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European Journal of Cancer 40 (2004) 881–889

European Journal of Cancer

www.ejconline.com

Modelling approaches for angiogenesis

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Received 5 December 2003; received in revised form 18 December 2003; accepted 12 January 2004

Abstract

The development of a functional vasculature within a tumour is a requisite for its growth and progression. This fact has led to the design of therapies directed toward the tumour vasculature, aiming either to prevent the formation of new vessels (anti-angiogenic) or to damage existing vessels (vascular targeting). The development of agents with different mechanisms of action requires powerful preclinical models for the analysis and optimization of these therapies. This review concerns 'classical' assays of angiogenesis *in vitro* and *in vivo*, recent approaches to target identification (analysis of gene and protein expression), and the study of morphological and functional changes in the vasculature *in vivo* (imaging techniques). It mainly describes assays designed for anti-angiogenic compounds, indicating, where possible, their application to the study of vascular-targeting agents.

Keywords: Angiogenesis; Angiogenesis inhibitors; Vascular-targeting agents; Endothelial cells; Tumour vasculature; Imaging; Preclinical assays

1. Introduction

Antineoplastic therapies directed towards the tumour stroma, and in particular against the tumour vasculature, are a promising new strategy [1–6]. Two main approaches have been identified: anti-angiogenic therapy, aimed at preventing the formation of tumour blood vessels, and vascular-targeting therapy, aimed at existing tumour vessels [6,7]. The anti-angiogenic strategy has led to the development of compounds designed to control a tumour's growth by blocking its ability to develop a blood supply. Vascular targeting is based on the finding that vessels within a tumour are phenotypically different from those in normal tissue, and are therefore selectively recognisable by antibodies or ligands of the target molecules. Anti-angiogenic and vascular-targeting agents can potentially be used as therapeutic tools *per se*, as vehicles for the delivery of toxic agents or vectors for gene therapy, and also in diagnostic imaging.

The preclinical analysis of several anti-angiogenic compounds has been successfully completed and they have entered clinical trials. Some of them have reached advanced-phase studies. Many drugs that were active in preclinical models of angiogenesis and inhibited tumour growth have, however, produced disappointing results once in the clinic. In relation to the many different reasons that might account for this (including inappropriate study design, choice of patients etc.), it is important to re-evaluate the significance and use of the preclinical models employed to select and measure the drugs' activity. Assays for the anti-angiogenic and vascular-targeting activity of compounds are now needed, not only to identify and validate molecular targets and to screen potential candidates but also to optimise dose scheduling and combination strategies, and to identify appropriate endpoints.

1.1. Choice of assay

The development of inhibitors of angiogenesis relies on a range of preclinical assays that mimic the various steps of the angiogenic cascade. Knowledge of the mechanisms of action of the tested compound will dictate the choice of assay. Alternatively, the behaviour of the compound in different assays may indicate its mechanism of action.

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doi:10.1016/j.ejca.2004.01.002

Ideally, assays of angiogenesis should by easy, reproducible, quantitative, cost-effective and permit rapid analysis. A typical analysis of anti-angiogenic activity is done with more than one assay, beginning with the more elementary assays *in vitro* that will provide easy, rapid and affordable data. *In vitro* assays cannot be considered conclusive, however, and the activity of a compound must be confirmed in other assays of increasing complexity, including *in vivo* assays of angiogenesis, angiogenic-dependent tumour growth and metastasis. *In vivo* assays are usually unsuitable for the quantitative screening of a large number of compounds, as they are often complex, expensive and may require specific surgical skills. Nonetheless, they are always required to confirm ultimately the activity of a potential drug.

2. *In vitro* assays of angiogenesis

In vitro assays are designed to recapitulate each of the multiple events that constitute the angiogenic process. Some of them are very specific in analysing a single event (proliferation, apoptosis, migration, production of proteases), whereas others provide a more complex picture of the process, involving multiple aspects, cell functions and interactions with the environment. Detailed descriptions of the methods can be found elsewhere [8–11].

In vitro assays for the activity of anti-angiogenic compounds are usually based on the use of endothelial cells. Though it is true that most anti-angiogenic/anti-vascular compounds do target endothelial cells, some do not and are instead directed against mural vascular cells (smooth muscle cells/pericytes) or the extracellular matrix. New potential targets for anti-angiogenic therapy have recently been proposed; these include lymphatic endothelial cells (given the recognised importance of intratumour lymphangiogenesis in tumour metastasis [12]) and endothelial progenitor cells (described as contributing to the formation of vessels in tumours [13]).

2.1. Choice of endothelial cells

A critical issue in setting up an *in vitro* assay is the choice of endothelial cells. Immortalized endothelial cells are sometimes used, as they provide an 'unlimited' source of cells. Although these cell lines have the obvious advantages of being easy to grow and relatively stable throughout *in vitro* passages and among batches, they have usually lost some of the characteristics of endothelial cells, including molecular markers, and exhibit changes in function. Not infrequently they are spontaneously activated and therefore unable fully to respond to angiogenic stimuli. If these cells are used it is therefore always better to confirm any relevant results on primary endothelial cells.

The most commonly used are endothelial cells from the human umbilical vein, as the source (the umbilical cord) is easily available and cell isolation is relatively simple. For the same reasons, bovine or murine aortic endothelial cells are often used too, but also these come from large vessels, and they have different phenotypic and behavioural characteristics from those of the microvessels that are more likely involved in angiogenesis. The most common sources of microvascular endothelial cells are the skin, brain, adipose tissue and adrenal gland. Endothelial cells derived from the microvasculature of different tissues/organs are often heterogeneous, imposing a further constraint on the choice of cell model [14]. Ideally, when developing inhibitors of tumour angiogenesis, tumour-derived endothelial cells should be used [15]. So far, however, practical difficulties in their isolation from tumour tissue and maintenance in culture have limited their use in preclinical studies.

Culture conditions are also critical. When grown in very rich media, endothelial cells proliferate rapidly, leading to a high rate of cell recovery, but often these over-stimulated cells become refractory to further stimuli and may therefore not be suitable for the assay.

An important consideration when using endothelial cells stabilised *in vitro* is that they usually lose the phenotypic, antigenic and functional characteristics imposed by their original environment. Culture conditions that more closely resemble the tumour environment of endothelial cells should be chosen.

2.2. Proliferation and apoptosis

The effects of compounds on cell proliferation and survival can be measured by mitogenic assays (incorporation of thymidine, 5-bromodeoxyuridine), by counting the increase in cell number (direct counts or indirect colorimetric evaluation). Apoptosis can be measured by TUNEL, or by analysing the expression of apoptosis markers such as the caspases and annexin V.

Although endothelial cell proliferation is often considered the leading assay for anti-angiogenic activity, the ability of a compound to affect proliferation is not sufficient to prove such activity. Indeed, any cytotoxic compound can be expected to inhibit the proliferation of endothelial cells, but only endothelial cell-specific antiproliferative compounds (i.e. those that do not affect the proliferation of other cell types at similar/lower concentration) can be considered selectively anti-angiogenic. This issue has been particular relevant in the definition of the anti-angiogenic activity of cytotoxic chemotherapeutics [16–19].

Finally, it should be kept in mind that the inhibition of endothelial cell proliferation is not a requisite for anti-angiogenic activity. On the contrary, a lack of antiproliferative activity may indicate low toxicity for anti-angiogenic compounds acting with non-anti-

proliferative mechanisms (e.g. inhibitors of cell motility, invasiveness etc.).

2.3. Motility

The Boyden chamber assay is the most commonly used test for endothelial cell motility. It can be performed in modified or unmodified chambers, or using disposable membrane inserts. The lower compartment of the chamber (containing the attractant) is separated from the upper (containing the endothelial cells) by a matrix-coated polycarbonate filter with pores small enough to allow only the active passage of the cells (5–12 μm pore size). Growth factors [e.g. vascular endothelial growth factor and fibroblast growth factor-2 (FGF-2)] or conditioned medium (e.g. from tumour cells or NIH-3T3 fibroblasts) can be used as the attractant, again depending on the mechanism of action of the inhibitor to be tested. Migration occurs rapidly, and in 4–6 h the cells have migrated through the filter and can be stained and counted.

In checkerboard experiments, the assay can be used to distinguish between chemotaxis (directional migration) and chemokinesis (random motility). Positive, negative and null gradient conditions are created by adding different amounts of attractant to the lower and/or upper compartments [20]. In addition, it is possible to create a gradient of substrate-bound attractant between the two sides of the filter to study haptotactic motility (migration induced by substrate-bound attractant [21]).

Other *in vitro* assays analyse the planar motility of endothelial cells, such as the healing of a 'wound' created in a monolayer of confluent cells.

2.4. Invasion/matrix degradation

Several compounds affect the ability of endothelial cells to degrade the extracellular matrix, therefore affecting their invasiveness. This is the typical case for inhibitors of matrix metalloproteinases [22]. Assays are available to measure the matrix-degrading activity of cells (radio- or fluorescent-labelled matrix degradation; the production and activity of proteolytic enzymes). Several assays are used to analyse the invasive ability of endothelial cells, measuring simultaneously both motility and matrix degradation (Boyden chamber invasion assay; phagokinetic tracks). The invasion assay is essentially as described above for the Boyden motility assay, except that the filter is coated with a thick layer of the matrix (usually Matrigel), which the cells must degrade before migrating through the filter.

2.5. Tube/cord formation

Following their proliferation and migration in the interstitial stroma, endothelial cells align and establish contact with each other, originating capillary-like structures. This event is recapitulated *in vitro* by assays in which endothelial cells, seeded on a permissive matrix substrate (usually made of type I collagen, fibrin or Matrigel), rapidly form capillary-like structures (reviewed in [8,23]). The presence of a lumen within these neocapillaries is considered proof that these assays truly recapitulate the morphogenetic events leading to capillary formation. The assays can be two- or three-dimensional, depending on whether the formed capillaries lie in one plane or are spatially organised within the whole matrix. To quantify the response, imagine-analysis techniques are used that measure the number and length of the tubes, the area covered by the capillary-like network, and/or the number and complexity of the connections.

This assay can also be used to evaluate the effect of agents that target newly formed vessels. The addition of such agents to newly formed cords causes rapid disruption of the capillary-like structures [24].

2.6. Embryoid body assay

This assay is largely used to study vasculogenesis, the differentiation of endothelial cell precursors to form a primitive vascular plexus; it is based on the use of murine embryonic stem cells [25]. These cells are maintained *in vitro* as totipotent and can be induced to differentiate into endothelial cells through a sequence of genetic events that closely resembles that of embryonic vasculogenesis. Although mainly used to dissect molecular events, this assay is also a useful tool for analysing the effects of compounds on angiogenesis [25–28].

2.7. Aortic ring assay

This *ex vivo* assay closely recapitulates the complexities of angiogenesis, forming a bridge between *in vitro* and *in vivo* studies. The cells are not subjected to the selection involved in isolation and growth *in vitro*, and, more importantly, they are not separated from their environment. This model allows the study of vessel formation in a complex system, because, as well as endothelial cells, other cell types (mural cells and fibroblasts) that play an important part in angiogenesis are present. Explants of rat or mouse aorta are cultured in biological matrices (collagen or fibrin) in the presence of the appropriate growth factors, where they generate outgrowths of branching microvessels [29].

The assay is sensitive, reproducible and quantitative [30]. Angiogenesis is quantified by visual counting or computer-assisted image analysis. Time-lapse videomicroscopy, immunohistochemical and ultrastructural studies can also be applied [29]. The drawback of using large vessels (which do not represent the normal source of tumour neovessels) can be overcome by using aortic

arches dissected from the chick embryo, which more closely resemble the endothelial cells of microvessels [9].

3. *In vivo* models of angiogenesis

The ideal *in vivo* assay for evaluating the efficacy of anti-angiogenic compounds should permit the scrutiny of vessel formation and maturation in a natural environment, and, at the same time, be suitable for rapid, easy and possibly quantitative analysis. Several *in vivo* assays of angiogenesis have been developed, but so far none of them completely meets these requirements. Most have the disadvantage of requiring surgical skills, of being difficult to quantitate, of inducing angiogenesis in irrelevant sites, and of lacking reproducibility. Finally, because tissue is often injured in the process, inflammatory reactions, which in turn cause vasoproliferation, can disturb the results.

Some assays allow for the detection and evaluation of vessels throughout the duration of the experiment, as angiogenesis is induced in sites that are naturally (cornea) or artificially (chambers) accessible. In other tests (Matrigel plug), the evaluation is made at one time-point only. In some cases, new vessels can be induced in a previously avascular site (such as the cornea, which is naturally avascular, or in exogenous supports such as Matrigel or sponges). In other assays the presence of a naturally occurring vasculature can hinder the evaluation (see Table 1).

3.1. Vascularisation into exogenous supports

Various *in vivo* assays have been developed in which the formation of new vessels is stimulated by angiogenic factors or tumour cells embedded in a biocompatible matrix made of synthetic polymers or natural materials.

Angiogenesis can be induced by tumour cells entrapped in a support of alginate polymer injected subcutaneously in mice (reviewed in [31]). The trapped tumour cells are isolated from direct contact with the host environment (including the immune system) and cannot proliferate, but the soluble angiogenic factors they produce are able to diffuse. Blood vessels form rapidly (3 days) and can be quantified by measuring the haemoglobin content or by histological analysis. The advantage of this assay is that it is not species-specific and can be used to test angiogenesis induced by tumour cells in syngeneic and xenogeneic systems.

3.2. Matrigel plug assay

This assay [32] takes advantage of the peculiar characteristics of Matrigel, an EHS sarcoma-derived, basement membrane preparation consisting of extracellular matrix components and growth factors. The nature of

Table 1
Advantages and drawbacks of major *ex vivo* and *in vivo* assays commonly used in the preclinical evaluation of inhibitors of angiogenesis

Assay	Easy to perform	Reproducibility	Quantitative	Evaluation in real time	Cost-effectiveness	High throughput	Natural site of angiogenesis	Avascular site	Availability of reagents	Representative of tumour angiogenesis
Aortic ring	+ + +	+ +	+	+ +	+ +	+ + +	−	−	+ +	−
CAM[a]	+ +	+ +	+ +	+	+ +	+ +	+	−	−	−
Cornea	−	±	+	+ +	−	−	−	+ +	+ +	−
Matrigel	+ + +	+	+ +	−	+ +	+	−	+ +	+ +	+
Chamber	−	+ +	+ +	+ +	+	−	±	±	+ +	+ +

[a] CAM, chorioallantoic membrane.

Matrigel enables it to act as a support for the development of new blood vessels. When enriched with angiogenic stimuli and injected subcutaneously in mice, it solidifies into a gel that can sustain the infiltration of host cells, and, in particular, of the endothelial cells that develop functional vessels. Some 5–10 days after implantation, the angiogenic response is quantified either by measuring the haemoglobin content of the Matrigel (as an index of functional, blood-containing vessels) or by immunohistochemical analysis of the pellet (i.e. by counting structures positive for CD31, a specific marker of endothelial cells). Different angiogenic stimuli can be employed in this system, particularly FGF-2, cell culture supernatants, or, directly, angiogenic cells.

This assay has been used in the preclinical evaluation of angiogenesis inhibitors that prevent new vessel formation [16,22,33]. In this case, treatment is usually begun early, when vessels are not yet formed, and continues throughout the whole duration of the experiment. In addition, the Matrigel plug assay can be used to study vascular-targeting agents [24]. Treatment with these agents, which act on existing neovessels, is done when vessels are already formed (5–7 days after Matrigel implantation). Shortly before autopsy, mice receive an intravenous injection of fluorescent tracers (such as fluorescein isothiocyanate-conjugated *Griffonia simplicifolia* isolectin B4) and the collected pellets are then analysed under confocal microscopy, where the three-dimensional structure of permeated, fluorescent vessels can be used as an index of vessel functionality.

The limitations of the Matrigel assay concern partly the complex nature and origin of Matrigel itself, and partly the procedure, e.g. the impossibility of following the angiogenic response with time. In addition, the vessels develop in an environment that is different from tumour tissue, which poses questions about the significance of the results for oncological applications. However, the technical simplicity, versatility and use of the mouse as the recipient host make this assay a convenient *in vivo* model.

3.3. Cornea assay

This is perhaps the most traditional assay of angiogenesis [34]. Angiogenesis is induced by the implantation of slow-release pellets (usually made of Elvax or Hydron) containing the test substances (including the angiogenic growth factors) into micropockets produced surgically in the avascular cornea of rabbits, rats or mice. Tumour fragments or cells can also be implanted and used as the source of angiogenic factors. It is possible to evaluate the interaction of multiple factors by either incorporating different substances into the same pellet or by implanting separate pellets into two adjacent pockets in the same cornea. Angiogenesis is quantified by subjective or computer-assisted analysis of the number and growth rate of newly formed vessels, using a slit-lamp stereomicroscope. This assay has recently been modified to study lymphangiogenesis [35].

An advantage is that the cornea is naturally avascular and hence the interpretation of result is clearer than in other bioassays such as the chorioallantoic membrane. Moreover, given the accessibility of the site, the angiogenic response can be monitored daily. The assay is also suited for investigating the regression of blood vessels once formed. Its drawbacks are the need for surgical skills, the difficulty in quantifying the response, the high costs and the limited number of performable tests, especially when using rabbits.

3.4. Chorioallantoic membrane (CAM)

This widely used assay allows the testing of angiogenic and/or anti-angiogenic compounds in the naturally occurring vasculature of the CAM [36,37]. Test substances (both stimulators and/or inhibitors) are added to a support (such as collagen or gelatin sponges or synthetic polymers such as Elvax 40 or Hydron) and placed on the extra-embryonic membrane, either *in ovo* or *ex ovo*. As in the cornea assay, single or multiple factors, cells or entire tumour fragments can be used to stimulate angiogenesis. The angiogenic response is measured by subjective or computer-assisted counting of the new vessels, or by histological analysis of the membrane.

Compared to other *in vivo* assays the CAM assay has the advantages of low cost, rapid throughput and relatively easy application. It does require some skill in evaluating the response, as the presence of natural vessels can interfere with the results. Attempts have been made to make this assay more quantitative [38,39]. A final drawback of this model is the scarcity of aviary-based reagents.

3.5. Chronic transparent chambers and intravital microscopy

In these assays, a transparent chamber is implanted in the ear, dorsal skin or cranium of rabbits, mice, rats or hamsters. Angiogenesis is stimulated by angiogenic factors or tumour cells, and can be quantitated by epi- or transillumination, depending on the thickness of the preparation (reviewed in [10]). These assays require a certain amount of surgical skill, and, unless performed on small animals, they are neither cost-effective nor high throughput. Their great advantage is that they allow visualization of angiogenesis across time, so evaluating phenomena associated with both onset and regression. The intravital microscopic techniques developed in these models have proved a powerful tool for the non-invasive, continuous monitoring of cellular and molecular

events. These imaging techniques cannot yet be used for extensive screening and they require expensive equipment, but this method for real-time analysis is providing essential information on the mechanisms of angiogenesis, the metabolic environment of tumours and the response to inhibitory compounds (reviewed in [11,40]). These and other imaging techniques will increasingly become indispensable to the preclinical development of anti-angiogenic compounds.

3.6. Zebrafish

The zebrafish (*Danio rerio*) is a recent addition to the *in vivo* models for studying inhibitors of angiogenesis [41,42]. This model of vascular development has several advantages: in this animal, embryogenesis occurs outside the mother's body, the embryos are transparent, vascular development is very rapid, and large numbers of embryos can be easily manipulated. The system is particularly suitable for the modulation of genes involved in angiogenesis. The homology between the molecular and signalling pathways that drive vessel development in the zebrafish and mammals is remarkable. Blood vessels can easily be quantified, for instance by using transgenic fish with fluorescent vessels [42]. Inhibitors of angiogenesis, including the inhibitor of vascular endothelial growth-factor receptor, have been successfully studied in this *in vivo* model [41].

4. Assays for studying anti-angiogenic/antivascular compounds in tumour models

4.1. Tumour models

Preclinical investigations of the antineoplastic activity of angiogenic inhibitors and vascular targeting compounds require final validation in *in vivo* tumour models. Murine tumours in syngeneic mice or rats and human tumours xenografted into immunodeficient mice can be used.

The classical model of the Lewis lung carcinoma has been extensively used to test anti-angiogenic compounds [43]. It has the advantage of growing in a syngeneic mouse in which the host response (including the vasculature) is intact; it is rapid, reproducible and metastatic. The tumour is, however, usually transplanted subcutaneously, which does not reflect its original site.

Xenograft models (in nude or severe combined immunodeficient mice) have the advantage of reflecting the biology of human tumours (e.g. the response to human tumour-derived angiogenic factors). Tumours are often transplanted orthotopically in the organ site of origin. Unfortunately, xenografts do grow in a xerogeneic and immunodeficient recipient, with consequent impairment of the host response.

As studies in the field of vascular targeting have progressed, the specificity and suitability of the *in vivo* tumour models has greatly improved. Angiogenesis-dependent tumours, in which the expression of angiogenic factors can be modulated [44], and models of angiogenesis-dependent escape from tumour dormancy have been developed. Genetically engineered mouse models of tissue-specific carcinogenesis have been used, as they allow to study the effects of anti-angiogenic therapies on all stages of tumour progression, particularly the early phases where a tumour acquires the capacity to induce angiogenesis (angiogenic switch). These multistage models have been particularly useful in demonstrating that anti-angiogenic treatments have a distinct effect at different stages of tumour progression, a finding with obvious clinical implications [45].

Endpoints for evaluating the efficacy of anti-angiogenic/vascular targeting therapies in *in vivo* models include an objective effect on the 'take', latency, growth rate and metastasis of the tumour. Analysis of the tumour vasculature is an important issue. A standard approach to quantifying angiogenesis in tissue sections is to measure the vascular density (maximal number of blood vessels per area unit) where blood vessels are detected by immunostaining of endothelial cells for von Willebrand factor, CD31 or CD34. Although the vessel count has been shown to be a factor for successful prognosis, it is now accepted that a reduction of vessel density does not always occur, and it is therefore not to be necessarily expected following anti-angiogenic treatments [46]. More subtle changes in the architecture/function/haemodynamics of the tumour vasculature are instead to be expected and can be analysed using different tools. These methods include the measurement of vessel structure, dimension and dilation (e.g. by corrosion casts), vessel maturation (e.g. staining for pericytes), permeability (e.g. using fluorescent tracers or dyes), and of changes in metabolic parameters and oxygen tension in the tumour tissue. With those vascular targeting agents that act by shutting down the tumour vasculature, vessel occlusion (permeation by fluorescent tracers) and tumour tissue necrosis (histological analysis) are to be expected.

Most of these assays for evaluating the vascular response are, however, invasive and can rarely be applied to the patient's tumour. Nevertheless, the need to develop endpoints for detecting responses to anti-angiogenic therapy in living animals and in patients is urgent, since, by definition, anti-angiogenic treatment is cytostatic and objective clinical responses (such as tumour regression) cannot always be expected. One way of overcoming this problem is to develop new ways of imaging that can monitor changes in vascular function and consequently the therapeutic response in patients.

4.2. Imaging of angiogenesis

Advances in imaging are transforming our understanding of angiogenesis and the evaluation of compounds that affect angiogenesis in preclinical models and in the clinic [47,48].

Cellular and molecular features of the microvasculature can be analysed by microscopic imaging methods (fluorescence, confocal, multiphoton and electron microscopy). These techniques are particularly powerful for the high-resolution analysis of tissue specimens. When applied intravitally with a tracer that can reach the vasculature through the circulation in preclinical tumour models, a real-time functional read-out of tumour blood vessels is produced [11]. Newer optical imaging techniques, such as fluorescent (the GFP reporter system) and bioluminescent (the luciferase reporter system) imaging, are of particular value for mapping specific molecular events in mice and for tracking cells [these methods are described in detail elsewhere in this issue of the journal].

Clinical methods, such as magnetic resonance imaging (MRI) and computed tomography following the administration of contrast media, or positron-emission tomography (PET) visualizing the distribution of a radiolabelled tracer, provide non-invasive methods of visualising angiogenesis deep within living tumour tissue. Compared with microscopy, however, they have a much lower resolution and in general cannot resolve the vessels of the microcirculation. Many of these non-invasive techniques, originally developed for human use, have now been scaled down to allow the high-resolution imaging of mice [48]. Mouse imaging models are increasingly used in preclinical work where tumours can be imaged at frequent intervals before and after drug administration. Because of its versatility, but despite its low sensitivity, MRI is now frequently used to monitor angiogenesis in drug-treated mice [49]. The higher sensitivity of PET, which allows measurement of tracer concentrations in the picomolar range, is promising for the visualization of specific molecular targets. Novel PET tracers (reporter probes) are being developed to image selective ligands on endothelial cells in live animals [48]. It is anticipated that these non-invasive ways of assessing the expression and functional activity of the target molecules of anti-angiogenic therapy will facilitate their further biological characterization.

5. Transcriptomics and proteomics

New tools for analysing the profiles of gene and protein expression will certainly open a new era in the development of anti-angiogenic and vascular targeting therapies. These strategies will allow the identification of new molecular targets for vascular targeting, examine the molecular signature of compounds in order to clarify their mechanisms of action, and classify them on the basis of the induced molecular response.

As discussed above, the loss of molecular characteristics, probably due to their removal from the host environment, imposes a limit on the use of *in vitro*-established endothelial cells or other supporting cell populations. New techniques are currently being employed to identify molecules expressed in the tumour stroma or directly on tumour endothelial cells. SAGE analysis of gene-expression profiles in endothelial cells purified from human tumours has led to the identification of several genes preferentially expressed on tumour-derived endothelial cells [14]. Putative cell-surface molecules, identified by sequence analysis of the differentially expressed genes, might represent candidate targets for antivascular therapies. In vivo phage display technology [50] has been recently used to identify vascular-specific motives associated to distinct tumour types and stages [51,52]. Laser capture microdissection to isolate endothelial cells, microarray analysis and affinity-based mass spectrometry (SELDI-TOF) are other examples of innovative technologies currently employed to identify transcriptomic and proteomic changes in tumour endothelial cells [53].

6. Conclusions

The field of study in angiogenesis is expanding and therefore the evaluation of the angiogenic response is becoming ever more relevant. We have reviewed some of the most frequently used models/methods for the preclinical evaluation of angiogenesis inhibitors and vascular targeting agents. When choosing an assay, it will always be useful to keep in mind the putative mechanism of action of the test compound and the desiderated endpoints. *In vitro* analyses in general rely on more endothelial types and are conducted with different assays. For validation, *in vivo* assays will be needed, but as yet no single assay seems adequate. They require technical skill, often lack of reproducibility and are difficult to quantify. The preclinical modelling of angiogenesis should therefore aim at assays that more closely recapitulate the complexity of angiogenesis and allow functional evaluations at the molecular level *in vivo*. The remarkable progress made in imaging technology might help to provide tools for translating preclinical results to clinical settings.

Acknowledgements

The Italian Foundation for Cancer Research has supported part of the studies described in this review.

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