Three-Dimensional Histoculture: Origins and Applications in Cancer Research

Robert M. Hoffman

The ability to grow cells in monolayer culture has afforded investigators the opportunity to study many aspects of cancer cell biology under carefully controlled conditions. Nonetheless, an important factor that has often been overlooked is that cells in this configuration undergo a loss of structural integrity that may significantly alter their functional properties. Three-dimensional histoculture represents a useful alternative approach to monolayer culture because it preserves the native architecture of cells while still allowing ease of experimental manipulation. This review discusses the origins of three-dimensional cultures, the potential application of these cultures to assays of tumor cell metastasis and drug sensitivity, and the evidence from gene expression studies that these cultures may be more realistic tumor models than cell monolayers.

In 1912, Alexis Carrel took a small fragment from the heart of an 18-day-old chick embryo and explanted it on hypotonic plasma.[1] Over a period of 3 months, he passaged the heart tissue 18 times, and observed that it not only remained viable but it also continued its rhythmic beating. This was the first demonstration that explanted tissue could retain normal function in vitro for an extended period of time.

Despite this evidence of biological activity, Carrel noted that the cultured tissue did not increase in size and that it assumed a spherical shape over time. He postulated that the proliferation rate of cells in the culture was determined by their accessibility to nutrients in the medium: Cells at the periphery would be expected to grow readily, whereas those positioned internally would tend to necrose. To circumvent this problem, Carrel began culturing tissue on a silk veil, which prevented the plasma of the culture from retracting and becoming spherical. Thus, as early as 1912, the main tenets of three-dimensional tissue culture—or "histoculture" as it is now known[2]—were set down.

EXPERIMENTAL APPROACHES TO THREE-DIMENSIONAL HISTOCULTURE

Sponge-matrix Culture

By 1951, monolayer cell cultures, in which cells grow as "sheets" on the surfaces of glass or plastic, had become the predominant culture technique. Such monolayer cultures had rather limited use in histogenetic studies or for pathological diagnosis, however, and in view of this Leighton[3] returned to Carrel's original idea of three-dimensional culture. Leighton improved the earlier system by using a tissue support consisting of cellulose sponges surrounded by plasma clots, contained within glass tubes. On each sponge, he placed 1–4 fragments of tissue, ranging in size from 1–5 mm^3, along with a single drop of chicken plasma and a drop of diluted chick embryo extract. After the plasma clotted and the tissue fragments firmly adhered to the sponge, he added a nutrient mixture and placed the cultures in a roller apparatus.

Leighton grew a number of tissues, including C3HBA mouse mammary adenocarcinoma, on this cellulose sponge matrix system. He observed that the adenocarcinoma outgrowth on the sponge matrix consisted of aggregations of cells that were organized much like the cells in the original tumor. Moreover, the cultures contained structures with distinct lumina, as well as stromal elements (mainly spindle-shaped fibroblasts and histiocytes) associated with some of the glandular structures. Such stromal elements are an important part of in vivo tissue architecture, presumably because they mediate critical interactions between various tissue constituents.

In other experiments, Leighton cultured fragments of chick embryo liver on the sponge system, and found that the proliferating epithelial cells formed filaments, irregular oval masses, and glandular structures. Such cultures contained masses of necrotic epithelial cells in the interstices in the central part of the sponge. Hepatoma cultures were found to behave differently than cultures of normal liver cells; the tumor cells grew in a loosely packed arrangement and, in areas where the plasma clots had been liquified, the cells were in direct contact with the liquid nutrient.[4]

Leighton also tested natural and gelatin sponges for their ability to support the organized aggregations of cells. Although gelatin sponges proved to be more conducive to histological sectioning than cellulose sponges, they were more prone to digestion by enzymes released by the cultured tissues and tumors. Overall, collagen-coated cellulose

Laboratory of Cancer Biology, University of California, San Diego, La Jolla, California 92093-0609 USA; and Anti-Cancer, Inc., San Diego, California 92110 USA

 © by Cold Spring Harbor Laboratory Press ISSN 1042-2196/91 $1.00

sponges were shown to be optimal for supporting the native architecture of tissue.[5] Table 1 summarizes the properties of the sponge matrix that are important for its ability to maintain the native structure of tissues in culture.

Collagen-gel Culture

A second approach to three-dimensional histoculture is to embed cells or tissue fragments in collagenous gels. In 1979, Nandi and colleagues developed a technique for embedding cells in gels prepared from rat tail collagen fibers.[6] They enzymatically dissociated cells from normal or malignant rat mammary tissue and embedded them in collagen, either as a cluster or dispersed throughout the gel. In the former case, the cells formed relatively thick duct structures that radiated into the matrix in three dimensions. The dispersed method of explantation also led to three-dimensional growth but the duct structures were not as thick. The cells in these collagen gel cultures grew and divided over a period of several weeks, with proliferation occurring mainly at the periphery of the cell mass. Very few fibroblasts grew in this system; this is in marked contrast to monolayer cultures, where fibroblasts often overgrow epithelial cells. Histologically, the mammary tissue grown in vitro resembled that in vivo, and the tissue could be recultured in new collagen gels. If the cells were plated in monolayer culture, however, there was a decrease in cell number as a function of time.

The authors postulated that a key reason for the success of cultures in collagen gels was that the *shape* of the mammary cells differed in collagen versus conventional monolayers.[6] Shape is critical in the control of epithelial cell growth[7,8] and, as discussed later, may also be important in eliciting hormone and drug responsiveness in epithelial cells.[9–13] In addition, many of the advantages of sponge culture detailed by Leighton (Table 1) may apply equally to collagen-gel culture.

TABLE 1
Properties of the Sponge Matrix that are Important for the Maintenance of Native Tissue Architecture in Culture[a]

- Displays a particular spatial arrangement of trabeculae that enables cells to grow and migrate in all directions
- Creates a large increase in surface area, which supports the growth of epithelial cells
- Reduces the diffusion rate within cell aggregates, which may allow retention of critical factors secreted by cells
- Permits the development of morphological diffusion gradients that may influence tissue differentiation
- Creates a "wick" that may substitute for capillary circulation in allowing intercellular exchange of metabolites

[a]For details, see ref. 4.

Filter- or Mesh-Supported Organ Culture

"Organ-culture" is a histoculture technique that was developed by Strangeways[14] and independently by Fell and Robinson.[15] Tissue explants were grown originally on lens paper in a watch glass[15,16] containing liquid culture medium, but more recently have been grown on filters or mesh. Elias was the first to demonstrate the potential importance of three-dimensional organ culture for the study of cancer.[17–20] In monitoring organ cultures of mouse mammary tissue, he noted that the ductal alveoli began to degenerate after 5 days in culture unless the medium was supplemented with hormones. If estrogen and progesterone were added, the histology of the alveoli resembled that seen in vivo. In contrast to the hormone dependency of normal and most preneoplastic alveoli for survival in culture, spontaneous mammary tumor tissue survived the 5- and 10-day culture periods without hormone supplements. The neoplastic epithelium also differed from normal or preneoplastic epithelium in that the central part of the tumor explants became necrotic after a few days in culture. This effect was not altered by any of the hormonal combinations added to the culture medium. Later work by Moretti showed that glucose uptake by normal and preneoplastic mammary tissue is stimulated by insulin, whereas that by tumor tissue is insensitive to this hormone.[21]

Other important features of three-dimensional organ culture were revealed in studies of gastrointestinal tissue.[22–26] Under normal culture conditions, the epithelium of such tissues became necrotic after a 15-min incubation at 37°C and had completely disappeared after 1 hr. However, Browning and Trier showed that it was possible to maintain fragments of human small intestine in culture for 24 hr simply by keeping the cultured tissue in contact with, but not immersed in, the medium.[23] Methods for preventing total immersion include attachment of the fragment to a matrix or platform that raises it above the medium level,[27–29] and a rocking culture chamber[30–32] that alternately exposes the culture to medium and atmosphere. With the use of a rocking chamber and an atmosphere containing 95% oxygen, survival of colonic mucosa was possible for up to 6 weeks.[33]

Spheroids

Multicell spheroids represent yet another system for histoculture. Developed 20 years ago by Sutherland and colleagues,[34] spheroids are three-dimensional configurations of cells formed by the aggregation of individual cells during rotation in spinner flasks.[35–37] Spheroids mimic tumors in at least two respects. First, they undergo necrosis, the initiation of which appears to be controlled by nutrient and oxygen

deprivation.[38] Second, as they enlarge, they acquire a genetic instability that correlates with the appearance of tetraploid cells; these tetraploid cells arise in the outer proliferating regions of the culture and gradually move deeper in the spheroid, where they become quiescent.[39] The quiescent cells regain their proliferative capacity after dispersal from the spheroid system and transfer to a more favorable growth environment.[35]

APPLICATIONS OF THREE-DIMENSIONAL HISTOCULTURE

Studies of Tumor Cell Migration and Invasion

Given its architectural resemblance to native tissue, three-dimensional tissue culture represents a unique model for investigating crucial events in tumor metastasis such as tumor cell migration and invasion. In his early studies, Leighton made several interesting observations that may be relevant to metastasis in vivo.[3,4,40–44] He noted, for example, that human tumor cell lines grown in three-dimensional culture seemed to migrate as cell aggregates rather than as individual cells. Conceivably, this physical interaction may reflect a metabolic cooperation between tumor cells that enhances their resistance to natural host defenses and to exogenously administered antitumor agents.

Leighton's work with three-dimensional cultures also led him to postulate that the invasion of some tumor cells into connective tissue may involve a mechanism of cell overgrowth. This proposal was based on his experiments with a combined plasma clot–cellulose sponge matrix that he inoculated first with connective tissue cells and then (after allowing time for proliferation) with tumor cells. When the inoculated tumor cells were HeLa cells, they increased in number, spread into the interstices of the sponge, and ultimately replaced the connective tissue cells. The most extensive invasion occurred where the connective tissue growth was the most dense, and the tumor cells seemed to follow the polarity of the fibroblasts during the invasion process.

Drug Sensitivity Assays

Another important application of three-dimensional culture is in the study of drug resistance. At present, the majority of in vitro assays for predicting drug resistance are based on the response of cells grown in monolayer culture. While these assays have played an important role in studies of resistance mechanisms, their ability to predict drug resistance in vivo has occasionally come into question, prompting the development of alternative types of assays.

There is now considerable evidence that cells growing in multicell spheroids—particularly those positioned in the inner-cell layers of the spheroids—are more resistant to cytotoxic agents than are cells in dispersed culture.[46–59] In comparative studies of drug response in spheroids versus single-cell suspensions, Bhuyan's group found that spheroids were much more resistant to antimetabolites such as cytosine arabinoside and methotrexate (>11-fold and >125-fold, respectively), and somewhat more resistant to vincristine and doxorubicin.[36] Alkylating agents were found to affect both cellular configurations equally. More recently, these workers have shown that monolayer cultures of colon carcinoma cells are sensitive to Piercidin C whereas colon tumors in vivo and, importantly, three-dimensional slices of the tumors grown in vitro are resistant to the drug.[52]

Many other studies have demonstrated an elevated level of drug resistance in multicell spheroids compared to cell monolayers. This has been reported for the combination of Chinese hamster ovary (CHO) cells and doxorubicin,[60] a human squamous lung cancer cell line and doxorubicin,[61] a human cervical carcinoma cell line and vincristine,[57] and CHO cells and mitoxantrone.[62]

As shown by Jääskeläainen et al.,[53] the elevated resistance of spheroids appears to extend to natural defense mechanisms as well as cytotoxic drugs. These workers compared the sensitivity of glioma cells, grown either as spheroids or as single-cell suspensions, to killing by lymphokine-activated killer (LAK) cells. Although the LAK cells penetrated equally well into the spheroids and cell suspensions, the tumor cells were lysed to a much lesser extent when they were in the spheroid configuration.

Investigators initially attributed the drug resistance of spheroids to two main factors: (1) poor diffusion of the drugs to the interior of the culture, and (2) the relatively high proportion of nondividing cells in the culture, a result of nutrient deprivation and hypoxia. It could be argued that such factors are not relevant in vivo because most tumors develop a rich vascular network to mediate transport of nutrients and oxygen. Elegant studies by Heppner and co-workers, however, have provided convincing evidence that the three-dimensional cultures are more accurate models of drug resistance in vivo than are cell monolayers.[63–65] These researchers embedded cell clusters or tumor fragments in a collagen gel matrix, exposed the cultures to various cytotoxic drugs, and then quantitated tumor growth by planimetry. Cells grown under these conditions were found to be less sensitive to the drugs tested than were cells grown as monolayers on collagen or plastic. In some cases, the difference in sensitivity was pronounced; for example, with mouse mammary adenocarcinoma cell lines, the IC_{90} for doxorubicin was 100-fold greater in three-dimensional cultures than in monolayers. The drugs melphalan, methotrexate, and 5-fluorouracil caused exponential reductions in the growth rates of mouse

mammary tumor cells in monolayer cultures, but they inhibited the growth of cells in collagen gel cultures to a much lesser degree (1,000-fold less for melphalan, 300-fold less for methotrexate, and 50-fold less for 5-fluorouracil). Some histocultures in collagen continued to grow in the presence of drug concentrations that reduced survival to <0.1% in monolayer cell cultures. If cells in collagen gel cultures were replated as monolayers, they then became sensitive to the drugs.

Interestingly, when Heppner and her colleagues treated the monolayer cell cultures with melphalan and 5-fluorouracil, and then transferred the cells to collagen gels, the cultures became resistant to the drugs. This result suggests that poor drug diffusion is not a key factor underlying the greater drug resistance of spheroids. A more likely possibility is that cells in the three-dimensional configuration assume particular shapes or attain degrees of differentiation that are important for the mechanism of drug resistance. Studies of the mechanism of drug resistance in these three-dimensional cultures may provide an explanation for the finding that tumors can sometimes be resistant in vivo even though cells prepared from the tumors are sensitive in monolayer cultures.

Our laboratory has adapted Leighton's method of three-dimensional histoculture on collagen sponge gels for use in predictive assays of chemotherapeutic response and for evaluation of new anticancer drugs.[66–70] As an endpoint for drug response, we measure [^{3}H]thymidine incorporation by histological autoradiography or monitor fluorescent dye exclusion by confocal microscopy. These collagen–sponge–gel histocultures closely approximate the in vivo state in that the tissues grow in three dimensions and contain not only the tumor cells but also stromal cells (e.g., fibroblasts, lymphocytes) and extracellular matrix components, all maintained in native architecture (Fig. 1). The main advantages of this system are summarized in Table 2.

We have successfully cultured over 25 types of human tumors and many corresponding normal tissue types in this way. In addition to its predictive ability regarding chemotherapeutic response, the degree of proliferation of cells within the histocultures has been shown to correlate with tumor stage and grade in the case of breast and ovarian tumors,[69] and with tumor phenotype (small-cell vs. non-small-cell) in the case of lung tumors.[70] Perrapato et al. have recently used this native-state method for drug-response analysis of urological tumors.[71] They reported a high culture success ratio, longevity in culture, maintenance of primary histopathology, and reproducible chemosensitivity.

As with any in vitro model, three-dimensional histoculture has certain limitations that must be considered when interpreting data. One drawback is the inherent heterogeneity of tumors as opposed to cloned cell lines. Another is the quantification of specific subpopulations of cells, which is technically more difficult in histocultures than in monolayer cultures.

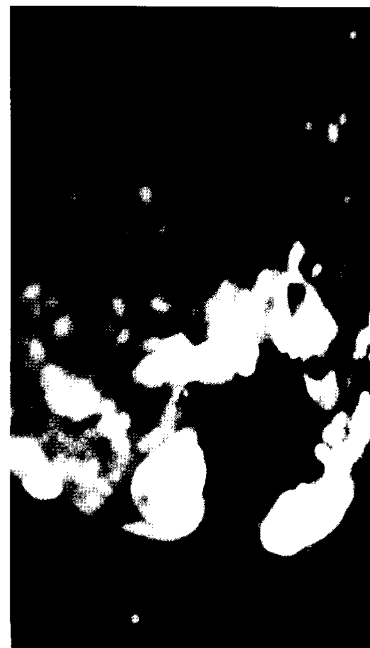

FIGURE 1
Micrograph of a human colon tumor 14 days in histoculture. Note the three-dimensional structure, including the mouth of a malignant crypt. Image was obtained by confocal scanning laser microscopy. Magnification, 1000×.

THE INFLUENCE OF THREE-DIMENSIONALITY ON GENE EXPRESSION AND MAINTENANCE OF SPECIFIC ANTIGENS

A variety of experiments indicates that tissues grown in three-dimensional culture are more likely to retain in vivo

TABLE 2
Advantages of Native State Histoculture for Drug Sensitivity Assays

- Allows growth of tissues in three dimensions and preservation of native tissue architecture
- Allows assay of cell proliferation by histological autoradiography and polarization microscopy, which yields high-resolution data
- Allows determination of cell viability by the use of fluorescent dyes and confocal scanning-laser microscopy
- Offers a very high rate of evaluation (≥80%) of drug response for all tumor types
- Allows simultaneous testing of multiple agents
- Requires relatively small amounts of tissue
- Produces a low frequency of false negatives[a]
- Allows normal and tumor tissue to be cultured in parallel, so that the tumor specificity of antineoplastic agents can be assessed
- Allows assay of wide range of antineoplastic agents (e.g., cytotoxics, biological response modifiers, activated immune cells)

[a]The frequency of false negative results was assessed by correlative drug response studies in vitro and in vivo (R.M. Hoffman, T. Kubota, and K.M. Connors, unpubl.).

patterns of gene expression than are cell monolayers. In 1977, Emerman et al.[9] made the observation that, in response to certain hormones, normal mouse mammary epithelial cells cultured in vitro undergo a specific differentiation-associated change, that is, secretion of the milk protein casein. Notably, this hormone-induced change occurred only when the cells were cultured on floating, detached collagen gels—not when they were cultured on attached gels or on plastic. The authors postulated that the *shape* of the cultured cells was important for their response to hormone. In further investigation of this phenomenon, Bissell et al.[72] reported that a significant fraction (30–40%) of mammary epithelial cells cultured on floating gels released β-casein after 6 days in culture, as compared to only 2–10% of control cells cultured on plastic.

Other workers have compared the expression of tumor-specific antigens in cultured tumor cells and tissue. Schlom et al.[73], for example, reported that 44% of human breast carcinoma and 80% of colon carcinoma biopsy specimens tested express the tumor-associated glycoprotein TAG-72, but only ~4% of breast cancer cell lines and ~6% colon cancer cell lines express the antigen. Moreover, in cell lines that were positive for the antigen, expression was traced to a small fraction of cells in each culture. When these workers grew the cell lines as monolayers on extracellular matrix proteins such as collagen, laminin, fibrin, and fibronectin (whether in serum-containing or serum-free medium), they saw no enhancement of TAG-72 expression. In contrast, they did see a significant increase in TAG-72 expression when they grew a human colon cancer cell line in three dimensions as spheroids or in agar. Upon subcutaneous injection into nude mice, these cells developed into tumors that expressed TAG-72 at a level comparable to that seen in the metastatic tumor mass from patients. Studies from our own laboratory have shown that when tissues are explanted and cultured on collagen–gel–sponge matrix in native-state histoculture, the levels of TAG-72 as well as carcinoembryonic antigen (CEA) expression are similar to those found in the precultured material derived from the patients.[74] Similarly, Menezes et al. (ref. 75 and pers. comm.) have shown that cells from a parotid-derived muco-epidermoid carcinoma were negative for mucin expression when cultured as monolayers, but were positive when grown in agar in three dimensions.

Recent experiments in R. Kerbel's laboratory (pers. comm.) have shown that MDA-MB-231 breast cancer cells in monolayer cultures constitutively produce high levels of transforming growth factor-β (TGF-β) mRNA, and these levels are not affected by the addition of exogenous TGF-β. In contrast, spheroid configurations of the same cells produce TGF-β mRNA only in response to exogenously added TGF-β. In addition, the TGF-β secreted from monolayers is inactive, whereas that secreted from spheroids is active. This and the other examples cited above illustrate that the pattern of gene expression in cultured cells can vary greatly according to their configuration, and that the three-dimensional configuration may more fully approximate the in vivo situation.

ACKNOWLEDGMENTS

This review is dedicated to the seventieth birthday of Professor Joseph Leighton for his founding role in three-dimensional histoculture. The manuscript was expertly word-processed by Polly Jayne Pomeroy and Susan Webb, whom we greatly appreciate.

REFERENCES

1. Carrel, A. 1912. On the permanent life of tissues outside the organism. *J. Exp. Med.* **15**: 516–528.
2. Sherwin, R.P., A. Richters, A.E. Yellin, and A.J. Donovan. 1980. Histoculture of human breast cancers. *J. Surg. Oncol.* **13**: 9–20.
3. Leighton, J. 1951. A sponge matrix method for tissue culture. Formation of organized aggregates of cells *in vitro*. *J. Natl. Cancer Inst.* **12**: 545–561.
4. Leighton, J. 1954. The growth patterns of some transplantable animal tumors in sponge matrix tissue culture. *J. Natl. Cancer Inst.* **15**: 275–293.
5. Leighton, J., G. Justh, M. Esper, and R. Kronenthal. 1967. Collagen-coated cellulose sponge: Three-dimensional matrix for tissue culture of Walker tumor 256. *Science* **155**: 1259–1261.
6. Yang, J., J. Richards, P.D. Bowman, R. Guzman, J. Enami, K. McCormick, S. Hamamoto, D. Pitelka, and S. Nandi. 1979. Sustained growth and three-dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. *Proc. Natl. Acad. Sci.* **76**: 3401–3405.
7. Folkman, J. and A. Moscona. 1978. Role of cell shape in growth control. *Nature* **273**: 345–349.
8. Gospodarowicz, D., G. Greenburg, and C.R. Birdwell. 1978. Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. *Cancer Res.* **38**: 4155–4171.
9. Emerman, J., J. Enami, D. Pitelka, and S. Nandi. 1977. Hormonal effects on intracellular and secreted casein in cultures of mouse mammary epithelial cells on floating collagen membranes. *Proc. Natl. Acad. Sci.* **74**: 4466–4470.
10. Yang, J., J. Enami, and S. Nandi. 1977. Regulation of mammary tumor virus production by prolactin in BALB/cfC3H mouse normal mammary epithelial cells *in vitro*. *Cancer Res.* **37**: 3644–3647.
11. Sakai, S., P.D. Bowman, J. Yang, K. McCormick, and S. Nandi. 1979. Glucocorticoid regulation of prolactin receptors on mammary cells in culture. *Endocrinology* **104**: 1447–1449.
12. Enami, J., J. Yang, and S. Nandi. 1979. Simultaneous production of casein and mammary tumor virus in mouse mammary epithelial cells grown on floating collagen gels. *Cancer Lett.* **6**: 99–105.
13. Michalopoulos, G., G. Sattler, and H. Pitot. 1978. Hormonal regulation and the effects of glucose on tyrosine aminotransferase activity in adult rat hepatocytes cultured on floating collagen membranes. *Cancer Res.* **38**: 1550–1555.
14. Strangeways, T.S.P. 1924. *Tissue culture in relation to growth and differentiation.* W. Heffer and Sons, Ltd., Cambridge, U.K.
15. Fell, H.B. and R. Robinson. 1929. The growth, development and phosphatase activity of embryonic avian femora and end-buds cultivated *in vitro*. *Biochem. J.* **23**: 767–784.
16. Chen, J.M. 1954. The cultivation in fluid medium of oganised liver, pancreas and other tissues of foetal rats. *Exp. Cell Res.* **7**: 518–529.
17. Elias, J.J. 1957. Cultivation of adult mouse mammary gland in hormone-enriched synthetic medium. *Science* **126**: 842–844.

18. Elias, J.J. 1959. Effect of insulin and cortisol on organ cultures of adult mouse mammary gland. *Proc. Soc. Exp. Biol. Med.* **101**: 500–502.
19. Elias, J.J. and E. Rivera. 1959. Comparison of the responses of normal, precancerous, and neoplastic mouse mammary tissues to hormones *in vitro*. *Cancer Res.* **19**: 505–511.
20. Elias, J.J. 1961. Stimulation of secretion by insulin in organ cultures of mouse mammary duct end-buds. *Anat. Rec.* **139**: 224. (Abstr.)
21. Moretti, R.S. 1960. M.A. thesis, "Effect of insulin on the maintenance and glucose uptake of normal and neoplastic mouse mammary tissues in organ culture." University of California, Berkeley.
22. Moog, F. and M.H. Kirsh. 1955. Quantitative determination of phosphatase activity in chick embryo duodenum cultured in fluid media with and without hydrocortisone. *Nature* **175**: 722–723.
23. Browning, T.H. and J.S. Trier. 1969. Organ culture of mucosal biopsies of human small intestine. *J. Clin Invest.* **48**: 1423–1432.
24. Hijmans, J.C. 1972. Effect of colchicine and hydrocortisone on invertase activity in the duodenum of the chick embryo. *Gastroenterology* **63**: 279–287.
25. DeRitis, G., Z. Falchuk, and J. Trier. 1975. Differentiation and maturation of cultured rat jejunum. *Dev. Biol.* **45**: 304–317.
26. Levine, R.R., W.F. McNary, P.J. Kornguth, and R. LeBlanc. 1970. Histological reevaluation of everted gut technique for studying intestinal absorption. *Eur. J. Pharmacol.* **9**: 211–219.
27. Defries, E. and L.M. Franks. 1977. An organ culture method for adult colon from germfree and conventional mice: Effects of donor age and carcinogen treatment on epithelial mitotic activity. *J. Natl. Cancer Inst.* **58**: 1323–1328.
28. Schiff, L. 1975. Organ cultures of rat and hamster colon. *In Vitro* **11**: 46–49.
29. Mak, K.M. and W.W. Chang. 1978. Inhibition of DNA synthesis by 1,2-dimethylhydrazine and methylazoxymethanol acetate in rabbit colon mucosa in organ culture. *J. Natl. Cancer Inst.* **61**: 799–804.
30. Autrup, H., G.D. Stoner, F. Jackson, C.C. Harris, A.K. Shamshuddin, L.A. Barrett, and B.F. Trump. 1978. Explant culture of rat colon: A model system for studying the metabolism of chemical carcinogens. *In Vitro* **14**: 868–877.
31. Autrup, H., L.A. Barrett, F.E. Jackson, M.L. Jesudason, G. Stoner, P. Phelps. B.F. Trump, and C.C. Harris. 1978. Explant culture of human colon. *Gastroenterology* **74**: 1248–1257.
32. Shamshuddin, A.K., L.A. Barrett, H. Autrup, C.C. Harris, and B.F. Trump. 1978. Long-term organ culture of adult rat colon. *Pathol. Res. Pract.* **163**: 362–372.
33. Reiss, N.B., N.T. Teland, and G.M. Williams. 1983. The application of organ culture to the study of colon carcinogenesis. In *Experimental colon carcinogenesis* (ed. H. Autrup and G. Williams). CRC Press, Boca Raton.
34. Inch, W.R., J.A. McCredie, and R.M. Sutherland. 1970. Growth of nodular carcinomas in rodents compared with multi-cell spheroids in tissue culture. *Growth* **34**: 271–282.
35. Durand, R.E. 1990. Multicell spheroids as a model for cell kinetic studies. *Cell Tissue Kinet.* **23**: 141–159.
36. Li, L.H., B.K. Bhuyan, and T.L. Wallace. 1989. Comparison of cytotoxicity of agents on monolayer and spheroid systems. *Proc. Am. Assoc. Cancer Res.* **30**: 2435. (Abstr.)
37. Sutherland, R.M. 1988. Cell and environmental interactions in tumor microregions: The multicell spheroid model. *Science* **240**: 177–184.
38. Franko, A.J. and R.M. Sutherland. 1979. Oxygen diffusion distance and development of necrosis in multicell spheroids. *Radiat. Res.* **79**: 439–453.
39. Olive, P.L., J.C. Leonard, and R.E. Durand. 1982. Development of tetraploidy in V79 spheroids. *In Vitro* **18**: 708–714.
40. Leighton, J. 1959. Aggregate replication, a factor in the growth of cancer. *Science* **129**: 466–467.
41. Leighton, J. 1960. The propagation of aggregates of cancer cells: Implications for therapy and a simple method of study. *Cancer Chemother. Rep.* **9**: 71–72.
42. Leighton, J., R.L. Kalla, J.M. Turner, and R.H. Fennell. 1960. Pathogenesis of tumor invasion. II. Aggregate replication. *Cancer Res.* **20**: 575–586.
43. Leighton, J., R.L. Kalla, I. Kline, and M. Belkin. 1959. Pathogenesis of tumor invasion. I. Interactions between normal tissues and "transformed" cells in tissue culture. *Cancer Res.* **19**: 23–27.
44. Leighton, J. 1962. In *Biological interactions of normal and neoplastic growth: A contribution for the host-tumor problem* (ed. M.J. Brennan and W.L. Simpson), pp. 681–702. Little Brown and Company, Boston.
45. Seftor, E.A., R.E.B. Seftor, and M.J.C. Hendrix. 1990. Selection of invasive and metastic subpopulations from a heterogeneous human melanoma cell line. *BioTechniques* **9**: 324–331.
46. Nederman, T. 1984. Effects of vinblastine and 5-fluorouracil on human glioma and thyroid cancer cell monolayers and spheroids. *Cancer Res.* **44**: 254–258.
47. Erlichman, C. and D. Vidgen. 1984. Cytotoxicity of adriamycin in MGH-U1 cells grown as monolayer cultures, spheroids, and xenografts in immune-deprived mice. *Cancer Res.* **44**: 5369–5375.
48. Frayer, J.P. and R.M. Sutherland. 1980. Selective dissociation and characterization of cells from different regions of multicell tumor spheroids. *Cancer Res.* **40**: 3956–3965.
49. Durand, R.E. 1981. Flow cytometry studies of intracellular adriamycin in multicell spheroids *in vitro*. *Cancer Res.* **41**: 3495–3498.
50. West, G.W., R. Weichselbaum, and J.B. Little. 1980. Limited penetration of methotrexate into human osteosarcoma spheroids as a proposed model for solid tumor resistance to adjuvant chemotherapy. *Cancer Res.* **40**: 3665–3668.
51. Nederman, T., J. Carlsson, and M. Malmqvist. 1981. Penetration of substances into tumor tissue—A methodological study on cellular spheroids. *In Vitro* **17**: 290–298.
52. Smith, K.S., G.J. Badiner, F.G. Adams, D.K. Wilson, L.H. Li, and B.K. Bhuyan. 1990. Modified 2-tumor (L1210, colon 38) assay to screen for solid tumor selective agents. In *Proceedings of the symposium on anticancer drug discovery and development*, Detroit, Michigan. (In press.)
53. Jääskeläinen, J., P. Kalliomäki, A. Pactau, and T. Timonen. 1989. Effect of LAK cells against three-dimensional tumor tissue. In vitro study using multi-cellular human glioma spheroids as targets. *J. Immunol.* **142**: 1036–1045.
54. Bhuyan, B.K., B.E. Loughman, T.J. Fraser, and K.J. Day. 1976. Comparison of different methods of determining cell viability after exposure to cytotoxic compounds. *Exp. Cell Res.* **97**: 275–280.
55. Kerr, D.J., T.E. Wheldon, A.M. Kerr, R.I. Freshne, and S.B. Kaye. 1986. The effect of adriamycin and 4′-deoxydoxorubicin on cell survival of human lung tumour cells grown in monolayer and as spheroids. *Br. J. Cancer* **54**: 423–429.
56. Soranzo, C. and A. Ingrosso. 1988. A comparative study of the effects of anthracycline derivatives on a human adenocarcinoma cell line (LoVo) grown as a monolayer and as spheroids. *Anticancer Res.* **8**: 369–373.
57. Wibe, E. 1980. Resistance to vincristine of human cells grown as multicellular spheroids. *Br. J. Cancer* **42**: 937–941.
58. Wibe, E. and R. Oftebro. 1981. A study of factors related to the action of 1-propargyl-5-chlorpyrimidin-2-one (NY 3170) and vincristine in human multicellular spheroids. *Eur. J. Cancer Clin. Oncol.* **17**: 1053–1059.
59. Wilson, W.R., G.F. Whitmore, and R.P. Hill. 1981. Activity of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide against Chinese hamster cells in multicellular spheroids. *Cancer Res.* **41**: 2817–2822.
60. Sutherland, R.M., H.A. Eddy, B. Bareham, N.K. Reich, and D. Vanantwerp. 1979. Resistance to adriamycin in multicellular spheroids. *Int. J. Radiat. Oncol. Biol. Phys.* **5**: 1225–1230.
61. Kerr, D.J., T.E. Wheldon, S. Hydns, and S.B. Kaye. 1988. Cytotoxic drug penetration studies in multicellular tumor spheroids. *Xenobiotica* **18**: 641–648.
62. Bichay, T.J. and W.R. Inch. 1987. Resistance of V79 multicell spheroids to mitoxantrone: Drug uptake and cytotoxicity. *Cancer Drug Delivery* **4**: 201–211.
63. Lawler, E.M., F.R. Miller, and G.H. Heppner. 1983. Significance of three-dimensional growth patterns of mammary tissues in collagen gels. *In Vitro* **19**: 600–610.
64. Miller, B.E., F.R. Miller, and G.H. Heppner. 1984. Assessing tumor drug

sensitivity by a new *in vitro* assay which preserves tumor heterogeneity and subpopulation interactions. *J. Cell. Physiol.* (suppl.) **3**: 105–116.

65. Miller, B.E., F.R. Miller, and G.H. Heppner. 1985. Factors affecting growth and drug sensitivity of mouse mammary tumor lines in collagen gel cultures. *Cancer Res.* **45**: 4200–4205.
66. Freeman, A.E. and R.M. Hoffman. 1986. *In vivo*-like growth of human tumors *in vitro. Proc. Natl. Acad. Sci.* **83**: 2694–2698.
67. Hoffman, R.M., K.M. Connors, A.Z. Meerson-Monosov, H. Herrera, and J.H. Price. 1989. A general native state method for determination of proliferation capacity of human normal and tumor tissues *in vitro. Proc. Natl. Acad. Sci.* **86**: 2013–2017.
68. Vescio, R.A., C.H. Redfern, T.J. Nelson, S. Ugoretz, P.H. Stern, and R.M. Hoffman. 1987. *In vivo*-like drug responses of human tumors growing in three-dimensional, gel-supported, primary culture. *Proc. Natl. Acad. Sci.* **84**: 5029–5033.
69. Vescio, R.A., K.M. Connors, T. Youngkin, G.M. Bordin, J.A. Robb, J.N. Umbreit, and R.M. Hoffman. 1990. Cancer biology for individualized therapy: Correlation of growth fraction index in native-state culture with tumor grade and stage. *Proc. Natl. Acad. Sci.* **87**: 691–695.
70. Vescio, R.A., K.M. Connors, G.M. Bordin, J.A. Robb, T. Youngkin, J.N. Umbreit, and R.M. Hoffman. 1990. The distinction of small cell and non-small cell lung cancer by growth in native-state histoculture. *Cancer Res.* **50**: 6095–6099.
71. Perrapato, S.D., H.K. Slocum, R.P. Huben, R. Ghosh, and Y. Rustum. 1990 Assessment of human genitourinary tumors and chemosensitivity testing in 3-dimensional collagen gel culture. *J. Urol.* **143**: 1041–1045.
72. Li., M.L., J. Aggeler, D.A. Farson, C. Hatier, J. Hassell, and M.J. Bissell. 1987. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc. Natl. Acad. Sci.* **84**: 136–140.
73. Horan-Hand, P., D. Colcher, D. Salomon, J. Ridge, P. Noguchi, and J. Schlom. 1985. Influence of spatial configuration of carcinoma cell populations on the expression of a tumor associated glycoprotein. *Cancer Res.* **45**: 833–840.
74. Guadagni, F., M. Roselli, and R.M. Hoffman. 1991. Maintenance of expression of tumor antigens in three-dimensional *in vitro* tumor histoculture. *Anticancer Res.* (in press).
75. Menezes, C.A., A. Shikhani, W. Koch, C. Griffin, V. Hyams, and W. Richtsmeier. 1990. Properties of a parotid-derived mucoepidermoid carcinoma tissue culture. *Otolaryngol. Head Neck Surg.* **103**: 226.