Letter to the Editor
LONG-TERM PASSAGE OF HUMAN TISSUES IN VITRO
AS THREE-DIMENSIONAL HISTOLINES

Dear Editor:

By 1951, monolayer cell cultures, in which cells grow as “sheets” on the surfaces of glass or plastic, had become the predominant culture technique and paradigm. Such monolayer cultures had rather limited use in histological studies or for pathological diagnosis, however, and in view of this, Leighton (1951; 1954; 1959a,b; 1960a,b; 1962; 1967) returned to Carrel’s original idea of three-dimensional culture (Carrel, 1912). Leighton improved the earlier system by using a tissue support consisting of cellulose sponges surrounded by plasma clots, contained within glass tubes. Leighton also tested natural and gelatin sponges for their ability to support the organized aggregations of cells.

Because of its architectural resemblance to native tissue, three-dimensional histoculture represents a unique model for investigating crucial events in tumor biology such as drug response, tumor cell migration, invasion and metastasis (Leighton, 1962). Elegant studies by Heppner and co-workers provided convincing evidence that three-dimensional cultures are more accurate models of drug resistance in vitro than are cell monolayers (Miller et al., 1984; Miller et al., 1985; Hoffman, 1991c). Our laboratories have 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. As end points for drug response, we measured [3H]thymidine incorporation by histological autoradiography, monitored fluorescent dye inclusion and exclusion by confocal microscopy as a measure of cell viability, determined the ability of tissues to reduce tetrazolium dyes such as MTT as a measure of cell metabolic activity and measured glucose consumption as a non-invasive multiple measurement end point. Results indicate approximately 90% of the patient cases of all tumor types can be evaluated with approximately 90% accuracy in predicting in vivo drug resistance and sensitivity (Freeman and Hoffman, 1986; Vescio et al., 1987; Hoffman et al., 1989; Guadagni et al., 1991; Hoffman, 1991a; Hoffman, 1991b; Hoffman, 1991c; Vescio et al., 1991; Furukawa et al., 1992; Chang et al., in press).

In addition to its ability to predict chemotherapeutic response, the histocultures show a strong correlation between the degree of cell proliferation and tumor stage and grade in the case of breast and ovarian tumors (Vescio et al., 1990a), and with tumor phenotype (small-cell vs. non-small-cell) in the case of lung tumors (Vescio et al., 1990b). Perrapato et al. have recently used the histoculture method for drug-response analysis of urological tumors (Perrapato et al., 1990; Schmittgen et al., 1991a, 1991b). The reports on urological tumors indicated a high culture success ratio, longevity in culture, maintenance of primary histopathology, and reproducible chemosensitivity.

We have also histocultured skin samples from mice and humans, retaining cell viability and tissue architecture. Viable hair follicles are maintained in culture with [3H]thymidine incorporated in follicle cells and hair shaft growth observed (Li et al., 1991a; Li et al., 1992a, 1992b). We have histocultured normal renal cortical tissue in collagen sponge gels while maintaining normal structure as well as the proliferation of new renal tubules (Chang et al., 1992). Tissue of the eye has also been histocultured on sponge gels with maintenance of corneal and conjunctival tissue structure and evidence of cellular proliferation (Li et al., 1991b).

In this report, we describe the passage of histocultures initiated from human tumor surgical specimens from sponge gel to sponge gel for up to 10 passages with continuous culture with maintenance of tissue histology of some of the specimens up to 2.7 years. Thus, the results of techniques described here open the possibility of long-term or indefinite in vitro studies of cells in a tissue environment. In other words, tissues have been established long-term, if not permanently, as lineages in vitro. Preliminary results of these studies have appeared in abstract form (Slocum et al., 1990).

Tissues were explanted as has been described (Vescio et al., 1991). Briefly, after tissues were surgically removed, they were divided into 1–2-mm diameter pieces. Six pieces of tissue were then placed on top of each gel. Eagle’s minimum essential medium (MEM) containing Earle’s salts, glutamine, 10% fetal calf serum, nonessential amino acids, and the antibiotic gentamicin was added to culture dishes such that the upper part of the gel was not covered. Histocultures were passaged by transferring the tumor to new gels. In some instances, the growing tumors were cut into smaller pieces before being transferred.

Cells within the three-dimensional cultures capable of proliferation were labeled by administration of [3H]thymidine (4 µCi/ml each) (Hoffman et al., 1989; Vescio et al., 1991). Cellular DNA is labeled in any cells undergoing replication within the tissues. After 3 days of labeling, the cultures were washed with phosphate-buffered saline, placed in histology capsules, and fixed in 10% formalin. The cultures were then dehydrated, embedded in paraffin, sectioned and prepared for autoradiography by standard methodology using Kodak NTB-2 emulsion and counterstained with hematoxylin.

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<th>Number of Passages</th>
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and eosin. Replicating cells were identified by the presence of silver grains, visualized as bright green with an epi-polarization lighting system, over their nuclei due to exposure of the NTB-2 emulsion to radioactive DNA (Hoffman et al., 1989). Labeled cells were counted either manually or semi-automatically with the “Fas-corn” system previously described (Hoffman et al., 1989) which quantifies the bright pixels of the light reflected from the silver grains.

The confocal microscopy system used was an MRC-600 Confocal Imaging System (BioRad) mounted on a Nikon Optiphot using a 10X PlanApo objective (Li et al., 1991a). Viable cells are selectively labeled with the dye BCECF-AM, which is activated to fluorescence by non-specific esterases present only in living cells (Li et al., 1991a). Non-viable cells, whose plasma membranes are leaky, are labeled with propidium iodide (PI), a dye which enters only cells

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**Fig. 1.** a, Pre-cultured surgical specimen of leiomyosarcoma (#5251) which is a highly cellular tumor composed of spindle cells. b, Leiomyosarcoma #5251 which was histocultured for 16 months (6 passages), then cryopreserved. After 5 months in the freezer, the tissue was re-exploated (Passage 7). This photomicrograph was taken after two weeks of histoculture which was initiated after the cultures were thawed. The tissue structure and cytomorphology are very similar to those seen in original surgical specimen. The collagen matrix has been partly destroyed due to the invasive character of the tumor.

**Fig. 2.** a, Pre-cultured surgical specimen of a moderately differentiated adenocarcinoma of the colon (#4073). Lumen formation and solid nests of the tumor cells can be seen. b, #4073 after 15 months histoculture on collagen gel (Passage 5). Note the presence of small gland-like structures (arrows).

**Fig. 3.** a, Pre-cultured surgical specimen of a melanoma (#4611) which is highly cellular, consisting of tumor cells of a spindle cell type. b, Melanoma #4611 after 9 months histoculture (Passage 4). Note that although the cytomorphology of tumor cells is still characteristic of melanoma, the spindle cell type is no longer dominant, and bizarre giant cells are present.
Fig. 4. The histoculture of leiomyosarcoma #5251 was transported from Buffalo, NY to San Diego, CA, and histocultured for an additional two months. The histoculture was double stained by BCECF-AM (green, live cells) and PI (red, dead cells). Note that most of the cells are viable, and that the tissue structure is well maintained as seen by confocal scanning laser microscopy.

Fig. 5. An autoradiogram of leiomyosarcoma #5251 labeled with $[^3]$Hthyridine after transport to San Diego. The autoradiogram was observed with simultaneous bright-field and reflected polarized light. Tritium-labeled nuclei appear bright green. Note the numerous proliferating sarcoma cells.
with non-intact membranes (Li et al., 1991a). Since the emission spectra of these two dyes are different, they can be used simultaneously on the same specimen. Both dyes are used at a concentration of 5 μM. The double-dye-treated cultures were analyzed by fluorescence and confocal microscopy within 30 minutes of staining.

Samples from 28 patients were successfully passed from sponge gel to sponge gel at least twice after initiation of histoculture. The highest passage number to date from sponge gel to sponge gel is 10 with a human sarcoma grown for 2.7 years. Table 1 indicates the numbers of passages and the number of cultures that have undergone that number of passages.

The tissues cultured long-term under multiple passages include renal carcinoma and normal renal tissue (Chang et al., 1992a), bladder carcinoma, testicular carcinoma (mesenchymal elements), colon carcinoma, malignant melanoma, and sarcoma. The results of Table 1 do not indicate a limit on passage number, as the histocultures continue to be passaged.

At indicated times, the cultures were washed with phosphate-buffered saline, placed in histology capsules, and fixed in 10% neutral-buffered formalin. The cultures were then dehydrated, embedded in paraffin, and sectioned by standard methodology and stained with hematoxylin and eosin.

Figure 1a shows a photomicrograph of a pre-cultured surgical specimen of leiomyosarcoma (#5251) which is a highly cellular tumor composed of spindle cells. Figure 1b shows the leiomyosarcoma #5251 which was histocultured for 16 months (6 passages), then cryopreserved. After 3 months in the freezer, the tissue was re-exploanted (Passage 7) and 2 weeks later, this photomicrograph was taken. The tissue structure and cyt morphology are very similar to those seen in original surgical specimen. The collagen matrix has been partly destroyed due to the invasive character of the tumor. Figure 2a shows a photomicrograph of a pre-cultured surgical specimen of a moderately differentiated adenocarcinoma of the colon (#4073). Lumen formation and solid nests of the tumor cells can be seen (arrows). Figure 2b shows a photomicrograph of #4073 after 15 months histoculture on collagen gel (Passage 5). Note the presence of small gland-like structures, which seem to be less differentiated than the original tumor (arrows). Figure 3a shows a photomicrograph of a pre-cultured surgical specimen of a melanoma (#4611) which is highly cellular, consisting of tumor cells of a spindle cell type. Figure 3b shows a photomicrograph of melanoma #4611 after 9 months histoculture (Passage 4). Note that although the cyt morphology of tumor cells is still characteristic of melanoma, the spindle cell type is no longer dominant, and bizarre giant cells are present indicating a possible more-malignant stage has been reached. The histoculture of leiomyosarcoma #5251 was transported from Buffalo, NY to San Diego, CA, and histocultured for an additional two months with an additional 3 passages. The histoculture was double stained by BCECF-AM (green, live cells) and PI (red, dead cells). Note that most of the cells are viable, and that the tissue structure is well maintained as seen by confocal scanning laser microscopy (Figure 4). Figure 5 shows an autoradiogram of leiomyosarcoma #5251 labeled with [3H]thymidine after transport to San Diego. The autoradiogram was observed with simultaneous bright-field and reflected polarized light. Tritium-labeled nuclei appear bright green. Note the numerous proliferating sarcoma cells.

The above samples of three tumor types, one carcinoma, one melanoma and one sarcoma, demonstrate the possibility of indefinitely passagable three-dimensional tissues, hence the proposal of the term histolines. The histolines maintain tissue organization throughout the passages although some may seem to progress to a seemingly more malignant state. The possibility now arises of expanding the amount of a histoline, cryopreserving and transporting it to other laboratories as demonstrated for leiomyosarcoma #5251, and the carrying out of multiple experiments over time on a single histoline, somewhat analogous to a cell line. The histoline, however, may offer a much more realistic in vitro model of the in vivo situation than a cell line.

This in vitro tissue model may be useful for investigating growth and therapeutic sensitivity of tissues over long periods of time, including multiple drug resistance of tumor tissue, the effects of cell-cell interactions and the effects of environmental toxins.

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