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Review

Methods for co-culturing tumour and endothelial cells: systems and their applications

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Abstract

Objectives The high levels of morbidity and mortality associated with cancer can be attributed to two main processes; the tumour's ability to rapidly proliferate and the process of metastasis. These key processes are facilitated by tumour-induced angiogenesis, which causes existing blood vessels to branch off and actively grow towards the tumour providing it with the nutrients and oxygen required for growth and the avenue through which it can metastasise to invade other tissues. This process involves complex interactions between tumour and endothelial cells and is at the forefront of modern biomedical research as anti-angiogenic therapies may hold the key to preventing tumour growth and spread. This review looks at modern co-culture systems used in the study of the tumour–endothelial cell relationship highlighting the applications and weaknesses of each model and analysing their uses in various tumour–endothelial cell investigations.

Key findings The tumour–endothelial cell relationship can be studied *in vitro* using co-culture systems that involve growing endothelial and tumour cells together so that the effects of dynamic interaction (either by direct cell contact or molecular cross-talk) can be monitored. These co-culture assays are quite accurate indicators of in-vivo growth and therefore allow more effective trialling of therapeutic treatments.

Conclusions The application of co-culture systems are of fundamental importance to understanding the tumour–endothelial cell relationship as they offer a method of in-vitro testing that is highly indicative of in-vivo processes. Co-cultures allow accurate testing, which is cost effective and therefore can be utilised in almost all laboratories, is reproducible and technically simple to perform and most importantly has biological relevancy. The importance of this form of testing is such that it warrants further investment of both time and money to enhance the methodology such as to eliminate some of the levels of variability.

Keywords angiogenesis; cancer; co-culture; endothelial cells; vascularisation

Introduction

Cancer represents a significant healthcare burden and is one of the leading causes of mortality and morbidity within Australia. Despite increasing research and therapeutic alternatives cancer still affects one in three Australians,[1] highlighting the urgent need to comprehensively understand the mechanisms of tumorigenesis, growth and metastasis. Cancer growth is a dynamic process largely induced and facilitated by the tumour itself as it secretes proangiogenic factors such as platelet-derived growth factor (PDGF), hypoxia-inducible factors (HIFs) and vascular endothelial growth factor (VEGF).[2,3] These cytokines induce angiogenesis (the branching off of new blood vessels from established vessels), which acts to directly support the continued growth of the primary tumour by providing it with the oxygen and nutrients it needs for survival.[4] This vascularisation also provides the avenue for metastasis whereby tumour cells migrate through the endothelium and travel around the body to form secondary tumours in other tissues.[5] The endothelial cell–tumour cell relationship is therefore of prominent scientific importance as it is pivotal to the maintenance and metastatic potential of the tumour.

The evolution of co-culture systems provides an extremely effective biomedical tool for assessing this relationship and examining the interactions between different cell lines as well as cells and their supporting substrate. Utilising direct and indirect methods of co-culturing affords a much better understanding of pathological diseases, such as neural stem cell migration towards glioma[6] or fibroblast influence over tumour growth,[7,8] as well as

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shedding light on normal physiological events such as the interaction of endothelial and smooth muscle cells during vessel formation.[9] The increasing accuracy of these systems is vital because all evolving candidate therapies must first pass a range of in-vitro tests before they are able to be applied experimentally *in vivo*. Therefore the relevance of these models is integral to trialling therapies that will have the safest and the maximum clinical benefits.

This review represents a collective analysis of the most common co-culture systems currently used to study the endothelial cell–tumour cell relationship. Each method is described in terms of its applications and is assessed based on its strengths and limitations in providing in-vivo relevancy.

Two-Dimensional Systems

Two-dimensional (2D) co-culture systems, often referred to as 2D monoculture systems, involve plating two different cell types cells directly on top of one another such that direct contact occurs between the two[10] (Figure 1). This allows visualisation of the effect that contact has on cell growth, both in tumour and endothelial cells respectively. Cells can be visualised for proliferation rate, cell morphology and for the presence of early angiogenesis, as indicated by endothelial cell migration into primary capillary-like networks.[11]

Monoculture systems are useful in that they are inexpensive and technically simple to perform with the possibility of high-throughput assaying. Cellular growth is easily monitored by various staining techniques and thousands of cells can be grown together within the one setup meaning that large-scale data sets can be obtained, as summarised in Table 1. This makes 2D monocultures beneficial in primary investigations such as studying tumour stromal interactions[12] or cellular cytokine and adhesion profiles.[13] These benefits are presented in a study of endothelial and tumour cells by Blaheta *et al*.[14] In their study, they showed that physical contact between the cells grown in a monoculture system reduced the expression of the adhesion molecule CD44 in endothelial cells while concurrently down-regulating the capacity of neutrophils to migrate towards the endothelial cell–tumour contact point. This suggests one mechanism by which tumour cells may metastasise *in vivo* and the tumour cell contact with the endothelium allows it to escape from the vessel while simultaneously decreasing the immune response which would otherwise inhibit it. Interestingly, only direct contact such as that which occurs in a 2D assay could induce this response and physical separation could not cause CD44 down-regulation. This research is supported by the work of Fan *et al*.,[15] who compared both contact and no-contact co-cultures and also found that the immune response of endothelial cells was down-regulated when in direct contact with tumour cells. This exemplifies the benefits of co-culture assays in understanding the physiological relationship between cell lineages and in particular highlights the necessity for a system that allows identification of those steps in the growth, migration and apoptosis pathways that are related to direct cell contact and those that are mediated by soluble factors.

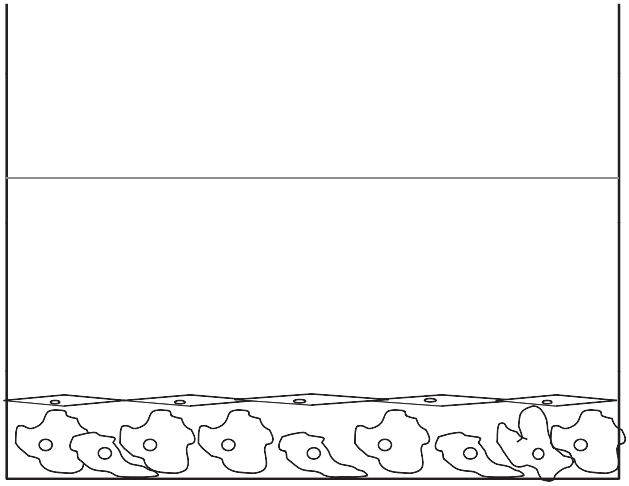

Figure 1 2D monoculture system. Cells are seeded on top of one another and surrounded by growth medium. Contact-mediated effects can then be examined.

Despite their beneficial applications, monoculture assays have been largely superseded by three-dimensional (3D) co-cultures due to the drawbacks seen in their application, all of which revolve around the fact that 2D systems fail to mirror a number of fundamental physiological conditions and therefore are not highly indicative of in-vivo growth. The most poignant limitation of the 2D monoculture assay is that it only allows the growth of cells within a 2D platform across the plate. This is a fundamental flaw because, like almost all tissue growth, the process of angiogenesis and tumour growth involves complex 3D cellular movements and interactions. This requires both intracellular and cell substrate contact combined with the ability of cells to perform molecular cross-talk with other co-cultured cells.[16]

The inability of the 2D monoculture system to allow 3D growth can change the properties of cells in terms of their proliferation rate, morphology and genetic expression, a process documented in both endothelial cells[17] (reviewed by Kim[18]) and tumour cells.[5] The changes in cell behaviour can be attributed to two facets of the monoculture system. Firstly, the lack of a supporting 3D medium, such as stroma, collagen or fibrin, ultimately means cells cannot migrate to form mature structures such as blood vessels and prevents cells from interacting with one another to form 3D connections. This drastically changes their genetic profile, alters their normal physiological behaviour and decreases survival, all of which dramatically reduces in-vivo relevance[19] (reviewed by Kim[18] and Olsen *et al*.[8]). Secondly, this method is a contact system that is based on physical interaction between different cell lineages. This contact is highly beneficial for understanding the role of adhesion molecule expression and interaction between cells such as those in the CAM family,[20] however, can impede certain investigations and alter the applicability of candidate therapies. Primarily this contact makes it very difficult to elucidate the specific origin of cytokines due to their uniform diffusion around the plate and due to the aggressive nature of tumour cells it is often difficult to elucidate whether the effects on the endothelial cells are due to the tumour-expressed cytokines or the proliferation and domination of the

Table 1 Summary of spheroid cultivation techniques

Assay	Brief methodology	Advantages	Disadvantages
3D spheroid co-culture Liquid overlay	A liquid overlay method involves suspending cells in a dish and then covering them with a thin layer of liquid such as methocel, which does not encourage nor facilitate adhesion of the cells. Therefore this encourages cellular contact rather than outwards migration resulting in spheroid formation.[23] The cell spheroids are then harvested and applied onto a monocultured cell lineage.	Inexpensive Large numbers of cells can be generated	The coating of cells with alginates or other nonadhesive media can disrupt normal growth and impede cellular proliferation. Heterogeneous spheroid size.[28]
3D spheroid co-culture 'Hanging drop'	Tumour cells are surrounded by growth medium and grown into spheroids in droplets along the lid of a small container where they are held by surface tension. The lid is then inverted and human microvascular endothelial cells are introduced into the medium. The level of vascularisation is then monitored as the endothelial cells grow into the tumour spheroid-droplets.	No addition of growth factors nor external growth medium such as fibrin or collagen is needed[29] This method can be used on a very wide range of cancer lines[29] High levels of angiogenesis and tumour vascularisation are seen often higher than the levels seen in a Matrigel system.[29,37,38]	High levels of lactate can accumulate within the spheroid when cultured for extended periods.[38] The system does not involve any supporting matrix so cell-substrate interactions can not be studied.[38] This method requires precision and care as the droplets must not be disturbed or burst while the experiment is underway which can be problematic particularly when trying to photograph/ analyse the medium at varying time points throughout the process and therefore this system only allows end point analysis[38]
3D spheroid co-culture Rotating wall vessel	Cells are placed within a growth medium filled vessel that is spun causing cells to be constantly suspended within the medium (never resting on the walls) leading to spheroid formation.	Mimics the in-vivo microenvironment of a tumour.[17] Allows for the movement and configuration of cells to form biological structures.[17] There is low shear stress placed onto the cells.[36] The circulation promotes nutrient delivery and waste removal.[36] Generation of spheroids by this method has been achieved in many cancer cell lines, including melanoma, prostate, breast and colon	Specialised machinery is required Cell viability will begin to reduce between 10 to 12 days in culture.[36]

tumour cells. Similarly this contact renders it difficult to induce experimental changes onto only one cell type.

Overall, the usefulness of monoculture plating is in the primary assessment of the growth and interaction of endothelial cells and tumour cells and as such it is still largely used in this application and as a comparison point between contact and non-contact co-cultures. However, the poor representation of in-vivo growth and function combined with the lack of reproducibility means that the 2D monoculture assay simply cannot accommodate the biological complexities of angiogenesis and is not the ideal assay system for more complex endothelial cell–tumour cell investigations.

Three-Dimensional Systems

Newer 3D co-culture systems far more accurately embody the natural in-vivo process of tumour and endothelial cell growth and of tumour-induced angiogenesis. This is largely due to the availability of continuously dividing endothelial cells, such as human microvascular endothelial cell-1, that can be cultured for extended periods[21] and the use of more complex 3D growth platforms such as Matrigel.[22] There are several standard and niche systems that have arisen to accommodate specific tumour types or processes within the angiogenic cascade, as represented in Table 2.

Table 2 Comparison of 2D and 3D co-culture systems

Assay	Classification	Brief methodology	Advantages	Disadvantages
2D Monoculture	**Contact**	One cell lineage is grown in a monoculture layer at the base of a medium filled well and is then covered by a second cell type[11]	Inexpensive Technically simple Can be adapted for high throughput assaying[18] Large cell numbers can be generated Allows for the study of primary angiogenic potential[11]	Poor representation of the complex in-vivo cell interactions[16] Cells have a limited confluence period Direct contact can change cellular function and expression[17,18] No matrix means that there is no cellular migration[18]
3D Spheroid co-culture	**Contact**	Cells are placed in a vessel containing specific growth medium, which enhances their propensity to naturally aggregate together. This causes them to form spheroids rather than grow on or into the medium. The two cells are either grown together in the one spheroid or a spheroid containing one cell type is seeded onto a monolayer of the other[24]	Inexpensive method Can generate large numbers of cells Dynamic interaction of the cells due to the 3D conform allows investigation of: • The effect of cell–cell interactions (also cytokine influences)[23] • The third dimension of the spheroid allows the configuration and orientation of cells to create complex structures such as blood vessels.[18,29] • The influence of the basal membrane on cell behaviours	Cell-to-cell contact can alter cell behaviour[18] Potential for one cell type to out compete the other creating a rate limiting step[10] Lack of control over the addition of treatments. Many spheroids do not mature and remain as large, undefined aggregates rather than growing into small well-defined spheroids.[23]
3D Boyden chamber	**Non-contact**	This assay is arranged with one cell type grown at the base of a medium-filled well with a small filter basket placed on top. The base of this filter contains a membrane and a second cell lineage is then grown on the surface of this membrane.	There is no direct cell contact and therefore the effects of molecular cross talk can be examined. Cells can be monitored for: • Migration or invasion through the filter membrane under the influence of the cellular cross-talk.[11,39] • Differentiation of cells under the influence of cytokines can be evaluated (this is achieved by incorporating smaller pores into the intervening membrane such that no cell migration can occur but cytokine migration is still possible[24] Cytokine release by the cells can be monitored by evaluation of the growth medium. Treatments can be readily introduced into the system via the growth media or membrane.	Expensive Time consuming to set up Highly variable results due to the nature of the chamber[39] Does not allow evaluation of contact mediated effects

Contact Assays

Multicellular spheroids

Spheroids were designed to enhance scientific understanding of tumour–endothelial cell relationships while improving on the many limitations of the monoculture system. Spheroids are 3D cellular conglomerates that are generated by exploiting the natural propensity of cells to form aggregates when grown within a non-adhesive medium (Figure 2). This medium causes cells to clump and form cellular connections rather than to grow onto, or into, a culture substance as is the case in 2D monocultures.[23] Spheroid design is dependent on the investigation being undertaken; they can be individual spheroids of a single cell type plated onto a second monoculture cell layer, such as a tumour spheroid plated onto a monocultured endothelial cell layer (Figure 2),[24] or they may be comprised of two different cell types in a mixed spheroid, such as a tumour–endothelial spheroid, which is then introduced onto a growth medium which may be embedded with a stimulatory or inhibitory substance. No matter how the spheroid is designed it is always a contact assay and is similar to the 2D monoculture in that two cell lineages are plated onto one another; however, the fact that one is present as an aggregate fundamentally changes the growth dynamics and makes this assay highly indicative of in-vivo tumour growth[17] (reviewed by Kim[18] and Ingthorsson *et al*.[24]). This system therefore

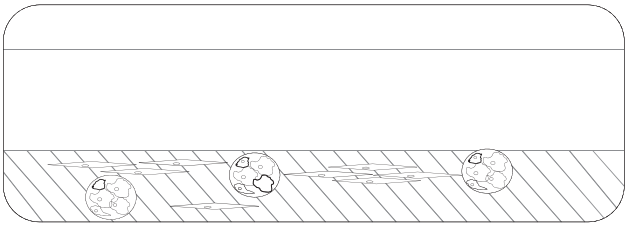

Figure 2 3D spheroid co-culture system. This figure shows the Matrigel insert, which allows 3D conformation and migration of cells. The endothelial cells are able to form connections and migrate into primary tubules while the spheroids consisting of tumour cells are able to readily proliferate and stimulate the surrounding endothelial cells.

incorporates the benefits of understanding contact-mediated cell interactions while being representative of the complex growth patterns *in vivo* by utilising a 3D conformation, a process that can not be trialled in other 3D systems such as the Boyden chamber.

Due to this in-vivo relevance the spheroid system is a method of choice in modern cancer research as it is relatively inexpensive and simple to generate, largely reproducible and allows for the evaluation of tumour–endothelial cell and tumour–endothelial cell–substrate interactions. Supporting this is the fact that the spheroid system manages to satisfy a primary physiological condition – that of direct cell contact. This contact allows cells to communicate and interact with one another which in turn enhances cell survival and correct functioning (reviewed by Kim[18]). This replicates the in-vivo environment where cells naturally seek connections to cells of the same and differing lineages to determine their location, their function and identity, illustrating why traditional 2D monocultures of both tumour and endothelial cells are ineffective as they do not allow proper contact and movement and so do not facilitate normal growth and function.[23,25]

Cellular contact is vital for both tumour and endothelial cell survival as tumour cells grown in a spheroid can be cultured for almost double the period of time compared with those grown in a 2D monolayer[25] and there is evidence that in cervical cancer a 3D co-culture system can produce five times the level of both endothelial cell and tumour cell growth when compared with a monoculture plated system.[17]

The in-vivo relevancy of this system is not confined to the aforementioned qualities but extends to the structure of the spheroid itself. In the endothelial spheroid the cells are able to connect (which decreases apoptosis) and migrate into capillary networks creating a vascular bed similar to that seen during in-vivo angiogenesis.[26] Korff and Augustin[23] have unequivocally shown that maturation into functional and mature blood vessels requires cell-to-cell contact, which renders the cells responsive to growth factors, such as VEGF, and that without this contact the cells undergo apoptosis despite the presence of any such factors. This need for direct contact is also shown in research by Pauduch & Kandefer-Szersze'n[27] in which direct contact between endothelial cells and breast cancer cells changed the responsiveness of the breast cancer to various supplements, which was also indicated in separate research by Mierke *et al*.[5]

This increased survival and growth is also mirrored in the tumour spheroid as it progresses through multiple stages to reach maturity. Initially cells form loose contacts, condense into a spheroid and finally compact into a tight group with a functional, mature outer layer and necrotic oxygen-deprived/hypoxic core (reviewed by Lin and Chang[28]). This creates a biologically relevant model of normal tumour formation and allows for the examination of how this central hypoxia may be a strong stimulus to drive tumour-mediated vascularisation[29] (reviewed by Lin and Chang[28]).

Another investigation made possible by the 3D spheroid co-culture is the role of dynamic interaction of cells in the progression of angiogenesis. Normally the endothelium is maintained in a non-active 'quiescent' state in which there is regular cellular integrity and morphology but no active proliferation or migration.[4] However, when a tumour is plated onto an endothelial cell monolayer it has the ability to directly alter the properties of these endothelial cells, altering their genotype and subsequently the phenotype via both direct contact and molecular cross-talk.[11,17,27] This causes the endothelial cells to become molecularly active and to up-regulate receptors, such as transforming growth factor (TGF)bRII and fibroblast growth factor receptorII, and secrete corresponding cytokines such as TGFβ3 and cysteine-rich fibroblast growth factor receptor-1 and VEGF.[11]

The role of direct contact in mediating this process cannot be underestimated in the functioning of cells, as research has shown that for a range of cancers, cell growth is enhanced by interaction with endothelial cells. Chronic lymphocytic leukaemia is one such cancer that only enhances its growth rate when in directly contact with the endothelium.[30] Research by Paduch *et al*.[27] demonstrated that in breast cancer the direct contact of the tumour with the endothelium caused the tumour to begin expression of matrix metalloproteinase 2, a substance responsible for degrading the extracellular matrix,[31] and therefore a possible mechanism of metastasis. Not only have spheroids greatly enhanced knowledge of tumour–endothelium interactions but they have also enabled a much more in-depth study of normal development such as how cells interact and migrate during primary vessel formation.[32] This demonstrates simultaneously the importance of biologically relevant contact co-culture systems and the usefulness of the spheroid system in meeting this need.

Another emerging benefit of the spheroid system is that it is an effective model for trialling new drug therapies because the complex aggregates that form in 3D cultures react to drug therapies in a similar way to tumours *in vivo*. The complex cellular connections formed within a spheroid alter drug effectiveness, particularly drug penetration, widely demonstrated in tumour cells, which more readily resist treatments when in a 3D culture.[27] Importantly research has shown that tumour cells grown in 3D are significantly more resistant to chemotherapeutic agents than 2D-cultured tumour cells;[33] in fact not only do tumours form more complex connections when in a 3D system but they are able to dramatically increase their tolerance to radiotherapy indicating the necessity of 3D systems for assessing potential drug efficacy.[41] Increasingly this is mirrored in non-tumour cell types such as keratinocytes, dermal fibroblasts and endothelial cells whereby 3D co-culture produces far more virulent cells that more aptly cope with external stresses and actively resist the actions of applied agents.[34] This phenomena is a manifestation of the

cell contact and aggregate morphology, which means that the cell connections protect the cells and ultimately means that spheroids are far more drug-resistant than monocultured cells. This makes them a prime test subject for assessing the level of penetration and effectiveness of a candidate in-vivo therapy.

While these applications are wide and highly beneficial, they do not satisfy all of the needs for endothelial cell–tumour research and are therefore only applicable within certain investigations. This is again where parallels can be drawn between the spheroid and 2D monoculture systems. The same problems encountered in the monoculture assay with applying and removing medium from only one cell type will also occur with this system due to the fact that cells are in direct contact.

Direct cell contact is so important in many endothelial-cancer investigations, including that of the metastatic process (including intravasation and extravasation), as well as how the adhesion or receptor profile of endothelial and tumour cells change when they are in contact does not allows the detailed study of the cytokine exchange and molecular cross-talk. The delicate interplay between endothelial cells and tumour cells is largely controlled by the exchange of soluble factors. This is particularly evident in the early stages of cancer-induced angiogenesis where cytokines and growth factors are present but there is not yet direct cell contact.[35] This means when using the spheroid system to understand cellular responses to an applied cytokine care must be taken to rule out that a false positive has not been encountered whereby the differential growth is occurring as a result of the tumour–endothelial cell contact rather than the applied substance. Therefore there is a fundamental need to understand solely the cytokine aspect of cancer growth and angiogenesis and this is the basis of newer 3D non-contact assays.

Non-Contact Assays

Boyden chamber

The Boyden Chamber is comprised of a deep, growth-medium-filled well in which a cell line is seeded. Into this well a small filter basket is inserted, which contains a gel membrane along its base onto which a second cell line is grown (Figure 3). The membrane covering the base of the filter insert is commonly made up of Matrigel, which contains growth factors derived from the Englebreth–Holm–Swarm tumour,[22] or collagen-1, which is better suited to the growth of bone cancers such as osteosarcoma or chondrosarcoma.[38] This setup allows for proper cell migration and interaction within a 3D conform, which means that biologically relevant structures are able to form and enables the examination of how soluble cytokines and molecular cross-talk impacts the growth and migration of cells.

This assay relies on cell interaction via chemical mediators shared through the growth medium surrounding both groups of cells, this is particularly significant when studying the early stages of cancer vascularisation during the period when the tumour is initiating angiogenesis via chemical mediators, such as VEGF, but direct endothelial-tumour cell contact has not yet occurred.[35] While research has already shown that direct tumour–endothelial cell contact abrogates normal endothelial functioning and direct cytotoxic stress,[39] what the Boyden chamber offers is a clearer insight into the mechanisms by which endothelial cells are stimulated and what impulses draw them towards the tumour cells *de novo*.

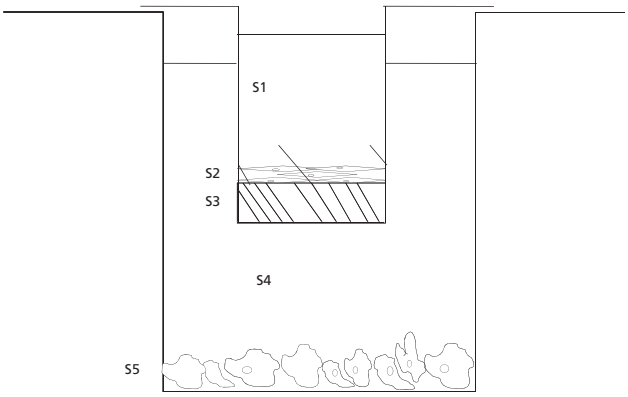

Figure 3 3D Boyden chamber co-culture. This figure shows the Boyden chamber set up in which tumour cells are seeded along the base of a well into which a smaller well is inserted. This insert is lined with Matrigel and endothelial cells. The five sample areas are demonstrated by the labels S1-S5.

Using the Boyden system is not only a better way to understand molecular angiogenic factors but also the most detailed way to understand early-stage angiogenesis. This is illustrated by research showing that the endothelial migration rate is up to 80% higher[11] and proliferation is at least doubled in the no-contact Boyden system when compared with a cell-contact/monocultured system,[11,15] providing powerful evidence of the effects of distant molecular cross-talk in generating angiogenesis. Tsujii *et al*.[40] used the Boyden system to co-culture colorectal and endothelial cells and to stud the molecular cross-talk that was occurring during angiogenesis. They discovered that cyclooxygenase (COX)-2 was a direct mediator of colon cancer progression and the means by which the tumour could stimulate the angiogenic process. Subsequently by examining endothelial cell expression they found that COX-1 was a proangiogenic factor active in endothelial cells that is up-regulated by the growing tumour, which shows the power of the Boyden chamber in allowing understanding of the dynamic two-way cross-talk between cells allowing each to mediate and influence the growth of the other.

The physical separation of cells enables scientists to elucidate not only the effects of tumour growth on endothelial cells but also the mechanisms by which endothelial cells are able to stimulate tumour growth by their own stimulatory mechanisms. Endothelial cells are usually found in a quiescent state within the body;[24] however, once stimulated they are able to change the microenvironment and induce or encourage the growth of both tumour and healthy epithelium. This was shown in recent research by Ingthorsson *et al*.[24] whereby they implemented this system to determine whether breast endothelial cells secreted soluble factors that could directly stimulate cancer cell growth. Interestingly, they found that both healthy and malignant breast epithelium showed increased growth, reinforcing the hypothesis that cancer progression is via a feedback system in which the endothelium does not play a subordinate, passive role but actively engages in the growth process.

Table 3 Findings from key tumour:endothelial cell co-culture studies

Reference	Key findings of study
Bishop *et al*.[16]	Two-dimensional co-cultures of tumour and endothelial cells provide a reliable assay system can be easily and quickly quantified by image analysis.
Montesano *et al*.[19]	In a two-dimensional co-culture system, the lack of a supporting matrix such as collagen or fibrin disallows cells from migrating and prevents cells from interacting with one another to form three-dimensional connections to form in vivo-resembling structures such as blood vessels.
Ades *et al*.[21]	Establishment of a continuously dividing (immortalised) human endothelial cell line that makes anti-angiogenesis assaying much easier to setup and evaluate.
Albini *et al*.[22]	Incorporation of the tumour-derived matrix – Matrigel – which leads to assays that are more supportive of cell growth and in turn allow longer-term effects of potential anti-angiogenic or anti-cancer drugs to be evaluated. However, one drawback is the price of commercially available Matrigel.
Chopra *et al*.[17], Ingthorsson *et al*.[24]	Spheroids comprised of two different cell types in a mixed spheroid such as a tumour–endothelial spheroid are presented as an assay system that is highly indicative of in-vivo tumour growth.
Ghosha *et al*.[25]	Tumour cells grown in a spheroid can be cultured for almost double the period of time compared with those grown in a monolayer.
Chopra *et al*.[17]	In cervical cancer, a three-dimensional co-culture system can produce five times the level of both endothelial and tumour cell growth when compared with a monolayer.
Alajati *et al*.[26]	In the endothelial spheroid, the cells are able to migrate into capillary networks creating a vascular bed similar to that seen during in-vivo angiogenesis.
Komoto *et al*.[33]	Tumour cells grown in three-dimensional systems are significantly more resistant to chemotherapeutic agents than two-dimension-cultured tumour cells.

The high level of control and the multiple testing sites within this system are where the benefits and accuracies lie. The chamber itself can be modified so as the pore sizes may allow cellular migration or be modified such that only cytokine migration is facilitated. This means that the system can be modified to suit the specific nature of the investigation and once completed five samples can be analysed (see Figure 3) so that the endothelial growth medium, endothelial cells, Matrigel, tumour growth medium and tumour cells can all be studied for the presence of cytokines and the cells themselves can be stained and examined to establish migration rates and also morphological or genetic changes. This offers a level of control not seen in any of the other co-culture systems and accounts for its popularity amongst scientists.

Outlining these benefits does not give the complete picture of the Boyden system as drawbacks in its application still remain. Firstly, the system is relatively costly to purchase, particularly when using Matrigel, and this often limits the number of tests that can be performed. The other issue is with the repeatability of the measurements and quantification of traversed cells during migration studies. Variability in the gel formation process can affect cell migration rates resulting in inaccuracies in cell counting whereby many cells may migrate during one run and yet, with no alteration in protocol, the next run may show little cell migration.[39] This is a prominent issue with the system as it fundamentally undermines the results and must be the focus of research in the future. The second issue is with the quantification of migrating cells. This involves correctly determining how many cells have migrated through the gel and is based on tedious counting, a process that can present its own level of assessor error and it is acknowledged that inevitably many of the cells will have migrated through the gel and, once penetrated, lost their adhesion and have began to float within the culture/ growth medium and therefore are not counted.[39] These shortcomings mean that one of the most modern areas of research is in fastidiously reducing these areas of variability by creating size-defined and repeatable pore sizes within the Matrigel and also improving staining and cell counting techniques.

Ultimately this system offers a stringent method of assessing cellular migrations and cytokine influence over tumour-mediated angiogenesis. As a 3D system, it allows intracellular contact and migration into complex structures and therefore in terms of the two 3D systems discussed here, it is solely the process being investigated, which will determine the most appropriate system to use. To date, several significant findings have been made using these various types of co-culture systems (Table 3).

Future Directions

Co-culture systems are vitally important to our increased understanding of the complex endothelial cell–tumour relationships that mediate the formation of tumour vascularisation.

Not only are newer 3D models enhancing this understanding but also their comparison with 2D models is illuminating the specific interactions that underlie the process *in vivo*. The different reactions of cells under contact and non-contact situations suggest that angiogenesis and metastasis need to be distinctively separated into the events that precede contact, the point at which the tumour draws the endothelium towards itself, seemingly by the very strong effects of cytokine communication, and the events post contact in which the interaction causes stress elements and phenotypic changes particularly within the endothelium.

The first line of co-culture research is already, and will continue to be, directed at modification and improvement of the current systems to eliminate many of the drawbacks and variables that are currently present. One such avenue may involve modifying the Boyden chamber to allow better drug or

substance administration to cells, which would involve a steady gradient-mediated release allowing multiple time-point assessment and preventing the need for multiple time-consuming and costly re-runs of the samples. The improvement of the intervening gel to one that is less variable in its results and less cumbersome to set up must be a priority and will allow for more reproducible results.

One of the most progressive and clinically beneficial applications of co-culture systems may very well be in 'personalised tumour analyses'. Because co-culture systems allow the cultivation of tumour cells and the examination of their receptor, cytokine and cytoplasmic constituents, in the future they may be modified and generated on a commercial scale such that a tumour can be analysed for aggressiveness, strength of growth, morphology, angiogenic potential and specific receptor profile. This would result in enhanced knowledge about the sub-categories of specific tumour types as well as cancer treatments that could be tailored to an individual's tumour and would therefore be far more accurate and devastating to its growth. However, for this to occur, the Boyden chamber has to be analysed at various strata – the invaded cells on the membrane, the Matrigel that contains invading cells, the medium above the Matrigel in the insert, the medium below the membrane, and finally, the cells at the bottom of the well below the insert (Figure 3). Sensitive methods for analysis will be required, such as the ability to perform protein profiling of the small number of cells extracted from the Matrigel or the small volume of medium below the membrane.

Conclusions

The application of co-culture systems are of fundamental importance to understanding the tumour–endothelial cell relationship as they offer a method of in-vitro testing that is highly indicative of in-vivo processes. Co-cultures allow accurate testing that is cost effective and therefore can be utilised in almost all laboratories, is reproducible and technically simple to perform and most importantly has biological relevancy. The importance of this form of testing is such that it warrants further investment of both time and money to enhance the methodology such as to eliminate some of the levels of variability. The use of 3D systems takes almost full precedent over 2D methods as it is able to encompass the complexities of the dynamic cellular relationship. The Boyden chamber and spheroid used in concert allow the mechanisms of both cellular contact and molecular cross-talk to be examined and enable the complex process of tumour-mediated angiogenesis to be more thoroughly understood.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Australian Institute of Health and Welfare & Australasian Association of Cancer Registries. *Cancer in Australia: An Overview*. Canberra: AIHW, 2008.
2. Nor J *et al*. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *Am J Pathol* 1999; 154: 375–384.
3. Manalo K *et al*. Pigment epithelium-derived factor as an impending therapeutic agent against vascular epithelial growth factor-driven tumor-angiogenesis. *Mol Carcinog* 2011; 50: 67–72.
4. Staton C *et al*. Current methods for assaying angiogenesis in vitro and in vivo. *Int J Exp Pathol* 2004; 85: 233–248.
5. Mierke C *et al*. Breakdown of the endothelial barrier function in tumor cell transmigration. *Biophys J* 2008; 94: 2832–2846.
6. Heese O *et al*. Neural stem cell migration toward gliomas in vitro. *Neurol Oncol* 2005; 7: 476–484.
7. Wang T *et al*. Fibroblasts promote breast cancer cell invasion by upregulating tumor matrix metalloproteinase–9 production. *Surgery* 2002; 132: 220–225.
8. Olsen C *et al*. Human mammary fibroblasts stimulate invasion of breast cancer cells in a three-dimensional culture and increase stroma development in mouse xenografts. *BMC Cancer* 2010; 10: 2–16.
9. Korff T *et al*. Blood vessel maturation in a 3-dimensional spheroidal co culture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J* 2001; 15: 447–457.
10. Albini A, Noonan D. The 'chemoinvasion' assay, 25 years and still going strong: the use of reconstituted basement membranes to study cell invasion and angiogenesis. *Curr Opin Cell Biol* 2010; 22: 677–689.
11. Khodarev N *et al*. Tumour-endothelium interactions in co-culture: coordinated changes of gene expression profiles and phenotypic properties of endothelial cells. *J Cell Sci* 2002; 116: 1013–1022.
12. Lang S *et al*. In vitro modelling of epithelial and stromal interactions in non-malignant and malignant prostates. *Br J Cancer* 2000; 82: 990–997.
13. Wallace CS, Truskey GA. Direct-contact co-culture between smooth muscle and endothelial cells inhibits TNF-alpha-mediated endothelial cell activation. *Am J Physiol Heart Circ Physiol* 2010; 299: 338–346.
14. Blaheta R *et al*. Tumor-endothelium cross talk blocks recruitment of neutrophils to endothelial cells: a novel mechanism of endothelial cell anergy. *Neoplasia* 2009; 11: 1054–1063.
15. Fan P *et al*. Testicular Sertoli cells influence the proliferation and immunogenicity of co-cultured endothelial cells. *Biochem Biophys Res Commun* 2011; 404: 829–833.
16. Bishop E *et al*. An in vitro model of angiogenesis: basic features. *Angiogenesis* 1999; 3: 335–344.
17. Chopra V *et al*. Three-dimensional endothelial-tumor epithelial cell interactions in human cervical cancers. *In Vitro Cell Dev Biol Anim* 1997; 33: 432–442.
18. Kim J. Three-dimensional tissue culture models in cancer biology. *Semin Cancer Biol* 2005; 15: 365–377.
19. Montesano R *et al*. In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. *J Cell Biol* 1983; 97: 1648–1652.
20. Haddad O *et al*. Tumor cell/endothelial cell tight contact upregulates endothelial adhesion molecule expression mediated by NFkappaB: differential role of the shear stress. *Exp Cell Res* 2010; 316: 615–626.

21. Ades E *et al*. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol* 1992; 99: 683–690.
22. Albini A *et al*. A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987; 47: 3239–3245.
23. Korff T, Augustin G. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J Cell Biol* 1998; 143: 1341–1352.
24. Ingthorsson S *et al*. Endothelial cells stimulate growth of normal and cancerous breast epithelial cells in 3D culture. *BMC Res Notes* 2010; 3: 1–12.
25. Ghosha S *et al*. Use of multicellular tumor spheroids to dissect endothelial cell–tumor cell interactions: a role for T-cadherin in tumor angiogenesis. *FEBS Lett* 2007; 581: 4523–4528.
26. Alajati A *et al*. Spheroid-based engineering of a human vasculature in mice. *Nat Methods* 2008; 5: 439–445.
27. Paduch R, Kandefer-Szerszen M. Vitamin D, tamoxifen and β-estradiol modulate breast cancer cell growth and interleukin-6 and metalloproteinase-2 production in three-dimensional co-cultures of tumor cell spheroids with endothelium. *Cell Biol Toxicol* 2005; 21: 247–256.
28. Lin R, Chang H. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol J* 2008; 3: 1172–1184.
29. Kelm J *et al*. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol Bioeng* 2003; 83: 173–180.
30. Buggins A *et al*. Interaction with vascular endothelium enhances survival in primary chronic lymphocytic leukemia cells via NF-κB activation and de novo gene transcription. *Cancer Res* 2010; 70: 7523–7533.
31. Morgunova E *et al*. Structural insight into the complex formation of latent matrix metalloproteinase 2 with tissue inhibitor of metalloproteinase 2. *Proc Natl Acad Sci USA* 2002; 99: 7414–7419.
32. Lilly B, Kennard S. Differential gene expression in a coculture model of angiogenesis reveals modulation of select pathways and a role for Notch signalling. *Physiol Genomics* 2009; 36: 69–78.
33. Komoto C *et al*. Three dimensional, but not two-dimensional, culture results in tumor growth enhancement after exposure to anticancer drugs. *Kobe J Med Sci* 2008; 53: 335–343.
34. Sun T *et al*. Culture of skin cells in 3D rather than 2D improves their ability to survive exposure to cytotoxic agents. *J Biotechnol* 2006; 122: 372–381.
35. Nör J *et al*. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *Am J Pathol* 1999; 154: 375–384.
36. Licato L *et al*. A novel preclinical model of human malignant melanoma utilising bioreactor rotating-wall vessels. *In Vitro Cell Dev Biol Anim* 2001; 37: 121–126.
37. Timmins N *et al*. Hanging-drop multicellular spheroids as a model of tumour angiogenesis. *Angiogenesis* 2004; 7: 97–103.
38. Clark J *et al*. New clinically relevant, orthotopic mouse models of human chondrosarcoma with spontaneous metastasis. *Cancer Cell Int* 2010; 10: 1–10.
39. Li Y, Zhu C. A modified Boyden chamber assay for tumor cell transendothelial migration in vitro. *Clin Exp Metastasis* 1999; 17: 423–429.
40. Tsujii M *et al*. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998; 93: 705–716.
41. Josson S *et al*. Tumor-stromal interactions influence radiation sensitivity in epithelial- versus mesenchymal-like prostate cancer cells. *J Oncol* 2010; doi:10.1155/2010/232831.