An Added Dimension: Will Three-Dimensional Cultures Improve Our Understanding of Drug Resistance?

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Do you routinely plate tumor cell lines as monolayers on plasticware for experiments? Although most investigators utilize standard monolayer cultures in their experiments, new insights have been garnered in the past few years from cells grown in three dimensions. Various three-dimensional culture techniques are in use, consisting basically of cellular aggregates in spinner cultures (1); on top of coated plastic surfaces, agarose, or extracellular matrix proteins (2); or embedded within agarose or matrix proteins (3). The article by Graham et al. (1) in this issue of the Journal describes an effect of three-dimensional culture on an important, but poorly understood, aspect of cancer progression: drug resistance.

The use of three-dimensional cell culture systems, typically with reconstituted basement membrane components, has revolutionized our understanding of the differentiation of many cell types, including endothelia, salivary gland epithelium, mammary epithelium, hepatocytes, Sertoli's cells, and others (2-4). In a study of mammary epithelial differentiation, for example, Petersen et al. (5) found that three-dimensional culture of human primary mammary epithelial cells within a basement-membrane extract induced the formation of acinus-like structures with central lumens, synthesis of new basement-membrane proteins followed by their secretion to the outside of the acinus, and production of sialomucin and its secretion to the inside of the acinus. None of nine breast carcinoma cultures or cell lines recapitulated these processes (5). Our laboratory also assumed that conventional monolayer culture on plasticware was sufficient until collaborative experiments with Dr. Mina Bissell's laboratory proved otherwise. We recently found that transfected with nm23-H1 complementary DNA, which had earlier been found to induce a 50%-90% reduction in metastatic potential in vivo (6), resulted in no change in cellular differentiation in two-dimensional culture (7). However, in three-dimensional culture, the nm23-H1 transfectants formed acinus-like structures, synthesized and directionally secreted basement-membrane proteins, and synthesized sialomucins. Such characteristics were not exhibited by similarly cultured parental cells or control transfectants (7).

In this issue of the Journal, a group headed by Dr. Robert Kerbel, Sunnybrook Health Science Centre, reports that a single exposure of either murine EMT-6 mammary cells or human MDA-MB-231 human breast carcinoma cells to either 4-hydroperoxycyclophosphamide or cisplatin induced rapid, transient drug resistance upon rechallenge, but only if the cells were exposed to drug as agarose-coated three-dimensional cultures (1). The extent of the observed increase in drug resistance of the EMT-6 cells, measured as surviving fractions, ranged from several fold at lower drug concentrations to several logs at higher drug concentrations. The drug resistance phenotype was accompanied by a more compact three-dimensional spheroid morphology. This report complements previous work by Miller et al. (8), in which murine mammary tumor cell lines were exposed to melphalan, methotrexate, or fluorouracil, either in monolayer culture or as three-dimensional cultures in a collagen gel matrix. The mammary cell lines were as much as several logfold more drug resistant in three-dimensional cultures as compared to the same cells treated as monolayers. Because the tumor cells were not pretreated with a chemotherapeutic agent in that study, Miller and coworkers measured intrinsic drug resistance; however, the principle of increased resistance in pretreated three-dimensional cultures is similar. Several other investigators (9-11) have also noted drug resistance in tumor cells in three-dimensional cultures, but did not directly compare two- and three-dimensional conditions.

Several questions must be addressed to understand the impact of these data. First, is this form of rapid drug resistance, named "multicellular drug resistance," relevant to the clinical course of human tumors? Many patients have tumors that are resistant to chemotherapy at the time of initial treatment or, alternatively, that respond to chemotherapy initially but rapidly develop a...
drug resistance phenotype. It can be hypothesized that the rapid induction of multicellular drug resistance in vitro may represent a similar type of drug resistance to that observed in patients, and it may precede or facilitate additional components of the drug resistance phenotype. Additional support for this hypothesis awaits in vivo implantation of multicellular drug resistant cells to determine if such cells are capable of producing the same drug resistance phenotype in animals.

In previous work published by these investigators (12,13), a correlation was observed between three-dimensional culture and in vivo drug resistance, utilizing a longer term drug resistance model system. Mice that carried EMT-6 tumors were sequentially passaged and treated with cisplatin, carboplatin, cyclophosphamide, or thiopeta over a 6-month period. Tumor cells were then re-implanted into mice and challenged with drugs, whereupon the expected drug resistance was observed. However, plating of these cells in monolayers revealed no evidence of drug resistance in terms of growth inhibition, generation time, or sulfhydryl levels (12). It was concluded that a form of drug resistance, evident only in vivo, had evolved. In a follow-up study, the drug resistance phenotype was observed when the in vivo resistant tumor cells were challenged in three-dimensional cultures on agarose-coated dishes (13). While this correlation of in vivo resistance with behavior in three-dimensional culture is striking, it cannot be assumed that a similar in vivo correlation will hold for the rapidly induced resistance phenotype described in this issue (7). Therefore, injection of the multicellular drug-resistant cells into animals is essential.

Additional questions pertain to what factor(s) cause the multicellular drug resistance phenotype to become manifest? When Teicher et al. (12) originally measured drug uptake and elimination from tumor cells and normal tissues in vivo, absorption of drug was slower and elimination was more rapid in drug-resistant tumors. The converse was true for the kidney, in which animals bearing resistant tumors eliminated drug more rapidly. The data suggested that differences in tumor cell as well as host drug pharmacokinetics may be operative (12). This hypothesis could be supported by the compact nature of the drug-resistant spheroids in three-dimensional cultures, which may impede drug penetration to cells inside (1,13). Another factor deserving consideration is the potential effect of selection in this model system. Do three-dimensional culture conditions select for the growth of an intrinsically more drug-resistant population? Other factors that could also play a role include cell cycle differences between cells in two- and three-dimensional cultures. Miller et al. (8) noted that three-dimensional cultures contained a higher percentage of cells in G0-G1 and fewer cells in S-phase. Hypoxia in the center of a three-dimensional mass may induce stress responses and other relevant phenotypic changes [reviewed in (8)]. Alterations in cell-cell adherence and communication may also be relevant, since doxorubicin-resistant spheroids of a murine mammary carcinoma cell line exhibited increased numbers of gap-junctions (11). Perhaps most interesting is the observation by Teicher et al. (12) that collagen fibers were observed in the extracellular matrix of all long-term resistant EMT-6 lines, but not in the parental line in vivo. These data suggest that the breast tumor cells may have induced their own three-dimensional matrix system in vivo, and that cell-matrix interactions may play a pivotal role in determining drug resistance.

Additional experiments involving other therapeutic agents, expanded dose-response testing, and evaluation of more cell lines will hopefully complement in vivo data to further assess the implications of rapid multicellular drug resistance. A potentially important application of this and other three-dimensional assays may be found in experimental drug testing. In addition to experiments using monolayer cultures, various investigators have utilized three-dimensional culture techniques that are closely related, such as spheroid culture and soft-agar colony formation assays, in experimental drug-testing efforts where careful control of assay variables and appropriate calibration have been achieved [e.g., (14-16)]. The Developmental Therapeutics Program of the National Cancer Institute is currently evaluating an alternate approach to achieving three-dimensional growth of breast cancer cells, as well as other types of human malignancy, for its potential utility in determining if a prospective antitumor agent is likely to have a favorable in vivo therapeutic index. Tumor cells are inoculated into capillary hollow fibers and fiber segments are implanted into mice intraperitoneally and subcutaneously; the mice are then treated with a potential antitumor agent, and subsequently fibers are removed for ex vivo evaluation of the tumor cells (17,18). The hollow fiber technique permits inoculation and/or growth over a wide range of cell densities in three dimensions, provides rapid quantitation indices of growth and drug sensitivity, and is amenable to histopathological evaluations.

The article by Graham et al. (1) presents the first description of the rapid acquisition of multicellular drug resistance by tumor cells exposed once as three-dimensional cultures to alkylating agents. Equally important, it serves to focus our attention on the careful assessment of specific cell cultivation parameters that influence tumor biology and expression of drug resistance phenotype(s). We remain hopeful that these and other experimental observations concerning manifestations of drug resistance will improve our understanding of human malignancies and lead to the identification of more effective therapeutic intervention measures for cancer patients.

References
(7) Howlett A, Petersen O, Steeg P, et al: A novel function for Nm23: overexpression in human breast carcinoma cells leads to the formation of base-
The detection of early-stage tumors followed by surgical resection remains the cornerstone of cancer therapy. Unfortunately, many patients present with advanced disease not amenable to surgical resection. Furthermore, efforts to screen patients for colorectal cancer through the use of stool guaiac tests remain controversial (1,2). Clearly, more reliable and effective means of early detection need to be developed.

Colorectal cancer became a model for cancer progression based on the elucidation of genetic changes that accumulate during histopathologic progression. Early steps in progression included inactivation of APC and activation of ras (3). It became evident that these genetic changes could serve as specific markers for cancer detection, eventually leading to the discovery that ras gene mutations could be identified in stool DNA from patients with colorectal cancer (4). This polymerase chain reaction (PCR)-based approach for cancer detection held the promise of providing cost-effective and reliable molecular screening for many patients.

In this issue of the Journal, Tobi et al. (5) report the ability to detect K-ras gene mutations prior to the development of colorectal cancer in high-risk patients. With the use of an enriched PCR technique, they demonstrated mutations of codon 12 in the K-ras gene in approximately 40% of high-risk patients. These included patients with a strong family history of colorectal cancer, some of whom had previously been found to harbor adenomas. Importantly, there was no evidence of clonal K-ras gene mutations in patients with inflammatory bowel disease or those without a family or clinical history of colorectal cancer. Tobi et al. did not report if more than one clonal population of cells harboring different K-ras gene mutations was occasionally detected. Kern et al. (6) have found more than one K-ras clone during analysis of neoplastic patches in colitis patients. The number of mutations may also have been under-represented, since the authors did not test for mutations of codon 13. Tobi et al. have also used a test that is moderate in sensitivity (one in 10^5 cells), similar to that used previously in other studies. Assays that detect rare gene mutations in excess of 1 in 10^6 may be too sensitive (7). These tests may detect the fixation of mutations before clonal expansion and may occur too early in clinical progression for intervention.

Following our initial identification of K-ras gene mutations in the stool, others (8,9) have demonstrated the ability to detect K-ras gene mutations in both gastric aspirates and stool specimens from patients with pancreatic cancer. Furthermore, K-ras gene mutations and p53 mutations have been detected before any clinical evidence of colorectal cancer. Although pilot studies (4,8,9) concentrated on the ability to document detection of gene mutations from patients with established cancer, the real value of these screening techniques will depend on their ability to accurately predict the occurrence of a neoplasm before clinical symptoms develop. The appropriate selection of high-risk patients and control subjects in this work now adds credence to the development of molecular diagnostic tests for asymptomatic patients.