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The three-dimensional question: can clinically relevant tumor drug resistance be measured *in vitro*?

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Abstract

In vivo-like drug responses are observed in three-dimensional culture but frequently not in two-dimensional culture, indicating that drug response may be a function of tissue architecture. Alexis Carrel introduced that *in vitro* culture of tissues in the beginning of the century utilizing a culture system that allowed the three-dimensional growth of tissues. Leighton improved upon this system by developing a substrate of sponge matrices. Other methods of three-dimensional culture include collagen gels and what are known as organ culture systems on filters or meshes. In addition, cell suspensions can be converted into multicellular spheroids, another form of three-dimensional culture. Comparison of the three-dimensional culture methods with two-dimensional culture methods has shown critical differences in drug response. The *in vivo* mechanism of drug resistance may involve alterations in cell-cell interaction which may occur in three-dimensional culture as opposed to monolayer culture.

Introduction

The origins of three dimensional culture

Alexis Carrel, in the early part of the century, was able to culture a small fragment of the heart of an 18-day-old chick embryo after explanation on hypotonic plasma [1]. The heart tissue continued to be cultured over a period of three months, during which it was passaged 18 times while remaining viable and, most importantly, continuing its rhythmic beating. Carrel's result was the first demonstration that tissues *in vitro* could retain normal function over a long period of time. Carrel understood at that time that it was difficult to induce a cultured tissue to increase in size and postulated that the proliferation rate of the cells was limited by accessibility of the cells to the nutrient medium. He observed that the peripheral cells grew most readily and that the internal cells became necrotic due to lack of access to nutrients. To overcome the lack of access of nutrients to the internal cells of the culture, Carrel began to culture the tissue on a silk veil which prevented the plasma of the culture medium from retracting and becoming spherical which would limit the access of the internal cells to the medium. Therefore, in 1912, Carrel set down the tenets of three-dimensional culture, or 'histoculture' as it is now known [2].

In the early 1950s, Leighton [3–10] improved on Carrel's idea of three-dimensional culture. Leighton's major contribution was the introduction of the idea of a sponge matrix as a substrate for the culture of tissues. Leighton's original experiments were on cellulose sponges which were surrounded by plasma clots contained within glass tubes. He placed one to four fragments of tissue, ranging in size from 1 to 5 mm^3 on the sponges, along with a single drop

of chicken plasma and a drop of diluted chick embryo extract. After the plasma clotted, the tissue fragment firmly adhered to the sponge, and Leighton then added the nutrient mixture and placed the culture in a roller apparatus to stimulate nutrient accessibility to the tissues. Leighton also used natural and gelatin sponges for their ability to support the organized aggregations of cells and found that collagen-treated cellulose sponges were optimal for supporting the maintenance and growth of native tissue architecture. Different tissue types were grown by Leighton on the sponge-matrix cultures. Leighton made a number of important observations; for example, when C3HBA mouse-mammary adenocarcinoma cells were grown on sponge-matrix histoculture, he found that the cells aggregated in a manner similar to that in the original tumor. Distinct structures were formed within the tumors such as lumina and stromal elements, with some of the glandular structures similar to the original tumor. Leighton was also able to grow in the sponge-matrix cultures normal tissues such as chick-embryo liver, and he observed that the epithelial cells proliferated and formed glandular structures. On the other hand, when Leighton cultured hepatoma cells in sponge-matrix culture, they behaved differently from the normal liver cells and grew in a loosely-packed arrangement as opposed to normal liver cells. It is important to note that, although stromal tissue is present and functional in sponge-gel histoculture, the fibroblast cells are relatively quiescent and do not dominate these cultures. Leighton has since taken three-dimensional sponge-gel histoculture to a higher level in what he calls histophysiologic gradient culture [11]. This type of culture is carried out in chambers such that the metabolic exchange between the pool of medium and the culture chamber takes place by diffusion across a collagen membrane roof of the chamber, thereby mimicking diffusion in tissues. Very realistic tissue formation takes place in such gradient cultures.

Another approach to three-dimensional culture is to embed cells or tissue fragments in collagenous gels. For example, Nandi and colleagues have utilized collagen prepared from rat tail to embed cells or tissues. When clusters of cells were used, Nandi and colleagues [12] observed the formation of duct structures that radiated into the matrix of the gel in three dimensions. Nandi and colleagues observed that the cells grew for a period of over several weeks, with proliferation occurring mainly at the edge of the cell mass, and they observed that very few fibroblasts grew in the system. When mammary tissue was cultured in the collagen gels, it resembled histologically the tissue *in vivo*. It is important to note that Nandi and colleagues observed that if the cells were plated in monolayer culture, unlike the collagen gels, there was a decrease in cell number as a function of time. Nandi *et al.* speculated that an important reason for successful growth of mammary tissue-like structures in collagen gels, as opposed to monolayer culture, is that the shape of the mammary cells differed in the collagen gels in three dimensions as compared with monolayer culture. Many of the advantages of sponge matrix culture described by Leighton may also be attributed to allowing the cells to maintain their normal shape.

Other approaches to three-dimensional culture include what is known as 'organ culture' or 'culture on filter or mesh supports'. Among the initial developers of this strategy in the earlier part of the century were Strangeways [13] and Fell and Robinson [14]. Originally, tissue explants were grown on lens paper in a watch glass which contained liquid culture medium, but more recently this approach has utilized filters or mesh. Authors such as Browning and Trier [15] found that it was critical for some tissues to keep the cultures at the air-liquid interface. Methods for preventing total immersion, include attachment of a fragment of tissue to a matrix or platform that raises it above the medium level or a rocking culture chamber that alternatively exposes the culture to the medium and the atmosphere.

Another approach to three-dimensional culture is the use of 'proto-tissues', which are aggregates of cells which form 'spheroids'. This approach was developed 20 years ago by Sutherland and colleagues [16] who formed aggregates of individual cells into a spherical structure by rotating the cells in spinner flasks.

Critical comparisons of drug resistance between three dimensional culture and two-dimensional monolayer culture

There is a substantial amount of evidence that cells growing in three-dimensional culture are more resistant to cytotoxic agents than cells in monolayer culture or dispersed culture. For example, Bhuyan compared cells in spheroids versus single-cell suspensions and found that spheroids were more resistant to cytosine arabinoside and methotrexate by greater than 11-fold and greater than 125-fold, respectively [17]. Bhuyan and colleagues have also shown that monolayer cultures of colon carcinoma cells are sensitive to Piercidin C in contrast to colon tumors *in vivo* and, importantly, three-dimensional slices of tumors grown *in vitro* which are resistant to the drug [18]. Many other studies have demonstrated elevated levels of drug resistance of spheroids compared with cell monolayers [19].

Initially, investigators attributed drug resistance of spheroids to poor diffusion of the drugs to interior cells of the structures and the relatively high proportion of the resting cells in spheroids, which could be a result of nutrient deprivation and hypoxia. However, Heppner and coworkers performed experiments that demonstrated that three-dimensional structure itself accounts for drug resistance rather than just simple inaccessibility to nutrients [20–22]. 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 increased resistance in the three-dimensional cultures could be up to 1,000-fold greater than in monolayer cultures, for example, with melphalan. In three-dimensional histocultures in collagen, the cells grew even in the presence of drug concentrations that reduced survival in monolayers to less than 0.1% of controls. Heppner and colleagues observed that if the cells from the collagen were repleted as monolayers they became sensitive again. The key experiment done by Heppner and colleagues which demonstrated that the tissue architecture itself is critical in determining sensitivity was when monolayer cultures were treated with melphalan and 5-fluorouracil and 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. But once the cells were transferred after drug exposure to a three-dimensional structure, the structure seemed to confer high resistance to the drugs.

In this light, an illustrative set of experiments is described comparing drug resistance in tumors grown in animals with the drug resistance of the tumors in monolayer versus three-dimensional culture. Teicher *et al.* [23] serially passaged the EMT-6 tumor in mice that were treated with cisplatin, cyclophosphamide and thiotepa over a six month period, with a total of 10 passages. This treatment induced highly-resistant tumors *in vivo*. However, when these tumors were grown as monolayer cultures, they were as drug-sensitive as the parental cells. It was concluded by the authors that certain types of drug-sensitivity may only be expressed *in vivo*. However, when Kerbel *et al.* [24] grew the same *in vivo* drug-resistant tumor cell lines as spheroids in three-dimensional cultures, resistance was observed up to almost 5,000 times that of the parent with certain drugs, for example 4-hydroperoxycylophosphamide (4-HO_2-CTX), 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. Kerbel and colleagues observed that, while the parental nonresistant tumor lines formed spheroids that were loose and grape-like multicellular aggregates that were ellipsoid in shape, every resistant tumor sub-line formed a very highly compact spheroid. It should also be noted that *in vitro* exposure of the parental EMT6 cells to 4-HO_2-CTX induced the formation of compact spheroids just as the *in vivo* exposure did as described above. Thus, the experiments by Kerbel reconfirm that cells in a three-dimensional configuration can be much more drug 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-phar-

macological doses of drugs by forming compact structures.

It should be noted that Leighton, in the early 1950s, observed that tumors grew as aggregates in histoculture and suggested that these aggregates were the units of metastasis rather than single cells by observing their migration in the sponge-gel matrices. In this light, Kerbel *et al.* (personal communication) have observed that the EMT-6 cells selected to be highly drug resistant *in vivo* are highly metastatic. These results have deep implications in that, if drug resistance generated *in vivo* is indeed mediated by formation of tight aggregates of the tumor cells, especially as they migrate as emboli throughout the body, then the tight aggregates may promote metastasis. Thus, the generation of drug resistance could greatly enhance the malignancy of tumors. Therefore, failure of drug treatment in patients may have a double ramification: not only will the tumor still remain viable despite drug treatment, but also the tumor would become more highly malignant because of the formation of highly aggregated emboli.

Greater resistance in three-dimensional versus two-dimensional culture extends also to radiation. Olive and Durand [25] observed that Chinese hamster V79 cells grown as spheroids in suspension culture are more resistant to killing by ionizing radiation than are the same cells grown as monolayers. They found that the rate of DNA repair to the radiation damage was faster in the DNA of cells in spheroids than in monolayers. Differential responses were found to be lost eight hours after separation of the spheroids into individual cells which corresponded to their loss of radioresistance. They also observed that spheroid cells showed fewer numbers of induced mutations per unit of radiation. Possibly DNA repair is also enhanced after chemotherapeutic-induced damage to the DNA in spheroids, or other three-dimensional configurations compared with monolayers, thereby possibly contributing to the enhanced drug resistance frequently seen in three-dimensional cultures.

This review has indicated that three-dimensional culture offers critical advantages with respect to measuring clinically-relevant drug sensitivity which may depend on tissue architecture. The review has also demonstrated that studies in drug resistance in three-dimensional culture may yield results relevant to the *in vivo* situation as opposed to monolayer culture. We have termed drug resistance in three-dimensional culture 'tissue resistance', which may critically depend on altered cell-cell interactions as observed by R. Kerbel's group [24], as opposed to 'cellular resistance' *in vitro*, which may depend on phenomena such as overproduction of cell membrane proteins such as P-glycoprotein to pump out drugs [26]. A critical question is whether altered cell-cell interactions determine drug resistance *in vivo* as well as in three-dimensional spheroids *in vitro*. The question also remains as to the relationship of altered tissue structure such as the tight aggregates seen in spheroids that accompany drug resistance to enhanced metastatic activity, which itself may depend on aggregate formation.

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