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Review

Tumour–Endothelial Cell Communications: Important and Indispensable Mediators of Tumour Angiogenesis

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Abstract. *Angiogenesis is an essential aspect of tumour growth and metastasis. Solid tumours cannot grow beyond 2-3 mm in diameter without inducing the formation of new blood vessels to support the energetic requirements of tumour cells. Angiogenesis is stimulated by cancer cells through a wide variety of cell-to-cell communication means. Cancer cells can induce endothelial changes by directly targeting cells via soluble factors, adhesion receptors, gap junctions and vesicles. They also can stimulate endothelial signaling pathways in an indirect way, e.g. by activating stromal cells, by secreting proteases into the extracellular space or even by changing the pH, temperature and availability of oxygen and nutrients. Anti-angiogenic drugs appear to be an effective cancer treatment in animal models but have been shown to have a limited effect in the long term. Resistance to anti-angiogenic therapies has been attributed to the ability of cancer cells to induce angiogenesis in a different way. We propose that cancer cells also change the way they communicate with endothelial cells in order to escape therapies that inhibit angiogenesis and that a better knowledge of this phenomenon will help us design more efficient drugs.*

Cell-to-cell communication is necessary for proper coordination of cell activity. It has been long known that the failure of cell communication can lead to severe diseases, such as immune disorders (1) and heart failure (2). Cells can send signals which can activate their own receptors (autocrine), target a neighbouring cell (paracrine) or target a distant cell (endocrine). There also exist different means of cell communication: it can occur through soluble factors, adhesion contacts, cell junctions or through vesicles (3).

Tumours are comprised not only of tumour cells but also of other cell types, called stromal cells, creating the perfect microenvironment for tumour development. Fibroblasts and macrophages are the two most common stromal cells in the tumour microenvironment. Tumour cells are able to attract and transform these stromal cells *via* communicating with them. Studies have shown that the aggressiveness and growth of tumours decreases when these communications are reduced or stopped (4, 5). For this reason, the development of strategies to block the communication between tumour and stromal cells has been an important area in cancer research.

Endothelial cells are another stromal cell type present in the tumour microenvironment. It has been shown that tumours cannot grow beyond 2-3 mm in diameter without proper vasculature to fulfil tumour cell energetic requirements. In order to continue their growth, tumour cells induce formation of new blood vessels from an existing one, which is called angiogenesis. Tumour angiogenesis not only provides the tumour cells with nutrients and oxygen, and allows removal of metabolic wastes, but also presents the metastatic tumour cells with points of entry to the circulatory system. The signal exchange between tumour and endothelial cells is critical to the development of tumour angiogenesis. The interruption of this signal exchange can lead to reduced vasculature within the tumour and reduced tumour size (6). This might serve as an additional target for anti-angiogenic strategy, even though the idea of anti-angiogenic therapy has been a long-standing area of interest (7).

In this mini-review, we summarize the different means by which tumour cells can communicate with endothelial cells in terms of tumour angiogenesis. We also briefly touch on the issues of anti-angiogenic therapy and drug resistance.

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Direct Cell-to-cell Communication

In this section, we review the different ways that cancer cells can target endothelial cells in a direct way through soluble factors, activation of a receptor by physical contact, gap junctions and vesicles (Figure 1).

Soluble factors. The most common means of cell communication is through soluble factors, where a signaling cell secretes a protein into the extracellular space which targets a neighbouring cell or travels through the bloodstream and targets a cell at a distant site. The soluble factor usually binds to a tyrosine kinase receptor and triggers an intracellular response but they can also activate ion channels. The vascular endothelial growth factors (VEGFs) are a very well-known family of vascular development modulators (8). There are five VEGF proteins secreted in mammals (VEGFA, -B, -C and -D and placental growth factor) that can bind to three tyrosine kinase receptors (VEGFR1, -2 and -3) (9). In vascular endothelial cells, VEGFA is the most well-studied ligand and as a result we have a clear idea of its role in cells. VEGFA binds to its receptors, VEGFR1 and VEGFR2, triggering an intracellular response that is responsible for modulating the proliferation, migration, permeability and tip cell filopodia induction (10). Interestingly, VEGFA has a high affinity to bind VEGFR1, but this receptor has a weak response to this ligand, generating weak tyrosine auto-phosphorylation (11). Endothelial cells can also express two different isoforms of this receptor, one that is the normal receptor localized in the membrane that can generate an intracellular signaling pathway, and a second one that is secreted into the extracellular space, binding VEGFA and working as an anti-angiogenic mechanism by inhibiting VEGFA (12).

Almost all tumours express VEGFA that it is considered the most significant angiogenic factor in tumours (7, 13). Since tumour angiogenesis is dependent on VEGFA, many drugs have been developed to target this pathway, among them, bevacizuma (Avastin®). This medication, which is an antibody against VEGFA, has limited effects in the majority of advanced malignancies. However, the administration of this drug in combination with chemotherapy has shown an improvement in the treatment (14, 15).

A study using frog mesenteric microvessels showed that VEGF increases the concentration of intracellular calcium, that was accompanied by an increase in endothelial permeability. The use of two activators of transient receptor potential channel 6 (TRPC6), 1-oleoyl-2-acetylglycerol and flufenamic acid, had the same effect, suggesting that VEGF might activate TRPC6 and increase calcium influx. TRPC6 and -7 have been identified in endothelial cells of human lung artery and both these channels are permeable to magnesium. Calcium and magnesium are both known to regulate endothelial functional, including angiogenesis (16). These findings suggest that VEGFA might regulate the activation of these channels in cancer, therefore contributing to angiogenesis.

Adhesion contacts/adhesion receptors. Under normal conditions, cells interact with their microenvironment, *e.g.* the extracellular matrix (ECM) and neighbouring cells. Cell adhesion receptors are essential proteins that contribute not only to the adhesive process between cells but also to the activation of intracellular signaling pathways. These receptors can activate enzymatic proteins located by the intracellular fragment of the receptor, and can also bind to adaptor proteins and to the cellular cytoskeletal. Adhesion receptors are regulated by intracellular signals as they exist in protein complexes that are dynamically adjusted in response to a stimulus, being translocated into the membrane sites of the adhesion. Adhesion receptors are critical in the regulation of migration. Therefore, the dynamic regulation of these adhesion complexes is a crucial feature of angiogenesis.

Another example of the importance of adhesion receptors in communication between cancer cells and endothelial cells is its role in tumour angiogenesis. Osteoprotegerin (OPG) is a pro-angiogenic protein that was found overexpressed in breast cancer tissue (17). *In vitro* studies showed that the direct contact between breast cancer cells and endothelial cells is partially responsible for the increase in endothelial OPG through integrin $\alpha_v\beta_3$ ligation and nuclear factor-kappa B activation (18). Chen *et al*. also demonstrated *in vitro* that JAGGED1 transmembrane protein expressed in breast cancer tumour cells can activate the NOTCH receptor in endothelial cells and trigger an angiogenic cascade (19).

Gap junctions. Another way that neighbouring cells can communicate with each other is through gap junctions, where they can exchange ions and small metabolites, providing a connection between the cytoplasm of the two cells (20, 21). Connexin is the primary group of proteins that form these channels. The connexin family comprises of 21 different proteins that have a tissue and cell type specific expression pattern. Connexin has been reported to have a part in tumour progression, but its role it is still not clear (22). The down-regulation of connexin 26 (CX26) has been shown to increase the aggressiveness of melanoma cells (23) and the knockdown of CX43 increased the growth and migration of breast cancer cells (24). Breast cancer cells treated with ACT1, a compound that stabilises CX43 gap junctions, had an adverse effect on the proliferation and survival of cancer cells (25). Although these studies suggest that gap junctions in cancer cells could be a good prognostic marker, CX26 and CX43 immunohistochemical analysis of breast cancer samples might complicate this assumption (26).

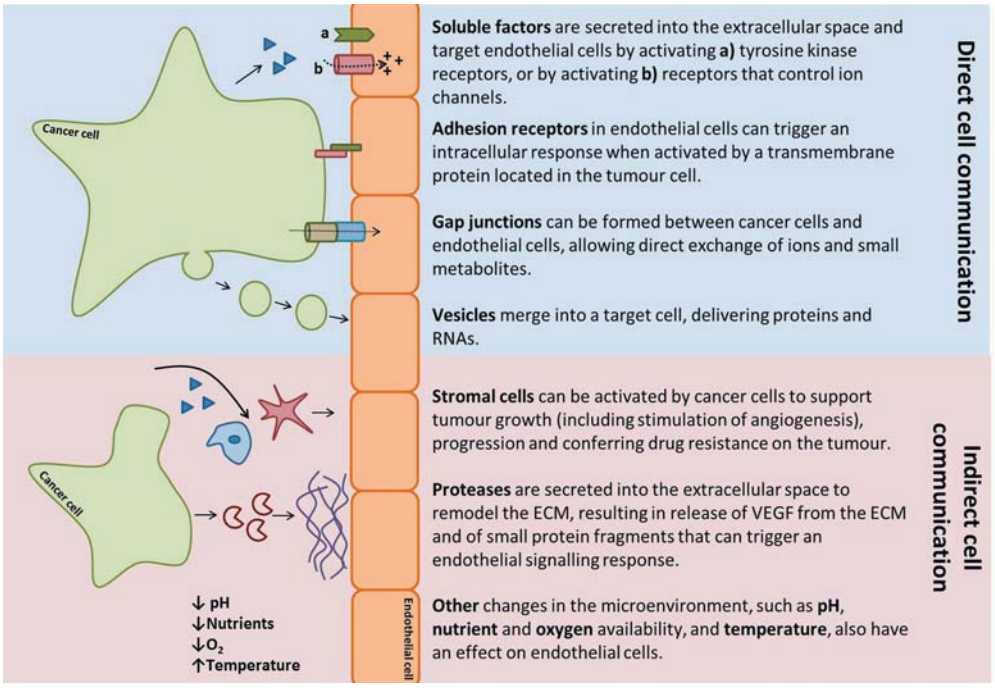

Figure 1. *Schematic representation of the different means of communication between cancer and endothelial cells. ECM: extracellular matrix; VEGF: vascular endothelial growth factor.*

Most primary tumours presented cytoplasmic expression of CX26, but not membranous. Although primary tumours exhibited cytoplasmic and membranous expression of CX43, it was predominantly cytoplasmic. When the expression of both connexins was evaluated in the matched metastases, membranous expression of CX43 was enhanced in the lymph node metastases and membranous CX26 was only present in the metastatic site (26). These findings suggest that connexins might have various roles in the different stages of the tumour progression. Several studies have shown that connexins are important in the adhesion of cancer cells to vessels and in the intravasation of cancer cells, suggesting that gap junctions between cancer cells and endothelial cells might be an important aspect of tumour progression (27-29). Confocal images confirmed that CX43 connections between cancer cells and endothelial cells are formed under *in vitro* and *in vivo* conditions and that those connections correspond to functional gap junctions, capable of exchanging fluid between the two types of cells (27-29). Moreover, Zang and collaborators demonstrated that glioma cells can establish gap junctions with endothelial cells, enhancing tube formation *in vitro* (30). Gap junctions can also propagate electrical signals between neighbouring cells and it has been shown that electrical stimulation induces angiogenic responses in endothelial cells by activating VEGFR (31, 32).

In summary, gap junctions between cancer cells and endothelial cells occur and seem to be important in the adherence of cancer cells to vessels and in the intravasation of cancer cells. Despite the lack of evidence that gap junctions between cancer cells and endothelial cells have an impact on tumour angiogenesis, they might play a significant role in the initiation of metastasis.

Vesicles. Recently, a different means of cell communication has been described which consists of secreted membrane vesicles that merge into target cells. There are several types of vesicles, including microvesicles, ectosomes, membrane particles and exosomes. Exosomes are one of the most studied vesicle types. They have an endosomal origin and are 30-100 nm in diameter. In the signalling cell, exosomes are present in multivesicular bodies which are released into the extracellular space, then merge with the plasma membranes of the recipient cells. These vesicles can have different cargo molecules, such as proteins, lipids and RNAs (33). The function of exosomes is still unclear, especially in the context of cancer. Many studies have demonstrated a role of exosomes in different aspects of cancer progression, such as angiogenesis, promotion of metastasis and modulation of the immune system (reviewed in 34). The role of exosomes seems to depend highly on their composition (35).

Several studies have shown that exosomes derived from cancer cells induce angiogenesis, *in vitro* and *in vivo*. Exosomes purified from leukaemia cells under hypoxic conditions induced approximately two-fold endothelial cell

tubule formation than those purified from leukaemia cells under normoxic conditions. MicroRNA-210 (miR-210) was shown to be up-regulated in exosomes from hypoxic leukaemia cells. The exosomal miR-210 was able to directly down-regulate the expression of the anti-angiogenic protein Ephrin-A3 by reducing its promotor activity (35). This demonstrates that exosomes carry functional molecules capable of modulating gene expression in the target cell. It also shows that the exosome cargo can differ according to the stress of the signalling cell. Exosomes can target neighbouring cells, but they also can travel through the bloodstream and modulate the gene expression of cells at distant sites. Human renal carcinoma cells expressing CD105 (stem cell marker) secrete exosomes that have been shown to trigger *in vitro* and *in vivo* angiogenesis (36). Profiling of exosomes revealed the presence of many pro-angiogenic mRNAs and microRNAs (36). Intravenous daily injection of exosomes into mice for 5 days enhanced lung metastasis which had been induced by injection of renal carcinoma cells (36). This could be due to angiogenesis stimulation which facilitates intravasation of cancer cells and also supports tumour growth. These findings demonstrate that exosomes induce angiogenesis and are important in the formation of pre-metastatic niches.

Urinary exosomes isolated from patients with high-grade bladder cancer promoted angiogenesis and cell migration (*in vivo*) (37). Epidermal growth factor-like repeats and discoidin I-like domains 3 (EDIL3) protein is overexpressed in many cancer types and has been associated with poor prognosis (37). This protein was found in exosomes derived from bladder cancer cell lines and patients. *EDIL3* knockdown demonstrated that this protein is essential for exosomes to promote angiogenesis *in vitro*. EDIL3 can activate epidermal growth factor receptor (EGFR) signaling and by using the EGFR kinase inhibitor Ag1478, it was demonstrated that exosomal bladder cancer cells induce angiogenesis in an EGFR-dependent way (37).

Tumour cells use exosomes as a way to modulate endothelial gene expression and induce angiogenesis. Furthermore, as exosomes are found in blood and urine, and they can reflect the aggressiveness of cancer, they have been studied as a predictive marker.

Indirect Cell-to-cell Communication

Tumour cells can also induce angiogenesis in an indirect way *via* stromal cells or by changing the tumour microenvironment, such as by secreting proteases into the ECM (Figure 1).

Stromal cells. For many decades, researchers had focused on cancer cells themselves, but nowadays we know that tumours comprise of tumour parenchyma and stroma, both distinct parts that communicate with each other to stimulate tumour progression. Growing evidence shows that stromal cells play an important role in tumour initiation, progression and metastasis. The most abundant stromal cells in connective tissues are fibroblasts, which secrete molecules into the ECM. Fibroblasts have been found to be activated in wound healing and fibrosis, which are characterized by an increase in the expression of alpha-smooth muscle actin and extra domain A (ED-A) splice of fibronectin (38). Recently, fibroblasts have been found to be activated within the tumour microenvironment. These fibroblasts are called cancer-associated fibroblasts (CAFs) and interestingly, they are very similar to those found in wounds and inflammatory sites (39). The origin of CAFs is still unclear but some studies suggest that they are activated local fibroblasts, bone marrow-derived mesenchymal stem cells or cancer cells after epithelial–mesenchymal transition. Cancer cells can activate fibroblasts by targeting them with cancer-secreted factors, such as transforming growth factor-β and C-X-C motif chemokine 12 (CXCL12)/ stromal cell-derived factor-1 (40).

There are many studies showing the involvement of CAFs in tumour angiogenesis, for example, Tang and collaborators (41) showed that CAFs highly express galectin-1, a pro-angiogenic protein. Co-culture experiments demonstrated that CAFs increase human umbilical vein endothelial cell (HUVEC) proliferation, migration, tube formation and VEGFR2 phosphorylation in a galectin-1-dependent manner. They also showed that galectin-1 accelerated tumour growth and promoted angiogenesis *in vivo*.

Proteases. The ECM is the structural support of endothelial cells, which under normal physiological conditions induces stable vessels by inhibiting angiogenesis through activation of endothelial receptors. In solid cancer, cancer cells and activated stromal cells secrete not only proteins into the ECM but also proteases which degrade ECM proteins (42). This way, a change in the composition of the ECM induced by cancer cells leading to a change in the endothelial phenotype, can be interpreted as another way of communication between these two cell types.

VEGF, as discussed above, is an essential pro-angiogenic molecule in cancer. It has been shown *in vitro* that most VEGF bind to ECM proteins secreted by cancer cells (43, 44) and the degradation of ECM proteins is required for access of VEGF to the endothelial cells in order to induce angiogenesis. Matrix metalloproteinases (MMPs) are the main group of proteases secreted by cancer and stromal cells that are capable of degrading ECM proteins. Many MMPs have been implicated in this process, for example, VEGF forms a complex with connective tissue growth factor (CTGF) and MMP3 and MMP7 were able to release VEGF from this complex by degrading CTGF (43). Interestingly, a set of *in vivo* experiments demonstrated that different concentrations of VEGF can regulate various aspects of

tumour angiogenesis (45), giving an even more important role to proteases in this process.

Degradation of collagen type IV, CXC chemokines and thrombospondin 1 also produces peptides that can have pro- or anti-angiogenic properties (46, 47).

The studies described above show that proteases have a major role in tumour angiogenesis and are an important method of communication between cancer and endothelial cells.

Other forms of indirect communication. Solid tumours are characterised by inadequate perfusion and high metabolic rates, leading to a transiently or chronically hypoxic and acidic environment. The pH of the tissue is usually related to glucose consumption (48). These changes in the microenvironment are created by the high metabolic rate of tumour cells and not as a means of communication, at least in our current understanding. However, these conditions will constrain and even change the behaviour of stromal cells within the tumour microenvironment. If we take the example of a single cancer cell that invades a new organ, it will create an acidic and hypoxic microenvironment that will affect the cells around it; this phenomenon could be interpreted as cell communication. For instance, Jiang *et al*. showed that culturing HUVECs under hypoxic conditions for 25 days completely changed the gene-expression profile of these cells and, more importantly, these cells enhanced their response to fibroblast growth factor 2 and VEGFA, essential angiogenic proteins regarding cell migration and proliferation (49).

Another characteristic of the microenvironment of tumours is the deprivation of nutrients, which has been shown to change the response of endothelial cells to other stimuli, such as tumor necrosis factor-alpha (TNFα). HUVECs increase superoxide production under starvation conditions, but more interestingly, the production of superoxide is even more increased if cells are treated with TNFα. However, TNFα alone does not affect the production of superoxide under standard conditions (50). The temperature is also increased in the tumour microenvironment and endothelial cells have temperature-sensing receptors that can modulate calcium influx or NO release (51).

We support the idea that a change in the tumour microenvironment, even as the secondary effect of cancer cell metabolism, can be interpreted as a means of communication and its role in cancer angiogenesis should be investigated.

Anti-angiogenic Therapy and Drug Resistance

The awareness that anti-angiogenic therapies could be one major step towards the prevention of cancer progression arose after studies showing that angiogenesis is essential for the growth of primary solid tumours and metastasis. Hypoxic cancer cells activate hypoxia-inducible factor-1α that triggers a signaling pathway inducing cells to express growth factors, *e.g*. VEGF, FGF2 and platelet- derived growth factor. VEGF can be up-regulated in cancer cells by oncogenes, such as *RAS* (52, 53), or after loss of tumour suppressors, such as phosphatase and tensin homolog (*PTEN*) and Von Hippel-Lindau (*VHL*) (54, 55). Growth factors can then target endothelial cells and induce proliferation, migration and capillary formation. The newly formed microvessels bring oxygen and nutrients to feed the cancer cells, allowing the tumour to grow.

As already mentioned, VEGFA plays a significant role in this process and many drugs have been developed to target its pathway. The most studied VEGFA drug is bevacizumab, which is a recombinant humanized monoclonal IgG1 antibody. It binds to VEGFA, neutralizing it and preventing it from activating its receptor. This antibody was capable of slowing tumour growth in animal models (56). Interestingly, it had a more beneficial effect when used in combination with chemotherapy (57), by increasing the stability of the tumour vasculature, contributing to better cytotoxic drug delivery (58).

Despite the efforts towards creating drugs to inhibit the VEGF signalling pathway, clinical trials showed that these drugs do not constrain angiogenesis for a long time. Anti-angiogenic treatments usually do not prolong the overall survival of patients with breast (59), renal cell carcinoma (60) or colon (61, 62) cancer. Moreover, an animal study demonstrated that anti-angiogenic drugs can actually increase the aggressiveness of tumours (63). A possible explanation for this is given by Conley and collaborators (64). They showed that when they used anti-angiogenic drugs, tumour cells activated hypoxia responses but more importantly, they activated the protein kinase B–β-catenin pathway which is involved in cell growth, making tumours more aggressive. Fascinatingly, tumours that were first sensitive to a therapy but developed resistance have been shown to become sensitive to that therapy following treatment with a different agent (65). This suggests that tumour resistance to therapy is an adaptation and not a result of a gene mutation or amplification. Another consideration regarding the use of this kind of agent is that studies conducted in mouse models showed that anti-angiogenic drugs increased the invasive potential of cancer cells from the primary site, increasing the number of metastases that occurred (66). This could be explained by the fact that a hypoxic environment is hostile to cancer cells, which might cause them to migrate to other tissues (67).

Tumours that respond well to single anti-angiogenic therapy might suggest that the tumour vasculature is very sensitive to that particular drug and also that the tumour cells are very dependent on the supply of oxygen and nutrients. The vasculature can also be sensitive to the drug but cancer cells adapt to the new conditions in order to survive. Lastly,

anti-angiogenic therapy may have no significant effect on tumour vasculature. The disease can be controlled for a period of time after anti-angiogenic therapy, but this is followed by tumour progression. In this case, at least one of two things happened, the tumour found a different way to drive angiogenesis, or tumour cells adapted to a less-vascularized environment (6).

Adaptation of tumours to anti-angiogenic drugs can be due to the switch of the VEGF pathway to a different pro-angiogenic pathway. Many other molecules secreted by cancer cells have been described as being pro-angiogenic, such as EGF (68), FGF2 (69) and interleukin-8 (70). Another mechanism used by tumours to overcome the effect of these drugs is to recruit stromal cells; fibroblasts have also been shown to confer resistance to anti-angiogenic drugs (71).

The type of cell-to-cell communication established between cancer cells and endothelial cells should also be considered in the phenomenon of drug resistance. Connexins have an important role in the development and progression of breast cancer. However, their role varies according to the type of connexin, type of cancer and stage of cancer progression. The prognostic potential of connexins was evaluated in a study involving patients with breast cancer, where high CX26 expression was correlated with a poor prognosis and low CX26 and high CX46 correlated with a good prognosis. The expression pattern of these connexions was also shown to change after chemotherapy (72). These findings suggest that cell communication, gap junctions in this case, is involved in the aggressiveness of the tumour and might be important in the prediction of prognosis. More importantly, it suggests that tumours can change their way of communicating after a treatment, perhaps enabling them to overcome therapeutic inhibition.

Conclusion and Future Directions

Herein we revised the different ways that cancer cells can communicate with endothelial cells and induce angiogenesis in order to fulfil the energetic requirements of tumour cells. We showed that cancer cells have a wide and complex variety of means of communicating with endothelial cells. Based on the information presented in this review, we argue that not only is a better understanding of the pathways of angiogenesis activated within tumour needed, but also a more comprehensive knowledge of how cancer cells trigger these pathways is essential. Furthermore, the investigation of the role of cell-to-cell communication between cancer and endothelial cells in different types of cancers and in the different stages of tumour progression will enable the development of better therapies. Finally, we also support the idea that cancer cells might change their type of cell-to-cell communication targeting endothelial cells in order to overcome anti-angiogenic therapy.

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