Histocultures and Organ Cultures

Introductory article

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Histocultures are three-dimensional tissues that are put into growth medium either with a collagen gel support or simply free floating.

Introduction

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 attention returned to the 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. He also tested natural and gelatin sponges for their ability to support the organized aggregations of cells.

Leighton's Culture Method of histologically intact tissue on supports such as collagen gels became known as histoculture. Histocultures can be made from many types of tissues, both cancer and normal. The unit of cultured tissue is approximately 1 mm^3, readily allowing the diffusion of culture medium nutrients and oxygen into the tissue, obviating the need for a circulatory system. Fragments of tissue can be placed on collagen gels, which are hydrolated by culture medium, or are freely floating in culture medium.

Because of its architectural resemblance to native tissue, three-dimensional histoculture represents a unique and *in vivo*-like model for investigating crucial events in tumour biology such as drug response, tumour cell migration, invasion, metastasis, immune response and HIV expression and antigen expression.

Why Use Histoculture or Other Three-dimensional Culture Systems?

Experiments have been performed that demonstrate that three-dimensional structure itself rather than just simple inaccessibility to nutrients accounts for the increased drug resistance of three-dimensional cultures over cultures in two dimensions. Heppner and colleagues embedded cell clusters or fragments in collagen gels, exposed the culture to various cytotoxic drugs, and compared the drug responses of the same cells in monolayers. They found that the resistance in the three-dimensional cultures could be up to 1000-fold greater than in monolayer cultures, for example with melphalan. In three-dimensional bolus cultures in collagen, the cells grew even in the presence of drug concentrations that reduced survival in monolayers to less than 0.1% of controls. When the cells from the collagen gels were replated as monolayers, they became sensitive again. The key experiment carried out by Heppner and colleagues, which demonstrated that the tissue architecture itself is critical in determining sensitivity, was to treat monolayer cultures with melphalan and 5-fluorouracil. When these cultures were subsequently transferred to collagen gels, the cells became highly resistant to these drugs. Thus, the cells were exposed to the drugs as monolayers, where the drugs can access the cells readily and diffusion is not limiting. However, once the cells were transferred after drug exposure to a three-dimensional structure, the structure seemed to confer high resistance to the drugs.

A set of experiments was carried out comparing drug resistance in tumours grown in animals with the drug resistance of the tumours in both monolayer and three-dimensional culture. The EMT-6 tumour in mice was treated with cisplatin, cyclophosphamide and thiotepa over a 6-month period with a total of 10 passages. This treatment induced highly resistant tumours *in vivo*. However, when these tumours were grown as monolayer cultures, they were as drug-sensitive as the parental cells. It was concluded that certain types of drug sensitivity may only be expressed *in vivo*. However, when these same *in vivo* drug-resistant tumour cell lines were grown as spheroids in three-dimensional cultures, resistance was observed up to almost 5000 times that of the parent with certain drugs, for example 4-hydroperoxycyclophosphamide (4-HC), the active form of cyclophosphamide used *in vitro*. High resistance was also observed to cisplatin and thiotepa. This resistance was not observed in monolayer culture, even when the monolayers were cultured on laminin or matrigel. While the parental nonresistant tumour lines formed loose spheroids and grape-like multicellular aggregates that were ellipsoidal in shape, every resistant tumour subline formed a very highly compact spheroid. It should also be noted that *in vitro* exposure of the parental EMT-6 cells to 4-HC induced the formation of compact spheroids just as the *in vivo* exposure did as described above. These experiments reconfirmed the view that cells in a three-dimensional

configuration can be much more resistant than monolayers of the same cells *in vitro*, and for the first time demonstrate that three-dimensional cellular configurations may become resistant to supra-pharmacological doses of drugs by forming compact structures. The compact nature of the spheroids may confer a 'tissue-based' resistance to drugs, as opposed to cellular resistance indicated by membrane pumps that reduce drug concentration.

In the 1950s it was noticed that tumours grew as aggregates in histocultures. The migration of these aggregates in sponge-gel matrices suggested that aggregates were the units of metastasis rather than single cells. It was later observed that the EMT-6 cells selected to be highly resistant *in vivo* are highly metastatic. These results have far-reaching implications in that if drug resistance generated *in vivo* is indeed mediated by formation of tight aggregates, then drug resistance may promote metastasis. Thus, the generation of drug resistance could greatly enhance the malignancy of tumours. Failure of drug treatment of patients may therefore have a double ramification: not only will the tumour remain viable despite drug treatment, but the tumour could become more highly malignant because of the formation of highly aggregated emboli.

The heterogeneous proliferation rate in solid tumours limits the initial therapeutic effectiveness of cytotoxic chemotherapeutic agents, most of which target rapidly proliferating cells. Such 'kinetic' resistance of solid tumour cells can be reproduced in tissue culture by growing tumour cell lines at high cell densities, such as in the form of multicellular spheroids or histoculture. For example, when cells were transferred from monolayer to three-dimensional culture, there was a strong upregulation (up to 15-fold) of $p27^{Kip1}$ protein in spheroids of human breast, ovarian and colorectal carcinoma. Antisense-mediated downregulation of $p27^{Kip1}$ in EMT-6 mammary tumour cell spheroids reduced intercellular adhesion, increased cell proliferation, and sensitized tumour cells to an activated form of cyclophosphamide. The results implicate $p27^{Kip1}$ as a major regulator of the drug resistance of solid tumours.

Why Use Histoculture for Individualized Drug Response Assays?

A 700-fold difference in doxorubicine sensitivity has been found among bladder cancer histocultures, indicating great individual variation among patient tumours. Such variations in patients cannot be effectively treated by empirical therapy. The clinical studies described below on the histoculture drug response assay (HDRA) indicate its potential survival benefit to patients.

To evaluate the histoculture drug response assay (HDRA) with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyitetrazolium bromide) endpoint for clinical use, chemosensitivity to MMC (mitomycin), doxorubicin, 5-FU (5-fluorouracil) and cisplatin of 107 advanced gastric cancer and 109 advanced colorectal cancers was determined *in vitro* in a correlative clinical trial. Of 216 patient specimens, 208 (96.3%) were evaluable in the HDRA. Thirty-eight patients with remaining measurable lesions after surgery were evaluable for comparison of the effects of chemotherapy in the HDRA with clinical outcome. Their overall response in the HDRA to all four drugs correlated to published historical data. Twenty-nine patients were treated with drugs shown to be ineffective in the HDRA and all 29 cases showed clinical chemoresistance. Of nine patients treated with drugs shown to be effective in HDRA, six showed clinical chemoresponse and three showed arrest of disease progression. The correlations rate of the assay to clinical drug sensitivity response was thus calculated to be 92.1% (35/38), with 100% (29/29) true-negative and 66.7% (6/9) true-positive rates, 100% (6/6) sensitivity, and 90.6% (29/32) specificity. Thirty-two patients with stage III and IV gastric cancer without any remaining measurable tumour lesions after surgery were treated with MMC and a fluoropyrimidine adjuvantly. The survival rate of 10 patients whose tumours were sensitive to either MMC and/or 5-FU in the assay was significantly ($p < 0.005$) better than that of 22 patients whose tumours were shown to be insensitive to both drugs. Twenty-nine patients with stage III and IV colorectal cancer without any remaining measurable tumour lesions after surgery were treated with fluoropyrimidines adjuvantly. The recurrence-free survival rate of seven patients whose tumours were sensitive to 5-FU in the assay was significantly ($p < 0.005$) better than that of 22 patients whose tumours were insensitive. Thus the HDRA with the MTT endpoint should be of clinical value in choosing optimal chemotherapy for response as well as for survival.

To further investigate the potential of HDRA to contribute to patient survival, 215 patients with gastric cancer from 45 medical centres were treated with the HDRA in a blinded study after resection of the primary lesion. One hundred and sixty eight patients received at least $20\,mg\,m^{-2}$ of MMC and a minimum of 30 g UFT, a mixture of tegafur and uracil at a molar ratio of 1:4, thereby making them eligible for the study. Of these cases, 128 were evaluable by the HDRA. Evaluable patient tumours were tested by the HDRA, with the [^{3}H]thymidine incorporation end point measured by microautoradiography, to be drug 'sensitive' or 'resistant'. The *in vitro* conditions for distinguishing sensitivity and resistance that matched the response rates for historical controls for gastric carcinoma were a 90% inhibition rate and $0.12\,\mu g\,mL^{-1}$ for MMC and a 70% inhibition rate and $1\,\mu g\,mL^{-1}$ for 5-FU, respectively. Most importantly in the blinded study, the overall and disease-free survival rates of the HDRA-sensitivity group were found to be significantly higher than those of the HDRA-resistant group tested

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under the same conditions. Data further indicated the importance of a three-dimensional tumour culture for obtaining accurate clinical information. The results demonstrate that the HDRA response is correlated to patient survival, which suggests that the HDRA could contribute to cancer patient survival when used prospectively.

A prospective trial was conducted to determine whether effective agents for each individual patient could be distinguished by the HDRA. Tumour tissues from 30 patients with advanced gastric cancer and 19 with advanced colon cancer were placed in collagen–sponge-gel histoculture, treated with chemotherapeutic drugs, and assayed for cell viability. Samples from 86% of gastric tumour cases and 100% of colon tumour cases were successfully cultured and evaluated for chemosensitivity. Patient tumours were scored as sensitive in the HDRA if there was a response to at least one agent. HDRA-sensitive or HDRA-resistant patients were in all other regards clinically indistinguishable. Patients with HDRA-sensitive tumours were treated with the drugs scored as effective in the HDRA, while the patient with HDRA-resistant tumours were treated by physician's choice. For patients with gastric cancer, the 50% survival of the HDRA-sensitive cases was 9.8 months compared with the 50% survival of 4.7 months for the HDRA-resistant cases ($p = 0.02$). In colon cancer, the 50% survival in the HDRA-sensitive cases was 16.3 months compared with the 50% survival of 7.4 months for HDRA-resistant cases ($p = 0.02$). This is the first prospective, controlled trial demonstrating an *in vitro* assay that can distinguish those agents effective for survival of the individual cancer patient.

Applications of Histoculture

Measurement of androgen sensitivity of prostate tissue in histoculture: the histoculture androgen sensitivity assay

A new, total-immersion three-dimensional histoculture (TIH) method was developed to evaluate growth of tissue containing a mixture of benign prostate hyperplasia (BPH) and prostate cancer *in vitro*. [^{3}H]Thymidine incorporation was 2-to 5-fold higher in tissue cultured in TIH than in sponge-gel-supported histoculture (SSH). The average inhibition by genistein at a concentration of 18 μmol L^{-1} was 73% in TIH versus 31% in SSH. TIH also appears to be more sensitive since the lowest concentration of genistein that significantly inhibited growth of BPH mixed with prostate cancer tissue was 2.3 μmol L^{-1}, while in SSH the lowest concentration was 9.2 μmol L^{-1}. Although the within-assay coefficient of variation was similar for both TIH and SSH, the between-assay coefficient of variation was better in TIH. These data suggest that TIH can be used as a discovery model for screening and evaluating inhibitors of prostate tissue growth *in vitro*.

In vitro skin histoculture toxicity assay

This method allows for the testing of toxicity *in vitro* on intact skin which contains hair follicle cells, which are probably the most sensitive cells in the body.

In vitro hair growth

Histocultured skin also allows for the measurement of hair growth *in vitro*, and the testing of drugs for their effect on the hair cycle. It should be noted that all procedures can be used to histoculture mouse skin as well as human skin.

Lymphoid histoculture for *in vitro* immunology and HIV studies

Histoculture architecture

Human tonsil histocultures are viable for at least 4 weeks in histoculture, as judged by morphology, live/dead confocal tests and production of IgG. Immunohistochemical analysis has revealed well-defined germinal centres formed by B cells with T cells concentrated around them. Numerous macrophages are concentrated in germinal centres. Extended networks of follicular dendritic cells are found inside germinal centres by three-dimensional reconstruction of confocal optical sections of histocultured blocks immunostained with fluorescein isothiocyanate-labelled anti-CD21 antibodies.

Virus production in histocultures

Viral particle production begins between days 5 and 6 after infection. Unlike HIV infection in cultures of peripheral blood mononuclear cells (PBMCs), efficient production of HIV in histocultures of human tonsil requires neither stimulation by PHA (phytohemagglutinin) nor activation with interleukin 2 (IL-2). Almost identical results to those above are obtained when histocultures of lymph nodes from recently deceased cadavers are infected with LAV:p24.

CD4 + T-cell depletion

Isolate-dependent depletion of CD4 + T cells is found in infected histocultures. The earliest decline of CD4 + T cells was noticed on day 4 after infection. On days 10–13 (when virus production for all tested isolates equalized and reached a plateau level), the CD4 + /CD8 + ratio in tissues infected with SI types LAV or patient isolate 302076 was less than 4% of the uninfected control. The level of depletion is not a consequence of the duration of infection: the CD4 + /CD8 + ratio for tissue blocks infected with the NSI isolate 302144 remained constant at day 20 after

infection. On day 13 after infection with LAV, there was a total decline in the number of T lymphocytes (CD3 +) to 40 ± 3% of the control. There was no statistically significant change in the CD8 + subset of T cells. The observed decrease in the number of T cells was mainly due to the depletion of the CD4 + subset to 4 ± 2% of the control.

Lymphoid histoculture has demonstrated that R5 HIV-1 isolates are highly cytopathic, but only for CCR5 + / CD4 + T cells. Because these cells constitute only a small fraction of CD4 + T cells, their depletion does not substantially change the total CD4 + T cell count. These results may explain why the clinical stage of HIV disease correlates with viral tropism.

Further Reading

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