Research

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Archives in Cancer Research
ISSN 2254-6081

2017
Vol.5 No.2:144

DOI: 10.21767/2254-6081.1000144

Tumour Dissemination and Assays Involved

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Received: June 14, 2017; **Accepted:** June 28, 2017; **Published:** June 30, 2017

Citation: Zraikat M, Shnyder S, Sheldrake H, Patterson L (2017) Tumour Dissemination And Assays Involved. Arch Can Res 5: 2.

Abstract

Tumour dissemination is a major reason for failure of therapy for many tumour types. Metastasis and angiogenesis result from the interaction between the tumour cells in the tumour microenvironment. The detailed picture of tumour and the tumour microenvironment interaction however is not fully understood due to a lack of representative models. This review shows a brief summary of the assays and models used to describe the tumour dissemination process.

Keywords: Cancer; Invasion; Migration; Integrins; Spheroids; Transwells; Pharmacology; Metastasis; Drug screening

Introduction

Cancer or neoplasia is a variety of distinct diseases that result in unregulated cell growth as a result of gene defects resulting in either loss or gain of gene functions. This leads to the formation of malignant cells that are able to invade and metastasise to nearby parts of the body [1]. Cancer is a major human health problem worldwide and cancer dissemination is the major cause of death for most kinds of cancer [2].

According to CRUK 2014 the number of people diagnosed with cancer was 331,487 in the UK in 2011. Furthermore in 2011, 396.2 people per 100,000 of the UK population were diagnosed with cancer. The cancers of the breast, lung, prostate and bowel in 2011 represented over 54% of new cancer cases in the UK.

According to the GLOBOCAN 2008 estimates, the cancer cases were about 12.7 million and the cancer deaths were about 7.6 million globally. 56% of the cancer cases and 64% of the deaths were in the economically developing countries [3]. A total of 1,638,910 new cancer cases and 577,190 deaths from cancer have occurred in the United States in 2012. In Europe alone, in 2006 3, 191, 600 cancer cases were diagnosed with 1, 703, 000 cancer related deaths occurring in the same year [4]. Furthermore, 3.45 million new cancer cases and 1.75 million deaths from cancer have been estimated in 2012 in Europe. [5]

Cancer is a process which needs steps to progress which have been described as the hallmarks of cancer. The hallmarks of cancer include six hallmarks required during the multistep development of human tumours. These are sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling immortality, inducing angiogenesis, and activating invasion and metastasis [1]. Furthermore four other hallmarks were recently added including the genome instability, the inflammation, metabolic energy reprogramming and evading immune destruction as reported by Hainaut and Plymothin in 2013.

The stages at which the cancer is diagnosed and its type are the main determinants for the selection of therapy. There are currently three main types of cancer therapy: surgery, radiotherapy, and chemotherapy. Other therapeutic modalities include immunotherapy, hormonal therapy, antibody and gene-based therapies. These tend to be highly disease-specific [6].

Whilst the current cancer therapies have demonstrated considerable success, there are still many fatalities from the disease. This can be explained by several reasons including inability to prevent or control tumour dissemination once it happens, the development of drug resistance, and the off-target toxic effects on normal cells [6].

Non-specificity is one of the major problems associated with cytotoxic anti-cancer agents as it results in a wide-range of toxic side-effects. The cells most affected by the non-specificity are the rapidly dividing epithelial cells like the cells lining the intestine, bone marrow and hair follicles and organs like the kidneys [6]. Immune system suppression and alopecia, digestive problems, nausea, vomiting, decreased appetite, nephrotoxicity, neurotoxicity and hepatic toxicity are among the common side effects that are associated with non-specificity of chemotherapy that result in limiting the therapeutic dose used in the clinic [7].

Multi drug resistance (MDR) is another drawback for the use of chemotherapies in cancer treatment as the tumour cells become resistant to a range of anticancer drugs not only limited to the initially used drug [8]. MDR is associated with molecular pumps in tumour-cell membranes that are

responsible for pumping chemotherapy drugs from the interior of the tumour cells to the exterior, making the tumour cells resistance to the toxic effect of the drug. There are many examples of efflux pumps such as P-glycoprotein pump, the multidrug resistance–associated protein (MRP) pump [8] and ABC transporters [9].

The Tumour Dissemination Process

The tumour dissemination process covers metastasis, including migration, invasion, adhesion, and angiogenesis. Metastasis is a multistep process which involves the detachment of cells from the primary tumour, movement of the cells on the basement membrane (migration), and penetration through the basement membrane by degradation of the ECM proteins (invasion). Tumour angiogenesis is a process which involves the formation of new blood vessels after the tumour size reaches 2 mm^3 where a state of cellular hypoxia begins due to the lack of vasculature and results in subsequent oxygen starvation [10,11].

One of the main requirements of metastasis is the loss of anchorage-dependent growth which allows the cell to leave the primary tumour, seed and start tumour growth in another part of the body [1].

Adhesion

Cell adhesion is an important part in the cellular migratory machinery. Adhesion occurs due to localized actin polymerization resulting in the formation of filaments. These filaments lead to the extension of cytoplasmic protrusions which adhere to ECM via adhesion molecules and form one or several focal adhesion sites. The proteases then become recruited to attachment sites and initiate proteolytic cleavage of the matrix [12,13].

The integrin family is an example of the adhesion molecules that are involved in the cell adhesion to ECM structures. Integrins participate in cellular adhesion by forming structural links between cells and the ECM [14].

Integrins attract adaptor molecules such as focal adhesion kinase (FAK), actinin, talin, tensin, paxillin and vinculin which work by connecting integrins to the filamentous actin cytoskeleton thus forming a focal adhesion. After binding to the ECM, integrins are associated with adaptor proteins and signalling molecules that allow phosphorylation and dephosphorylation events for the regulatory molecules causing downstream signalling known as outside-in signalling. Depending on the nature of the ECM structures surrounding the migrating cells, forward movement, cell adhesion, proliferation and survival could be the result of this signalling [15].

The cadherin family molecules are also important examples of the molecules which are involved in the adhesion process. Cadherins such as catenin mediate the intercellular adhesion at sites termed adherens junctions [16]. Adherens junctions are highly expressed at connections and gap junctions and work to support cell–cell adhesion during the collective cell invasion process; the most observed type of tumour invasion [17]. In addition to regulating the cell-cell adhesion, the E-cadherins are involved in regulating the contact inhibition of cell growth which is considered an important property involved in tissue maintenance and its loss is an important characteristic that leads to loss of cell cycle regulation and inducing tumour growth.

Migration and invasion

Migration is defined as the directed physiological or pathological movement of cells on a substrate such as the basal membrane or ECM fibres on a 2D level with no restructuring effects [18]. Tumour cell movement or migration involves five interdependent steps repeated in a cyclical manner including: i) protrusion of the initial pseudopodia; ii) focal contact formation through attachment of integrin to ECM; iii) secretion of protease in the sites of focal contact; iv) cell body contraction, and v) movement of the cell body as tudied by Friedl in 2004. Invasion is defined [18] as the physiological or pathological movement of the cell through a 3D matrix, accompanied by a restructuring of the 3D environment [17].

There are two main types of morphological or functional cell migration termed collective and single cell migration. The latter can further be divided into fibroblast-like spindle-shaped migration, mesenchymal migration and leukocyte-type 'amoeboid' migration [19]. In the absence of cell–cell adhesion, tumour cells invade as single cells [17]. Cells that migrate in a mesenchymal fashion originate mostly from connective-tissue sarcomas or dedifferentiated carcinomas. [20]. Some cells like stem cells, leukocytes and cells of haematopoietic origin, utilize more short lived and less adhesive cell–matrix interactions displaying a migration type termed amoeboid migration [17,21]

Collective migration results from the movement of cells that retain cell–cell junctions as they move, either remaining connected to the primary tumour or as detached cell groups or clusters. Collective migration can be seen in basal cells [22]. The migrating cells can switch between mesenchymal and amoeboid modes in what is called mesenchymal-amoeboid transfer [MAT] resulting in a switch from elongated, polarized morphology to a roundish shape [23].

Molecules involved in the tumour dissemination process

During cancer progression, the expression of molecules such as integrins, cadherins, proteases and oncoproteins are relevant to invasion [1].

Integrins are the major focus of this thesis and covered in detail below. changes in integrin expression are associated with the migration process, for example the αvβ3 integrin is highly expressed on actively invasive melanoma cells, but weakly expressed on quiescent cells [24]. Induction of the expression of α6β4 integrin on thyroid cancer cells is

associated with the transfer of the thyroid cells towards the invasive mode [25].

Proteases, such as MMPs are a family of enzymes that are either secreted or anchored to the cell membrane and are involved in cell migration and invasion in addition to integrins. MMPs are typically absent in many normal adult cells, but a variety of stimuli, such as cytokines, growth factors, and alterations in cell-cell and cell-ECM interactions, can induce their expression. The expression of MMPs is frequently localized to the stromal cells surrounding malignant cells [26]. MMPs start the local proteolysis towards ECM proteins to allow the forward-expanding of the cell body [27,28].

Over-expression of the epithelial growth factor receptor (EGFR) family and c-Met are important characteristics of metastatic cells [29]. Transformation of the cell by the v-Src, v-Jun, v-Myc, and k-Ras, induces activity of proteases such as calpain which promotes focal adhesion remodelling and induce cell migration [30]. Downregulation of cadherins like catenins could lead to the detachment of single cells from the primary mass and invade through the basement membrane and entering the bloodstream through intravasation process resulting in epithelial–mesenchymal transition (EMT) from collective to mesenchymal migration [31]. When tumour cells form micrometastases the tumour cells exit the blood and go through mesenchymal–epithelial transition (MET) in the new sites of metastasis. Consequently, EMT and MET are considered as the start and the end of the metastasis process [32].

Assays for Screening Therapies Targeted At Aspects of the Tumour Dissemination Pathway

Whilst standard chemotherapeutic agents that have a cytotoxic effect are readily monitored, it is difficult to evaluate the molecules which interfere with the tumour dissemination process such as dual integrin antagonists as they may work at several points in the process, or may not even target the tumour cells themselves. Studying the molecules which interfere with the tumour dissemination process needs appropriate assays that can cover different stages of the process. Such assays can be concerned with adhesion, migration or invasion of tumour cells, or may evaluate effects on tumour angiogenesis or the extracellular matrix. I will give an overview of the various assays below, focusing on the assays relating to tumour invasion.

Angiogenesis Assays

There being a large complement of assays for angiogenesis which cover *in vitro, ex vivo* and *in vivo* studies. The following sections explain some of the angiogenesis assays used at different levels of testing [33].

In vitro assays of angiogenesis

Endothelial cell differentiation assays are considered *in vitro* assays of angiogenesis and simulate the formation of capillary-like tubules. The main principle of this assay depends on the plating of endothelial cells onto a layer of extracellular matrix, stimulating cell migration and formation of tubule-like structures [34]. The formation of tubules and tight junctions by endothelial cells is observed over a specific period of time using light or electron microscope [35].

An essential part of the study of angiogenesis is the interaction between endothelial and tumour cells. The simplest co-cultures involve seeding the cells simultaneously or allowing one cell to adhere first and then seeding the second cell type on top. In order to assess the influence of one cell type on the others behaviour, it is necessary to label at least one population prior to seeding [36].

Ex vivo assays (organ culture)

The rat aortic ring assay is an example of an *ex vivo* assay that depends on the isolation of rat aorta and cutting it into segments placed in Matrigel which is a gelatinous protein mixture represents the ECM that presents in many tissues. Over a 7–14 day period, the explants are monitored for the outgrowth of endothelial (and other) cells [37].

The chick aortic arch assay is another example of an *ex vivo* assay in which the aortic arches are dissected from 12–14 day old chick embryos, cut into rings and placed on Matrigel. Outgrowth of cells is measured within 48 h [37].

The chorioallantoic membrane (CAM) is formed during the chick embryo incubation and described as a developed ectoderm composed of small capillaries network that connects the venous blood vessel with arterial ones providing a tumour inducing environment through being a source for angiogenic vessels formation [38]. Furthermore the CAM with its surrounding capillaries network provides dissemination inducing environment that induce the tumour cells to extravasate in an aim to form micro metastatic foci. The CAM assay importance arises from its inclusion of many steps of the metastatic cascade in a short time period [38]

In vivo assays

The corneal angiogenesis assay is an *in vivo* angiogenesis assay which is based on the fact that the cornea itself is avascular. Thus, any vessels seen in the cornea after stimulation by angiogenesis-inducing tissues or factors are new vessels. The original method was developed on rabbit eyes [39], but has been adapted to mice [35]. The method involves making a pocket in the cornea, introducing tissues into this pocket, and observing the growth of new vessels. Slow-release materials such as ELVAX (ethylene vinyl copolymer), Hydron or sponge materials are used to introduce test substances into the corneal pocket [40].

In the sponge Matrigel assay, Matrigel containing test cells or substances is first injected subcutaneously, where it

solidifies to form a plug. A sponge or tissue fragment is then inserted into the plug. This plug can be recovered after 7–21 days in the animal and examined histologically to determine the extent to which blood vessels have entered it [41].

The hollow fibre assay developed by the US National Cancer Institute has been reported to be an *in vitro* solid tumour model that can be easily translated *in vivo* [42]. The hollow fibres are composed of a biocompatible polymer (polyvinylidenefluoride) that is formed into cylindrical fibres with a diameter of one millimetre and walls with pore size cut off of 500kDa. The hollow fibre assay involves preparing the tumour cells and seeding them into compatible hollow fibres which are then sealed into 1-5 cm segments, divided and cultured [43]. The hollow fibre assay has been used to observe formation of new vascular networks surrounding the hollow fibres containing tumour cells implanted subcutaneously compared to control hollow fibres that contain no tumour cells [44].

Cell Adhesion Assays

Adhesion is an important cell property that is related to the maintenance of tissue structure, and the promotion of cell migration. It involves different molecular interactions, such as receptor-ligand binding, changes in the intracellular signalling pathways, and changes in the structure of the cell cytoskeleton [45].

Adhesion assays measure the cell-extracellular contacts and cell-cell adhesion to give essential information about other cellular properties such as cellular flattening, and differences in signalling leading to changes in cellular fluxes. Adhesion assays are usually used for testing the ability of a specific cell line to adhere to a specific substrate, [46]. The attachment assay is among the important adhesion assays which detect bound cells [47]. The spreading assay is another kind of adhesion assays that uses phase contrast microscopy to determine the flattening of adherent cells [48].

Spreading assays

The spreading assay is an example of adhesion assays which measure the flattening of adherent cells on an immobilized substrate. The assay includes seeding the cells of interest on a cover slip for 1 hour before taking microscopic images of the speeded cells to be analyzed later using a computer based software [49]. Spreading assays take longer to perform but they are more specific compared to other adhesion assays for reasons such as the fact that many molecules can mediate attachment of cells even in non-physiological conditions but very few of these molecules are able to mediate spreading [50,51].

Attachment assays

The attachment assay is an example of adhesion assays which detect the ability of cells to bind to an immobilized surface. The cell attachment assay includes applying different concentrations of the protein of interest such as fibrinogen in the proper well plates. Blocking agent such as BSA are used to block the unbound sites. Before seeding the cells of interest in the coated wells they are treated with different inhibitors. The coated wells are washed to remove unbound cells after 1-4 hours incubation depending on the experiment. The adherent cells are noticed through colourimetric quantification and counted by microscope 10X objective lens [52]. In many cases the attachment assay is considered as the only and best choice as some cells are unable to spread at all, whereas other cells can only spread on specific substrates. The level of adhesion depends on the cell type and adhesive substrate under study [45,51].

Cell Migration Assays

There are many migration assays, the most common of which are described below.

Transwell migration assay (Boyden chamber assay)

The transwell assay or Boyden chamber assay was first introduced to analyze the chemotactic responses of leukocytes [53]. The Boyden chamber assay depends on the presence of two chambers containing medium and separated by a porous membrane through which cells migrate. The required pore size of the membranes is determined by the size of the cells to be analyzed. The pore diameter chosen should be suitable to the cell size to avoid unspecific dropping of the cells through the pores rather than invasion through the matrix. Pores are available with diameters between 3 µm and 12 µm. Cells are seeded in medium in the upper part so they can migrate in a vertical direction towards an attractant in the lower compartment, through the pores of the membrane. The cells which pass the membrane are fixed, stained with cytological dyes such as haematoxylin, and quantified. The incubation time is usually overnight but it could be optimized depending on the cell type [54-58].

Wound-healing assay (scratch assay)

This assay is one of the most widely used 2D migration assays. The scratch assay simply involves a confluent plate of any attached cell type which is ‘‘wounded’’ by scraping off an area of the cells. Cell migration can then be monitored microscopically while the cells are travelling through the scratched region [57]. Cell migration can be analyzed by measuring the decrease in wound area. The plates can be coated with e.g. fibronectin, collagen I or collagen IV before cell seeding to allow the study of migration on different substrates [59]. Cell migration in scratch assays appears as either single cells, loosely connected population or collective sheets of cells [60]. There are many advantages of the scratch assay such as simplicity, cheapness, easy readout, rapid setup, and analysis. Among the main drawbacks of the assay however, is that the migration speed of cells just prior to wound closure increases resulting in variations in results [61].

Cell exclusion zone assay (stopper based assay)

The cell exclusion assay depends on creating cell exclusion zones by fitting small silicone stoppers into each well of a 96-well plate. These stoppers are inserted before seeding the cells to create an exclusion zone with the tip of the stopper. After cell adhesion the stoppers are removed, resulting in circular cell-free areas of 2 mm in diameter wherein the cells will then migrate. The advantages of the cell exclusion zone assay are that the wound sizes are similar and with sharp borders. Also there is no damage to the cells from mechanical scraping as in the scratch assay [62].

Fence assay (ring assay)

The fence assay involves seeding the cells in the inner area of Teflon, glass or metal ring placed on a cell culture dish after detaching the ring, the non-attached cells are removed by gentle washing. The cells in the ring are then allowed to migrate out from the circular area in a radial way. The cell migration is measured as an increase of the area covered by migrating cells [63].

Microcarrier bead assay

The microcarrier bead assay measures the cell migration from microcarrier beads onto 2D cell culture vessel surfaces. The microcarrier beads are coated with cells grown to confluence on the surface of the beads. These are then placed on the culture dishes and incubated for a specific period of time. The beads are then removed and the cells which have migrated from the beads to the vessel surface area are fixed, stained and microscopically analysed. An advantage of this assay is that it allows the cells to perform close cell–cell interactions on the beads to mimic the tight contact of cells *in vivo*. Another advantage of this assay is that relatively equal numbers of cells are present on the bead surface when confluence is reached, making results comparison easier [64].

Single cell motility assay (colloidal particle assay, colloidal gold single cell migration assay)

This assay aims to measure migration of single cells through colloidal gold particle coated surfaces [65]. In this assay the cells are seeded at low density onto tissue culture plates coated with colloidal gold, seen as a homogenous layer of small dark dots under the microscope. White tracks appear under the microscope as a result of cell migration [66]. The main advantage of this method is that it is suitable for automation and for monitoring single cell migration, chemokinesis movement and real-time path to determine the absolute speed of cell migration [18].

Invasion Assays

Cell invasion assays test the ability of the cell to transfer through an ECM or basement membrane extract (BME) barrier by enzymatically degrading the barrier in a new location, thus mimicking invasion during tumour dissemination. There are many invasion assays, which can be classified as 2D or 3D, the most common of which are described in the following sections below.

The 2D invasion assays

Modified Boyden chamber assay (Transwell invasion assay): This assay is a modification of the transwell migration assay described previously in 1.6.3.1 and is carried out in transwells, into which membrane filters are inserted. These filters have 5–12 μm size pores, overlaid by a thin layer of ECM. The cells are seeded into the top chamber. The filters present between chambers restrict the movement of the cells from the upper chamber to the lower one. Only the invasive cells can degrade the matrix and adhere to the bottom of the filter where they are stained with haematoxylin. The stained cells are counted with a light microscope or analysed using fluorimetric detection. The invasion level is defined as the percentage of cells passing through the filter and so to calculate the invasive index, the ratio of invaded versus the migrated cells is calculated [56,67,68].

The advantages of the method are the availability of different insert sizes, the relative ease of the experimental setup and the relatively small chemokine gradient between the upper and the lower compartments [69-72].

Cell exclusion invasion assay: The cell exclusion invasion assay involves small silicone stoppers fitted in 96-well plates coated with thin layer of BME. As a result, the stoppers form exclusion zones when cells are seeded. The stoppers are removed after the cells adhere to the first layer of BME. The cells and the formed cell-free circles are then, overlaid by another relatively thick layer of BME. As a result of that, a layer of cells embedded between two layers of ECM and a centre of cell-free ECM are formed. Cell invasion towards the centre can be seen using light microscopy over time as well as using confocal microscopy after immunofluorescent staining. Among the main advantages of this assay is possible kinetic analysis and live-imaging. At the end of the assay the gels can also be fixed and processed for immunofluorescence analysis [73].

The 3D invasion assays

The use of animal and humans models in research is usually hindered by the availability of test subjects, difficulty of testing procedures, and ethical concerns regarding pain caused to live subjects. The two-dimensional (2D) monolayer models have been used as an alternative to animal models and have been a huge addition to research; however there have been limitations for using those models. One of the biggest problems associated with the 2D model is the inability to reflect the real situation in the tumour microenvironment. The 3D models arise as an alternative to the previously used assays. Unlike the 2D models, the 3D models are able to provide a cellular microenvironment that is able to mimic the original microenvironment found in tissues or represent the single cell behaviour on the 3D level more closely. This feature is vital for more comprehensive drug testing experiments.

3D cell tracking: This assay involves 3D analysis of single cell invasion through ECM using computer-aided time-lapse video microscopy. There are many ways to analyse either labelled or unlabeled cells at the 3D level using different microscopy techniques such as confocal microscopy, contrast or digital microscopy. Among the major advantages of this assay is the possibility to analyse the cell tracking in real time the possibility to determine the exact length of the invading cell paths [74].

Gelatine degradation assay: The gelatine degradation assay allow the analysis of invasion at the sub-cellular level rather than the whole cellular level [75]. In this assay cells are seeded on top of a thin layer of a fluorescently labelled matrix. The fluorescence is lost in the regions where the matrix is degraded by the invading cells consequently the sub-cellular invading structures can be detected by high resolution pictures [76]. The main advantage of this method is that it provides high resolution data of sub-cellular structures of invading cells although cells cannot be followed as a whole during their movement [77].

Vertical gel 3D migration/invasion assays: The vertical invasion assay aims to investigate the downward movement of cells from a monolayer through a layer of ECM. This assay involves preparation of a thick collagen layer with the cells seeded on the top of the gel surface. Stromal subcutaneous fibroblasts are embedded in the collagen gel to investigate the effect of fibroblasts on the invasion of tumour cells [78]. The vertical invasion of the cells from the cell layer can be followed by monitoring the optical sectioning and counting the cells by radioactive labelling or quantified by immunohistochemical staining or by using image analysis software [79,80].

Spheroids

Spheroids are considered as one of the best examples of 3D culture models as they better represent the *in vivo* tumour structure in comparison to 2D cell monolayer cultures [81]. In this thesis I am going to investigate the use of tumour spheroids as a 3D invasion model to study the effect of novel cancer therapeutics.

Spheroids are defined as spherical cell aggregates which are formed in dishes spontaneously or by culturing on treated substrates. The spherical model is applied on normal and tumour cell lines [82,83].

The 3D spheroid structure is one of the best ways to reflect the solid microenvironment. This is because spheroids are characterized by heterogeneous cell populations with intermediate, necrotic areas mimicking the avascular stages of solid tumours including transport of oxygen, nutrient, and metabolite inside the microenvironment [84-86].

Spheroids mimic the proliferation gradient of solid tumours as the outer layer of the spheroid contains the proliferating cells while the innermost layer contains non cycling, differentiated or dead cells due to necrosis [87,88].

Methods for generating spheroids

There have been many methods reported for culturing spheroids based on the nature of the cells and their ability to form spheroids. These methods include the hanging drop method [89], spinner flasks [90], spontaneous aggregation [91] and growing cells in methylcellulose containing media [92]. The expression of certain molecules such as E-cadherin [93] and integrins such as αv integrin subunit [94] on the surface of the cells could promote of spheroid formation.

Hanging drop method

This method results in formation of relatively small uniformly sized spheroids. It involves setting up 20-30 µL drops containing certain cell concentrations on the inner surface of the lid of a Petri dish and leaving this in an incubator for a few days until the formation of spheroids occurs (**Figure 1A**). The main advantage of this method is that it produces homogenous sizes of spheroids in relatively short period of time. However the main disadvantage associated with this method is that it is not suitable for all cell lines; in addition to that this method is not suitable to form multilayer spheroids [89,95].

Spinner flask method (rotary systems)

The cells are grown as suspensions in culture medium in Petri dishes coated with agar to prevent the attachment of the spheroids to the surface of the flask, leading to the production of high numbers of micro-spheroids. The spheroids are then transferred into spinner flasks in which the spheroids grow until they reach the required diameter size [90]. The cell suspensions could also be directly transferred to a rotating 6-well plate or a 250 ml spinner vessel stirred by a magnetic stir bar until the spheroids are formed **(Figure 1B)**. The main advantage of this method is that is suitable to produce multilayer spheroids. However the main disadvantage is the resulting spheroids are heterogonous in size.

Spontaneous aggregation

The cells are cultured at a high seeding density in non-adherent culture dishes or coated with agar until they spontaneously form spheroids. The spheroids of certain diameter size are then selected and grown in new dishes **(Figure 1C)** [91]. The main advantage of this method is that it is relatively easy to perform compared to other methods. The main disadvantage is that it is not applicable to all cell lines.

Spheroids grown in methylcellulose containing media

The cells are plated in non-adherent round bottom wells in methylcellulose-containing medium to form spheroids of defined cell number and composition. At this stage, the spheroids can be transferred to collagen to be observed for different characteristics such as invasion **(Figure 1D)** [92]. The main advantage of this method is that it produces homogenous sizes of spheroids. However, the main

disadvantage associated with this method is that it is not suitable for all cell lines.

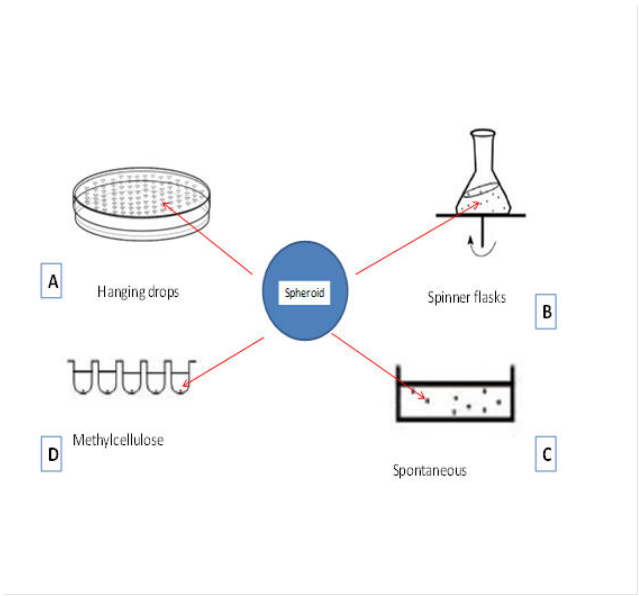

Figure 1 The different ways to make spheroids adapted from [96].

Spheroid use in 3D tumour models of invasion

Spheroids are among the best *in vitro* options to mimic the 3D tumour structures *in vivo* [97]. In this section I will discuss some of the 3D spherical models which are used as invasive models.

Spheroid/mono dispersed cell invasion assay

The main principle of this assay is that non-invasive spheroids are co-cultivated with a single cell suspension of invasive cells, which attach to the spheroid surface and start to invade into it [98]. Fluorescently labelled cells can be used to demonstrate the 3D invasion with (confocal) fluorescence microscopy imaging. The analytical quantification of cells attached to the spheroids can be done using flow cytometry or by immunofluorescence staining of the spheroid sections. The importance of this assay is that it mimics the cell–cell interactions and multicellular 3D structures of the tissues through which the tumour cells invade [99].

Spheroid confrontation assay

This assay studies the invasive behaviour at a 3D level through co-cultivating spheroids composed of invasive cells side by side with spheroids of non-invasive cells. The invasive cells start to invade through the non-invasive ones and the behaviour of these cells is investigated. The invasive spheroid may invade as single cells, collectively or display a non-invasive behaviour [100]. Among the important advantages of this method is the consideration it takes of the cell–cell interactions and the 3D structure of the tumour tissue and the surrounding tissue, leading to a more successful reflection of the *in vivo* situation when studying the cancer cells invasion to surrounding tissues and organs [100].

Spheroid gel invasion assay

This assay involves embedding spheroids into 3D ECM such as collagen I. If the spheroid is composed of non-invasive cancer cells it will stay as a compact spheroid but if the spheroid is composed of invasive cells it will start to invade into the surrounding matrix and demonstrate cell-line specific modes of invasion.

Spheroids that result from hanging drops are homogenously sized making them very suitable for 3D invasion assays [101-103]. Briefly, spheroids that result from hanging drops are allowed to invade into neutralized collagen layer. The invasion is observed over 4-7 days and is analysed or by simply calculating the number of cross points covered by invading cell using a grid **(Figure 2).** The gels containing the invaded spheroids could also be fixed, immunofluorescently stained and analysed by confocal microscopy [98].

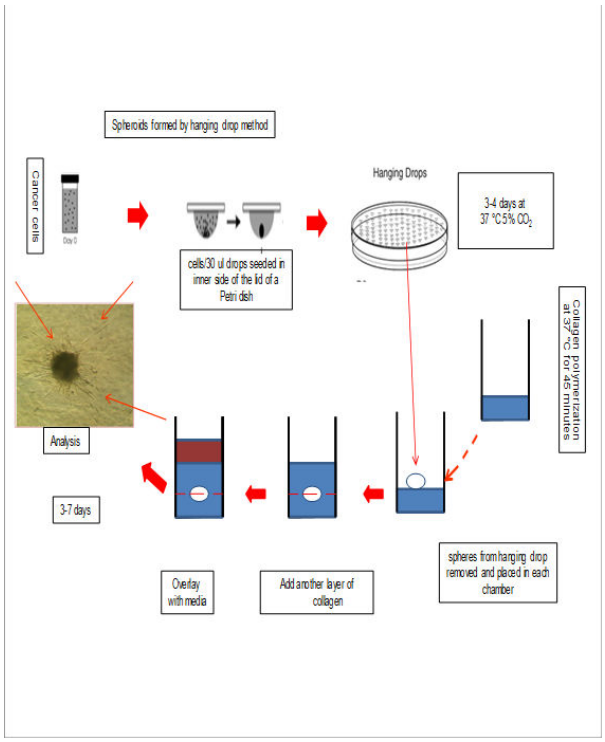

Figure 2 Schematic presentation of 3D spherical invasion assay [102].

Among the main advantages of this method is that cell invasion within the 3D matrix mimics very closely the invasion *in vivo*, especially as this assay takes in to consideration the cell-cell interactions between the tumour cells in the tumour mass *in vivo* [104].

Spherical co-cultures

Co-culturing tumour cells with different kinds of stromal cells is the best way to understand the cell crosstalk inside the

tumour microenvironment. The co-cultures can be made by preparing mixed spheroids of both tumour cells and stromal cells [105]. The tumour spheroids also could be co-cultured with fibroblast monolayers or fibroblast suspension or the fibroblast cells could be incorporated into the tumour spheroids to form multicellular spheroids [106,107].

Conclusion

The detailed picture of tumour and the tumour microenvironment interaction however is not fully understood due to a lack of representative models. This review shows a brief summary of the assays and models used to describe the tumour dissemination process.

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