The relationship between neoplastic cells and blood vessels in primary and secondary brain tumours.

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Abstract

Cancer associated angiogenesis has been regarded for many years as essential for a tumour to grow. However the discovery that some neoplasm can also grow in absence of angiogenesis has demonstrated that the relationship between cancer and blood vessels is much more complex than previously believed. These non-angiogenic tumours grow by co-opting and exploiting the pre-existing vessels. Alongside lung, liver and lymph nodes, brain is one organ where both primary and metastatic malignancy can grow in this way. Among the primary tumours of the central nervous system those from the glia have been more extensively investigated. Anti-angiogenic treatments have been tested but the results have not kept up with the expectations, one of the reasons being the ability of these tumours to exploit the normal brain vessels. Metastatic tumours to the brain are a common clinical event among oncological patients with poor prognosis and non-angiogenic growth has been observed also in brain secondary. Initial data from literature have started to show that vascular co-option by both primary and secondary malignancy is an active process involving pathways related to cell adhesion, motility and apoptosis. Detailed understanding of this process will possibly lead to new therapeutic approaches aimed to disrupt this modality of neoplastic progression. In this review it is presented a synthesis of our current understanding of this intriguing relationship between vessels and tumours in the brain.

Key words:
Brain primary tumours, brain metastases, angiogenesis, vascular co-option

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1. Cancer and blood vessels: an introduction

According to the general rule enunciated by Folkman (1) angiogenesis was believed to be essential for any type of tumour to grow. Following the discovery that tumours can also grow without angiogenesis (2) the relationship between vessels and cancer turned out to be much more complex than thought before. It is now recognised that there are multiple ways (summarised in **Table 1**) in which a tumour can be vascularised.

Table 1. Mechanisms of tumour vascularization

Formation of new vessels: "angiogenic tumours

A) Angiogenesis
   i. Sprouting angiogeneses
   ii. Intussuceptive angiogenesis
   iii. Glomeruloid active

B) Vasculogenesis

Without formation of new vessels: "non angiogenic tumours"

A) Vascular co-option
   (including glomeruloid passive)

B) Vasculogenic mimicry *****
   (including Glioblastoma-Endothelial Cell Transdifferentiation)

Mixed: including more than one mechanism.

*****It is now coming up that in some tumours vasculogenic mimicry appears to be guided by pathways usually inducing angiogenesis (7): it can therefore be argued that cancer cells in some tumours with vasculogenic mimicry are actually angiogenic cells but trigger mimicry rather than classic new vessel formations.

Angiogenesis is defined as formation of new vessels growing from the pre-existing one and there are three types described (3). The first one is "sprouting angiogenesis", in which the new vessels grow as a branch from a pre-existing one; the second is called "Intussusceptive Microvascular Growth" or "Splitting angiogenesis" which is defined as the longitudinal splitting in two of the pre-existing vessel producing two vessels, and the third is the "Glomeruloid Microvascular Proliferation (active type)" in which new microvessels fold to form the glomeruloid vascular bodies. Vasculogenesis is instead the formation of new vessels originating from normal stem cells (3).

Vascular co-option is instead defined as the hijacking and exploitation of pre-existing vessels by cancer cell which fails to trigger angiogenesis and/or vasculogenesis (non-angiogenic tumours) (4).

Vasculogenic mimicry (VM) is defined as the ability of tumour cells to form vessel-like networks (5): it was reported as another way for tumours to receive blood in the absence of angiogenesis (6). However, as discussed later on in this paper, it has subsequently emerged that vasculogenic mimicry is due to the "stem cell" capacity of the cancer cells to differentiate. This capacity is variable from cell type to cell type so it leads to a broad spectrum of events: from crude channels lined up by clearly morphologically neoplastic cells up to normal looking

vessels. However these latter, have been demonstrated to equally be made up by neoplastic cells, as they contain genomic alterations like the other tumour cells, but are able to achieve a very good differentiation. As it is now emerging that in some instances this second type of vasculogenic mimicry appears to be driven by angiogenic pathways (7), it is well possible that some tumours with vasculogenic mimicry are actually angiogenic but trigger new vessels formation from cancer cells rather than from pre-existing vessels or normal stem cells.

2. The primary brain tumours

Tumours originating from the glia cells, broadly defined as glioma, are the most common in the Central Nervous System (CNS). Several different types are named in the latest WHO classification, and each of them is graded, from 1 to 5, according to their malignancy, the glioblastoma being the most aggressive among the glial tumours (8). Most of the studies on the role of blood vessels in primary brain tumours are concerned with tumours of the glia (9). As all the other neoplasia, gliomas need vascular support and this can be provided by angiogenesis and vasculogenesis. However some tumour cells can be non-angiogenic and can grow without triggering new vessels formation but by exploiting pre-existing vessels. In a minority of cases vascular mimicry is found.

2.1 The formation of new vessels: angiogenesis

Following the observation that factors secreted by tumours can induce angiogenesis (10) Brem and colleagues concluded, in a purely morphological investigation(11), that angiogenesis is present in gliomas using a Microscopic Angiogenesis Grading System, based on histological observation, of three criteria: vasoproliferation (i.e the number of vessels present), endothelial cell hyperplasia (i.e. hyperplastic capillaries) and endothelial cytology (i.e. endothelial morphological deviations including presence of mitosis). Results showed that low grade gliomas (astrocytomas) where comparable to normal brain for vasoproliferation and endothelial cell hyperplasia, but endothelial cytology was atypical while high grade gliomas (glioblastoma) scored highly for all three criteria. Subsequently, as the mechanisms driving angiogenesis were unveiled, further studies on human samples of glioblastomas demonstrated in these tumours an increased number of vessels associated with endothelial cell proliferation(12), and with expression of Hif1, VEGFA (12, 13), VEGFB (14)the VEGF receptor Neuropillin1 (15), tenascin C (16), Angiopoietin 2 (17)and CXCR4 (18). In gliomas presence of the anti angiogenic protein Thrombospondin-2 has been found to be inversely correlated with vascular density, but not with VEFG detection, and the authors suggest that Thrombospondin-2 action could be VEGF independent (19). In a comparable fashion three other angiostatic factors BAI1, BAI2 and BAI3 were equally expressed in normal brain and low grade glioma with BAI3 expression decreased in higher grade tumours (20). Glioblastoma associated endothelial cells, but not the endothelial cells in normal brain or low grade gliomas, are frequently positive for proliferating markers (12) and for the FAK tyrosine kinases, associated with cell migration: the authors suggest that its expression could be a marker of endothelial migration linked to angiogenesis(21). Angiogenesis in gliomas has been also indirectly demonstrated by revealing, using imaging, the physiological consequences of angiogenesis, which are vessels permeability increase, changes in vascular perfusion and tissue oxigenation levels (22).

A special type of vascularization has been described as frequent in glioblastoma,

although can be also be found in other tumours, the so called Glomeruloid bodies, also known as Glomeruloid Microvascular Proliferation, as their morphology reminds the kidney glomeruli (23). They are made up by small vessels lined by high endothelial cells, surrounded by a discontinuous layer of pericytes. Each capillary loop has a basal membrane around it. In the centre of the glomeruloid formation there are also a very few capillaries with flat, rather than high endothelium, possibly the vessels from which the glomeruli originate (24, 25). There are two models of how glomeruloid bodies form, one called "active" and the second called "passive" and they can co-exists (23). In the first (active), described above, there is angiogenesis with formation of new microvessels that form the glomeruloid bodies. (26, 27). The second type "passive" has been defined as made up by the folding of pre-existing rather than newly formed capillaries and contains only flat endothelium (23)

The first experimental model of angiogenesis in glioblastoma was published by Brem (28): he illustrates how human glioblastoma cells elicit formation of new vessels when implanted in the cornea of a rabbit. Through the following years "in vitro" and "in vivo" animal studies have shown that in these models, sprouting angiogenesis is associated with glioma (reviewed in (9, 29-31)).

However in other animal studies a difference in the relationship between vessels and tumour cells in the initial phases of tumour growth has been reported (32, 33). While Folkamn's theory was that all tumours have an a-vascular phase, in which grow no more than 1 or 2 millimetres in diameters, until angiogenesis is triggered (34), in this case there is first an a-vascular phase in which the tumour growth by exploiting pre-existing brain vessels, "vascular co-option", but only as a transient phenomenon in the early stages of tumour growth, before the subsequent triggering of angiogenesis. Adult mouse blood vessels express high levels of Angiopoietin1 which antagonises Angiopoietin2 on the Tie2 receptor, and maintain the mature vessel stable by inducing anchorage to the basal membrane and protecting them from apoptosis. The first event observed following co-option was an increase in the levels of Angiopoietin2 in the pre-existing vessels surrounded by tumour cells. Higher levels of Angiopoietin2, without increases of VEGF expression, induced than vascular regression by detachment of the endothelium from the basal membrane and subsequent apoptosis. However in this model, VEGF expression was induced on the hedge of the tumour triggering neo agiogenesis developing from the Angipoietin 2 regressing vessels. (32, 33).

2.2 The formation of new vessels: Vasculogenesis

Vasculogenesis. i.e the formation of new vessels from circulating endothelial progenitors or other non-neoplastic stem cells, is another mechanism that provide new vessels to tumours(9). Animal models revealed a role for vasculogenesis in experimental gliomas. Endothelial Progenitor Cells (EPS) isolated form human umbilical cord blood (HUCB) have been injected in mice bearing a sub cutaneous implant of human glioma cells (35) while in another experiment rat glioma cells have been orthotopically implanted in murine brains and naïve bone marrow cells have been injected (36, 37). In both experimental situations these precursor cells have been found to generate the endothelium of the intra-tumour vessels. Inhibition of vasculogenesis, rather than angiogenesis, has been discovered to prevent glioblastoma recurrence in animal models after irradiation (37) suggesting an important role for vasculogenesis in resistance to treatment. However its

importance appears to be not the same in all the models published (38). More limited the evidences available from patients-based studies but an increased number of circulating EPS in the peripheral blood has been detected (39). Therefore, on the ground of the experimental evidences discussed, the hypothesis is emerging that vasculogenesis could be a major player in resistance to radiotherapy (40) (41)and anti-angiogenic drugs (41, 42)

2.3 The non angiogenic tumours and the mechanisms of vascular co-option

The possibility that malignant lesions of the brain could be not entirely angiogenesis-dependent was firstly raised by a study looking at microvessels density in glioblastoma patients (43). In this study Wesseling et al. described in human specimen, surgically removed before any other treatment that in several histological fields of the tumour the number of vessels observed was in the same range as in the normal cerebral white matter. On this ground, and on the morphological pattern of distribution of the intratumour vessels, the authors proposed that probably only a few new vessels had formed in such areas, although no conclusive evidences demonstrating the existence of non-angiogenic tumours were presented. Following the formal description and demonstration in lung of non angiogenic tumours which grow by exploiting preexisting vessels (44, 45) the interest for these type of tumours has slowly but steadily increased.

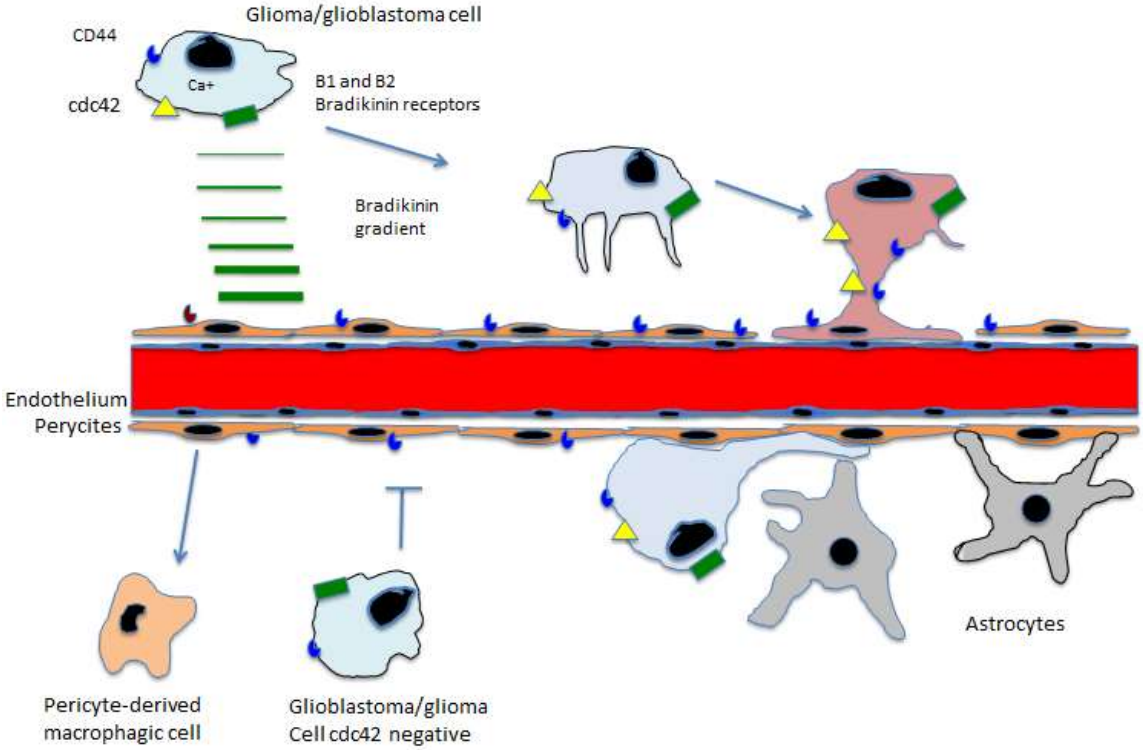
In animal models, vascular co-option in the brain, although temporary, has been demonstrated for the first time by Holash et al (32) as previously discussed. The role of non angiogenic growth, as one of the mechanisms of resistance to anti angiogenic drugs has been shown by Rubenstein et al (46) and Kunkel et al (47). Both groups described that, post anti VEGF treatments, numerous nodules of glioblastoma cells, distinct form the primary mass, appeared in the normal tissue surrounding the main tumour. All these nodules contain a central pre-existing blood vessel (so called "perivascular cuffing"). The ability of glioblastoma cells to growth along pre-existing brain vessels has been confirmed in mouse models using in vivo multiphoton laser scanning microscopy, which allows to visually monitor the movements and proliferation of neoplastic cells in relationship to the brain vessels (48). The occurrence of vascular co-option in human glioblastoma after anti-angiogenic treatment has been further supported by immunohistochemical studies (49). The newly formed vessels associated with the neoplastic mass have a basal membrane positive for CollagenIV while the endothelium is PDGFR beta positive. Instead the co-opted vessels, like the normal brain vessels, display CollagenIV presence in the basal membrane, are surrounded by PDGFR beta positive pericytes but the endothelial cells are PDGFR beta negative. Using "in vivo" multiphoton laser scanning microscopy, which can monitor the movement and proliferation of cells as they interact with the brain vessels, Winkler et al (48) demonstrated, in mouse models, that glioblastoma cells grow along the pre-existing brain vessels.

But why some cells do not trigger angiogenesis? A full understanding is still far from being achieved but some initial data are emerging. Using a murine orthotopic model of glioblastoma and performing "in vitro" experiments exploiting the chorio-allantoic membrane (CAM) model , Auds et al. demonstrated that inositol-requiring enzyme 1 (IRE1), a proximal endoplasmic reticulum (ER) stress sensor and a central mediator of the unfolded protein response involved in angiogenesis, is important in dictating the type of blood supply (50). U87 wild type (wt) glioma cells, which express wild type IRE-1, grew as a well-defined, very

angiogenic lesion. However U87 cells, in which IRE-1 has been silenced, grown into an ill-defined, highly infiltrative lesions which appears to co-opt pre-existing vessels. IRE1 action is mediated by IL-6, whose levels diminished following IRE-1 silencing. If IL-6 is transfected into the IRE-1 deficient cells, the angiogenic phenotype is restored and vascular co-option is stopped, but the increased tumour infiltration is maintained. These IRE1-negative U87 cells expressed fewer pro angiogenic genes. Currently, there are no studies on human tumours correlating IRE1 mutations and/or levels of expression to angiogenic patterns.(50)

Some studies have started to address more in details the mechanisms which allow the neoplastic cells of primary brain tumours to co-opt the vessels (**Figure 1**).

Figure 1. Co-option of brain vessels by primary CNCS tumours.



The neoplastic cells are directed to the vessels by a vessels along a bradykinin concentration gradient. Two different events have been described in cells reaching the vessels. 1) The neoplastic cells produce pseudopodia which, through the mediation of cdc42, fuses with the pericytes forming an hybrid cells. The fusion is prevented by blocking cdc42: in this case some pericytes can become mobile and acquire an an anti-tumour activity. 2) The malignant cells squeeze between astrocytes and pericytes and, by blocking their physiological interaction, alter the pericytes function.
Original picture based on: (52) (51) and (53)

The first question is how the neoplastic cells are attracted towards the vessel. In one model (51): the authors show that this is mediated by bradykinin signalling pathways: the glioma cells expresses Bradykyinin 2 receptor (B2R) which is activated by the Bradykyin secreted by the endothelial cells. Once activated B2R induces intracellular Ca2+ oscillations triggering cell motility and the migration

toward the vessel along the gradient of secreted Bradykyinin. In a second study Caspani et al (52) unravel the interaction between glioblasoma multiforme (GBM) cells and the pericytes of the brain vessels both in animals and in vitro models. They describe that GMB cells produce cytoplasm extensions, denominated flectopodia, which relay on cdc42, a GTPAse which regulates the actins dependent cytoplasm extension. These flectopodia than adhere to the pericytes trough the adhesion molecule CD44. Following the co-option, the pericytes contract inducing changes of the vascular structure from linear to convolute. Fusion between the cytoplasm of the GBM cells and the pericytes occurs, with some hybrid cells formed "in vitro". Inhibition of cdc42 results in impaired vascular co-option and differentiation of some pericytes into macrophage like cells with anti-tumour activity. The possibility that this process could be actually occurring in humans is supported by immunohistochemical stainings demonstrating increased levels of cdc42 and CD44 expression in perivascular GBM cells on histological sections. Finally, Watkins et al. reported that glioblastoma cells slip between the astrocyte end feets and the vessels interrupting the astrocyte-blood vessel coupling. Moreover, the glioma cells can take over from the astrocytes the ability to influence vascular tone (53). Under these conditions, when glioblastoma cells have taken the place of astrocytes, drugs able to induce vasodilatation in normal brain tissue, cause instead vascular constriction. Whether the glioblastoma cells replacing the astrocytes also fuse with brain pericytes was not said.

An experimental example of how angiogenic and non-angiogenic lesions appears at different times of the neoplastic disease is illustrated in the work of Sakariassen et al. (54) who transplanted fragments of human glioblastoma into the brains of rats. Initially the lesions produced were non-angiogenic. However, following further passages in rats, the lesions returned to angiogenic behaviour. Transcriptomic studies showed that cells from the non-angiogenic tumours in the early rats displayed some neuronal stem cell markers like musashi1, nestin and vimentin and so the authors concluded that this ability to change the type of growth, when re-implanted in other animals, was possibly related to cancer stem cells phenotype. However, as also stated by the same authors, it is not clear whether these cells are derived from transformed neural stem cells, from stem cell fusion events, or from otherwise restricted subpopulations within the tumour.

2.4 Vasculogenic mimicry

In a minority of instances, tumours that do not trigger formation of new vessels, by angiogenesis or vasculogenesis, can grow obtaining blood supply by vasculogenic mimicry (VM), which is defined as the capacity of tumour cells to form channels networks in which blood can flow (5, 55). VM was discovered in uveal melanoma and has now been seen in many different tumours (7, 56). The difference from vascular co-optation is that the neoplastic cells rather than exploiting existing vessels, form functional channels made up by the very same tumour cells. VM appears to recapitulate vasculogenesis as cancer cells with an embryonic-like phenotype become competent to mimic endothelial cells (5). Therefore the appearance can go from a channel lined by rather neoplastic looking cells (5) up to formation of cells which are morphologically indistinguishable from normal endothelium as derive for more indifferentiated cancer stem like. One example is the Glioblastoma-Endothelial Cell Transdifferentiation (9) in which normal looking endothelium is actually derived from the neoplastic cell pool as it contains the same genetic abnormalities.

Vasculogenic mimicry, with features similar to the original VM described by Maniotis (5), has been seen in glioma biopsies: channels were present lined by cells variably stained for CD34, positive for glial gibrillar acid protein and containing EGFR amplification typical of the glioblastoma cells, with underneath a PAS positive membrane (57-60). Similar channels formed by human glioma stem cells were observed in murine models of glioblastoma (61). As in other tumours, cancer cells with various degree of stemness are driving this process in gliomas (62-64)

3. The brain metastases

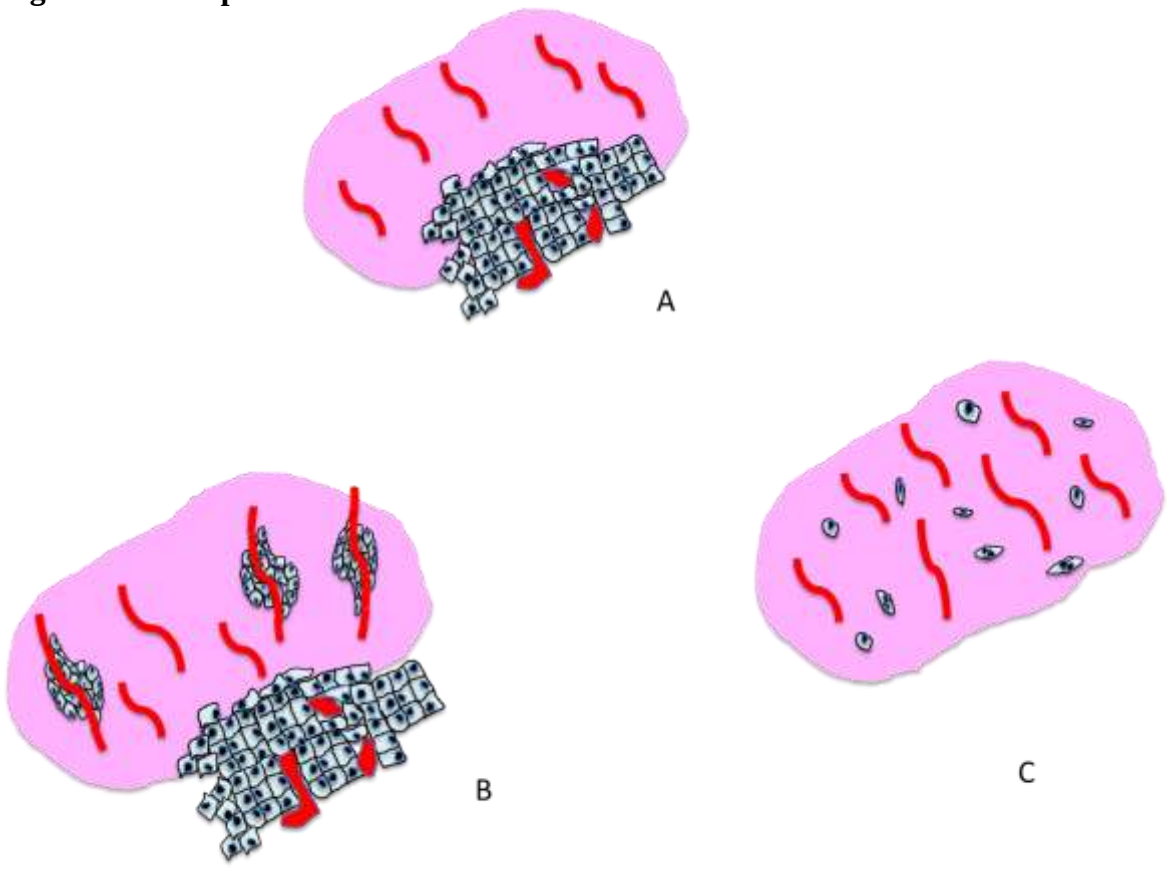
The largest clinical problem, as far as brain malignancies is concerned, is not that of the primary brain tumours but that of the metastatic lesions. As matter of fact, it is the latter that account for the highest number of malignant growth in the brain as in clinical practice they are 10 times more frequent than primary brain tumours. Whatever their origin, they represent, so far, an almost impossible therapeutic obstacle and their prognosis is abysmal with a survival of months rather than years (65) (66, 67). As for any other type of tumours, metastases were thought to be completely angiogenesis dependent (68) but this was not the case as they can grow in absence of angiogenesis (2). The first step towards the establishment of a brain metastasis is the extravasation of a neoplastic cell (69). Recently the concept of brain metastatic niche, i.e. the micro environment in which the extravasated cell can survive, has been proposed (69). Such a niche is perivascular and the interaction with the nearby abluminal surface of the vessels appear to be mediated by a series of adhesion molecules, L1CAM, E-selectin and beta1 integrins and to be essential for the cell to survive. Some cells, once in this location, appear to remain dormant (70) rather than immediately start to growth.

3.1 Vascular patterns of growth

Once the metastatic cells have successfully extravasated and start to moltiplicate, they have three major possible ways in which to obtain a blood supply: by inducing classic angiogenesis, by co-opting pre-existing vessels or by exploiting both mechanisms (65). In addition, as in the primary tumours, also in the brain metastases vascular mimicry has been reported (71) but not properly described and investigated yet, although it is likely to be quite important as it has been found to be present in many tumours known to metastasise to the brain, like melanoma, and, as discussed above, in primary brain malignancies.

We still do not have very reliable markers available to decide whether a mature vessel inside a brain neoplasia is a pre-existing or a tumour-induced vessel who has achieved maturation. However this is not the case on the edge of the tumours and here is where the work has concentrated on human tumour samples. Three types of invasion patterns (**Figure 2**) have been described on the edge of brain metastases in a study including secondary lesions from different tumours: well demarcated, vascular co-option, and diffuse (72).

Figure 2. Main patterns of infiltration in brain metastatic carcinomas.



A) Well demarcated pattern: the metastasis has a reasonably well demarcated border with the normal brain. **B)** Vascular co-option pattern: cuffs of neoplastic cells (perivascular cuffing) grow along normal vessels away from the tumour. **C)** Diffuse infiltration pattern: scattered cells, single or in small aggregates, are present in the parenchyma way from vessels, no new vessels are seen. Original picture based on: (72)

In the first type, present in 51% of the lesions examined, a well demarcate border between the neoplastic lesion and the normal brain is seen. Higher levels of alphaVbeta6 were found compared to the other two patterns but no comments on the angiogenic status of this pattern is made in the paper. The vascular co-option (18% of the patients) is instead characterized by the neoplastic cells growing along the pre-existing vessels in a non angiogenic fashion (so called peri-vascular cuffing). The pre-existing nature of the vessels is established as the vessel is inside the normal brain tissue and, only in some tracts, is "cuffed" by cancer cells. The third and last pattern (diffuse infiltration, reported by the authors in 32% of the cases) it is characterized by single neoplastic cells scattered throughout the nearby brain tissue, in absence of vascular sprouting. No association between pattern of growth and nature of the primary tumour or with outcome was found. (72)

3.2 Clinical correlations

High levels of microvessel density were initially reported as a surrogate marker of high angiogenesis and consequently of poor outcome (73) although this concept has not hold the test of time (74). Microvessel density, by staining with an anti CD105 (endoglin) or anti CD34 antibody revealed variable values between metastases from different tumours (75) (76, 77) with an high rate of mature

vessels present (76) suggesting a widespread role for vascular co-option also in presence of angiogenesis.

In a series of matched primary and brain nSCLC, the metastases contain a number of vessels comparable to that of the correspondent primaries but the percentage of mature vessels is, again, higher in the brain secondary lesions than in corresponding lung primaries. Expression of CA9, an hypoxia surrogate marker, is comparable in matched primary and metastatic lesions while no correlation could be found between the expression of VEGFA in the primaries and in the secondary, Finally the vascular pattern of the primary lesion was independent of the pattern present in the secondary (78). High MVD has been found, unexpectedly, to correlate with better overall survival in a series of 230 metastases from nSCLC (79) and in a subsequent wider study on 639 metastases from 5 different types of primary carcinomas (77);

3.3 Biomarkers

Some first clue on the underlying biology of the relationship between metastatic cancer cells and brain come from a study looking at 14 genes over-expressed in Malignant Glioma vessels. It is shown that the phenotype of the intra metastatic vessels is very much similar, as far as these 14 genes is concerned, to the phenotype of the intra glioma vessels leading to the assumption that the vascularization of metastases is similar to that of brain primary tumours which, the authors assume, is angiogenic. (80) Another study compares the transcriptome of nSCLC, SCLC and Melanoma metastases to non-neoplastic temporal lobe tissue from patients undergoing neurosurgery for intractable epilepsy. Analysis showed that the bulk of the differentially expressed genes was common to all three types of cancers and these genes included VEGFA and other involved in endothelial cells proliferation and growth factors activation. Other genes where instead differentially expressed according to the type of tumours. In nSCLC FLT1/VEGFR1R was up regulate together with an inhibitor of VEGF, the SEMA3F gene. Also in melanomas a set of anti-angiogenic genes was selectively up regulated like ANGPT2 and SERPINF1, possibly starting to explain why some tumours do not trigger angiogenesis. Using the Omicsnet protein interaction network THBS1, MMP2 and FN1 were identified as the three genes with the highest network capacity in all three types of metastases (81) highlighting the very important role possibly played by Thrombospondin in the differentiation between angiogenic and non angiogenic processes (82, 83). Differential transcription of miRNA regulating angiogenesis has been described as well, with miRNA-378 associated with lung cancer nSCLC brain Meta (84). These results could start to explain why angiogenesis is more common in lung brain metastases while melanoma secondary rely more on vascular co-option (70, 85).

3.4 Animal models

Animal models are proving to be useful in studying the mechanisms of both classical sprouting angiogenesis and non-angiogenic mechanisms of tumour vascularisation (3). Accordingly, there are several studies that have used animal models of brain metastasis to study the vascularization mechanisms. Non angiogenic metastatic growth by means of vascular co-option in an animal model is described by Bugyik et al. (86). Cells from five tumor cell lines (C38 murine colon carcinoma, HT25 colon, H1650 lung and ZR75 mammary carcinomas and HT1080 fibrosarcoma) have been implanted directly in the brain. All the tumours which developed had a lower MVD than the surrounding nervous tissue. No vessels sprouting were observed but one cell line (HT1080) resulted into tumours with intussuceptive, rather than

sprouting angiogenesis. The incorporated vessels retained their normal structure, with the exception of the astrocyte foot processes that were replaced by tumor cells. Despite the lack of angiogenesis the malignant cells had higher expression of VEGFR2, PDGFRbeta and Tie2 than the surrounding normal brain tissue.(86).

In a previous study from the same team, the formation of glomeruloid structure in mice experimental brain metastases has also been scrutinised (23). Vascular glomeruloid bodies are vascular formation morphologically reminiscent of the renal glomeruli and are commonly described both in primary and secondary brain neoplasia(25, 87-89). In this study the researcher report the novel finding that these glomeruloid structures are, in some instances, made by preexisting vessels co-opted by cancer cells rather than from newly developed. The co-opted brain capillaries have a passive role in being arranged in this glomerular fashion and have relatively low level of endothelial cell proliferation. No morphological evidence of sprouting activity was seen. The tumor cells adhering and proliferating on the surface of basement membranes of cerebral microvessels pull the capillaries into looping and coiling leading to the appearance of these microvascular structures. (23)

Most of the animal studies are centred on the role of the VEGF pathway, a well-known regulator of angiogenesis. Yano et al (90) looked at the relationship between VEGF and metastatic growth in a mouse model. Six human cancer cell lines injected trough the carotid of nude mice produced brain metastases, growing at variable speed: some fast and others rather slowly. The rapid enlarging contains numerous vessels and high levels of VEGF mRNA and protein while their levels are lower in the slowly growing lesions. Inhibition of VEGFA expression in the fast growing lesions causes slowing down but transfection with VEGF of the slow growing cells does not induces any change suggesting that VEGF is necessary but not sufficient for fast growth of brain metastases. Brain meta from a prostate adenocarcinoma cell line secreting VEGF showed vascular dilatation but the intratumour MVD remained comparable to surrounding brain. (91)

Brain metastases in mice using the melanoma line Mel57 produces non-angiogenic growth with an infiltrative pattern. As this cell line has low levels of VEGFA, it was stably transfected with VEGF165 and this Mel57-VEGF165 line was tested in the same animal model. Tumours formed by the transfected Mel57-VEGF165 cell line exhibited a more diffuse growth pattern, with the exception of the rime that was always infiltrative.However the vessels were still positive for Brain Blood Barrier markers Glut1 and Zo1 with a good pericyte coverage, suggesting that despite the VEGF transfection these vessels were pre-existing. No obvious angiogenesis was see suggesting that high levels of VEGF expression alone does not necessarily lead to angiogenesis As for the wild type cell line, the microvessel density in the metastases was lower than in the surrounding brain but the morphology of the vessels was changed: both intra and peritumoural vessels were dilated with high expression of KDR and CD105 (92).

Neoplastic melanoma lines derived by sub-cutaneous melanoma injected either directly inside the brain or through an artery grew more in the leptomeninges and ventricles than in the brain parenchyma, whatever the route of injection. The three lines D-12, A-07 and U25 have higher levels of IL8 than R-18. VEGFA secretion was higher in the A-07 and U25 lines. Still, despite these differences, all the four cell lines grow as angiogenic in the leptomeninges and ventricles but in a non-angiogenic way in

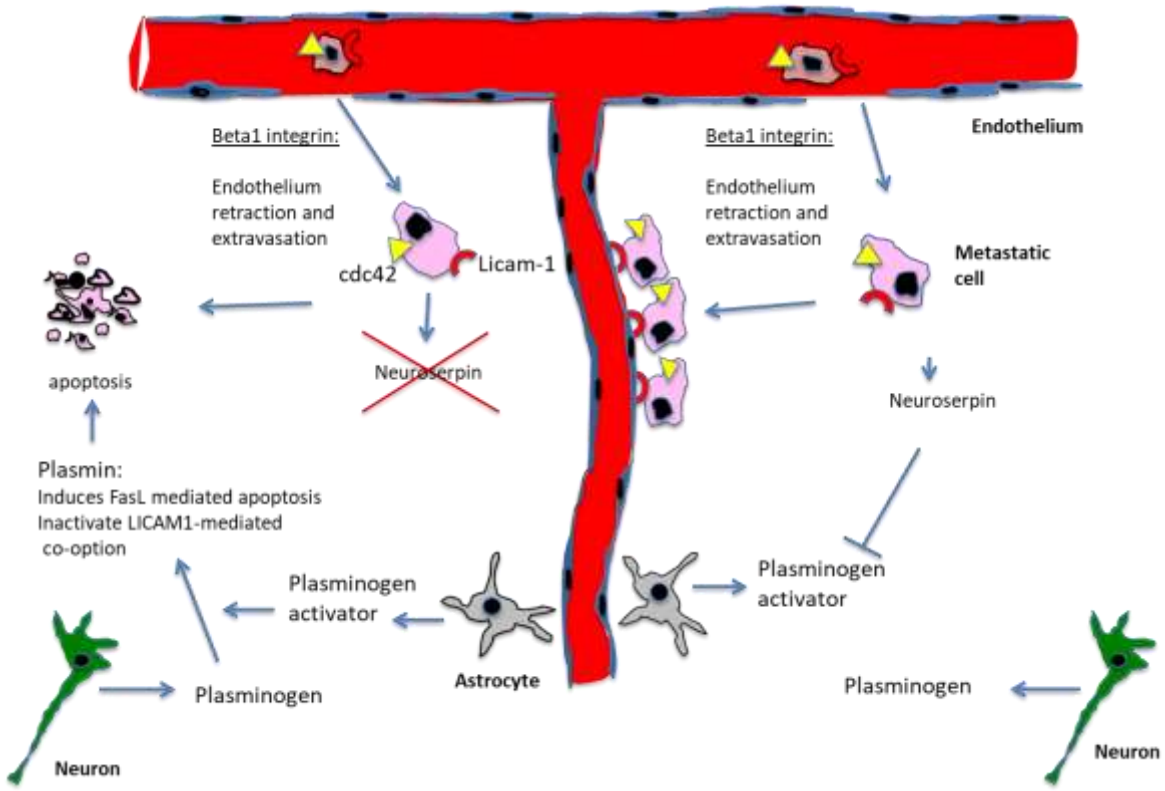
intracerebral location demonstrating, once again, that VEGF alone does not dictate angiogenesis and the site can be very important in allowing non-angiogenic growth. However, if the metastases are angiogenic, the action of VEGF is highlighted by the fact that the lines with more VEGF show more growth and higher MVD in the ventricular and leptmeningeal angiogenic location. (93). VEGF appears to be under the control of STAT3 expression as seen in a mouse model of angiogenic breast cancer metastases (94).

The expression of alpha V beta3 and STAT3 in metastatic tumour cells enhances angiogenesis in brain Metastases (94, 95). The effect of alphaV beta3 appears to be site specific as does not affect angiogenesis when the same cell line is implanted in the fat mammary pad(95)

3.5 Mechanisms of vascular co-option

A second line of investigation on animal models focused on the mechanisms by which the neoplastic cells co-opt a pre-existing brain vessel (**Figure 3**). In a mouse model neoplastic cells form 5 different cell lines were injected inside the brain parenchyma. The neoplastic cells and the brain structures were visualised by immunofluorescence.

Figure 3. Co-option of brain vessels by metastatic cells.



First the neoplastic cells must move from the blood lumen into the parenchyma (exravasation) a process mediated by cdc42. In the model here presented, the successful non-angiogenic metastatic cells are defined by the secretion of neuroserpin and expression of LIcam1. Neuroserpin prevents the activation of Plasminogen Activator; therefore plasmin cannot be produced. In absence of Plasmin the apoptosis of the cancer cell (trough FasL) is prevented and the LIcam1 adhesion molecule cannot get blocked, remains active, allowing adhesion of the tumour cell to the abluminal surface of the vessel.
Original picture based on: (96) and (69)

As the neoplastic cells started to co-opt the vessel, the surrounding astrocytes were detached while the desmin positive pericytes remained in place and became incorporated by the tumour. Once surrounded by the newly formed neoplastic lesion, the trapped vascular structures started to show some change as the pericytes started to express Smooth Muscle Actin and, around the vascular basal membrane, a new layer of membrane was produced, this time containing laminin 5 which provided an anchorage for the neoplastic cell expressing alpha3-integrin. In the case of the ZR75 breast cancer cell line, the cells surrounding the vessels would be differentiated into a cell with two distinct poles: the apical pole, EMA and Claudin3 positive, and the basal pole providing adhesion to the basal membrane throughout alpha3-integrin. (86)

In a more detailed study "in vivo", on the brain of nude mice using multiphoton laser-scanning microscopy, the neoplastic cells were followed as, after internal carotid injection, arrested in the brain vessels, extravasated and started to grow. Cells from the two melanoma (MDA-MB-435 and A2058) and the two lung carcinoma (PC14-PE6 and HTB177) cell lines arrested only in vessels with a diameter equal to theirs, therefore because of mechanical rather than biological reasons. Cells not able to extravasate died but the cells moving into the parenchima were still alive at day 14. Over time the extravasated cells that moved away from the abluminal site of the vessels also died and the only one viable left were the one maintaining a direct cell-vessel contact. Than the path of the melanoma cells diverged from that of the lung cancer ones. The lung cancer cells start to proliferate causing dilatation and tortuosity of the blood vessels quickly followed by angiogenesis and accelerated growth. The melanoma cells instead kept growing alongside brain vessels leading to capillary (glomeruloid) loop formation. Occasional foci of angiogenesis could be found only in the larger lesions.(70)

Valiente and colleagues (96) looked at the changes occurring inside the metastatic cells as they co-opt brain vessels (**Figure 3**). Through analyses of human tumours and data obtained from mouse models, they showed how metastatic cells resist apoptosis and co-opt brain vessels by expressing the protein neuroserpin, which blocks the generation of plasmin. Plasmin protects the brain from metastasis by promoting the apoptosis of cancer cells and by inhibiting their spread along the vasculature, a defensive reaction of the brain tissue to malignant cells entering the parenchyma. Linking these two events (the entrance of metastatic tumor cells and the production of plasmin) is the astrocyte which, in response to inflammation, parenchymal damage and the passage of metastatic cells across the brain barrier, expresses high levels of two proteins: Fas ligand (FasL) and plasminogen activator (PA). Increased levels of PA lead to cleavage of plasminogen (which is secreted by neurons) into the active form, plasmin, which can then act on several other proteins, including FasL and the adhesion molecule L1CAM. FasL is bound to the membrane of astrocytes, but plasmin is able to cleave and release it as soluble form, sFasL, which in turn can link to its receptor, Fas, on the tumor cell and trigger apoptosis. However, in the presence of cancer cells expressing high levels of neuroserpin, the astrocyte production of PA is inhibited, thereby blocking the release of plasmin from plasminogen, and suppressing the secretion of sFasL and thus apoptosis of the tumour cell. Once the survival of the cancer cells is achieved there is the problem of how it will adhere to the co-opted vessels and a group of studies has been investigating the relationship between neoplastic cells and different components of the vessels: endothelium, basal membrane and pericytes. One mechanism follows again

neuroserpin expression: by inhibiting generation of plasmin, the L1CAM molecule expressed by the tumour cell is not cleaved and remains intact allowing the metastatic cells to adhere to the outbound surface of the vessels. (96)

4. Conclusions

The ways in which tumours cells can relate to blood vessels are surprisingly numerous and varied in nature. Among them, and quite possibly the most important, is vascular co-option by non-angiogenic tumours. These findings question the validity of "Inducing angiogenesis" as an Hallmark of Cancer (68) as tumours can also grow by exploiting pre-existing vessels in absence of angiogenesis (97). Understanding these mechanisms and especially the impact of vessel co-option and the biology of the non-angiogenic neoplastic cell, may open up promising new ways to improve the therapeutic impact of targeting the interaction between tumour and vasculature and pathways specific for the non-agiogenic cancer cell.

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