

A New Perspective on Tumor Endothelial Cells: Unexpected Chromosome and Centrosome Abnormalities

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Abstract

It has been shown that endothelial cells in solid tumors are cytogenetically abnormal. These cells are aneuploid with multiple chromosomes and multiple centrosomes. Unlike normal endothelial cells which remain diploid in long-term culture, the aneuploidy of tumor endothelial cells is exacerbated in culture suggesting that these cells are inherently unstable. It is speculated that this instability might compromise the effectiveness of antiangiogenesis therapy. (Cancer Res 2005; 65(7): 2507-10)

Endothelial Cell Biology

Blood vessels are needed to supply oxygen and nutrients to the various organs and to get rid of waste products. These blood vessels are generated by several mechanisms. One is the differentiation of precursor cells into vascular endothelial cells, which assemble to form a vascular plexus that supports blood cell circulation and matures into a vascular network, including arteries and veins, a process termed vasculogenesis (1). A second mechanism is angiogenesis, which is characterized by the sprouting and branching of capillaries from preexisting arteries and veins (1). These capillaries undergo extensive pruning and remodeling and mature by attracting pericytes and smooth muscle cells (SMC) that regulate blood flow and permeability. In addition, it is now recognized that there is another vascular process, the differentiation of circulating endothelial progenitor cells (EPC) into mature endothelial cells and their incorporation into new blood vessels (2).

Tumor Blood Vessels Are Abnormal

One subclass of endothelial cells are those found in tumors (tumor endothelial cells). These blood vessels are primarily capillaries that invade tumors or are coopted from the host by the tumor. Tumor angiogenesis results in enhanced vascularization and subsequently enhanced tumor progression. The newly formed tumor capillaries also become a conduit for cells to escape from the tumor and to metastasize to distant organs (3). It is very apparent that tumor blood vessels differ rather dramatically from normal vessels (4–6). In fact, an abnormal vasculature is a hallmark of solid tumors. Tumor vessels are organized in a chaotic fashion instead of the hierarchal branching pattern of the normal vasculature. Tumor blood vessels are often tortuous in appearance with uneven vessel diameters due in part to compression of the immature vessel wall by tumor cells. Tumor vessels exhibit chaotic

blood flow and vessel leakiness due to loose endothelial cell interconnections. The high interstitial fluid pressure in a tumor causes blood vessel collapse and impedes blood flow. Tumor endothelial cells do not form regular monolayers and thus do not have a normal barrier function. Tumor endothelial cell basement membranes have structural abnormalities including loose associations with endothelial cells and variable thickness of type IV collagen layers that are usually not seen in normal endothelial cells. Pericytes are present on tumor endothelial cells but have abnormally loose associations with these cells and extend cytoplasmic processes deep into the tumor tissue. In addition, tumor-associated stromal cells in the tumor microenvironment such as fibroblasts, macrophages, pericytes, and SMC undoubtedly influence the tumor vasculature.

Tumor Endothelial Cells

The phenotypic abnormalities in tumor endothelial cells compared with normal endothelial cells suggest possible alterations in gene and protein expression patterns. One of the first reports describing these differences used a serial analysis of gene expression (SAGE) to compare gene expression patterns of endothelial cells derived from blood vessels of normal and malignant human colorectal tissues. SAGE revealed that 46 transcripts that predominate in the endothelium were elevated in tumor-associated endothelial cells (7). Some of these, designated as tumor endothelial markers (TEM), are transmembrane proteins associated with the endothelial cell surface. Several different TEM genes have been identified, including *TEM1*, *TEM5*, *TEM7*, *TEM7R*, and *TEM8*. *TEM7* is the most specific for tumor endothelial cells. It is alternatively spliced and a variant that is secreted may be a serum marker for tumor angiogenesis (8).

There are also several reports of tumor endothelial cells being cultured and characterized for differences compared with normal endothelial cells. Endothelial cells isolated from renal cell carcinoma, for example, did not undergo the senescence which is typical of normal endothelial cells, and were resistant to serum starvation and vincristine-induced apoptosis (9). They exhibited higher proliferation rates in low serum, enhanced Akt activation, and decreased expression of the tumor suppressor, PTEN. Tumor endothelial cells isolated from murine Lewis lung carcinoma were characterized by elongated morphology, fewer cell-cell contacts, a tumor-specific extracellular matrix, and increased levels of endothelial cell markers (10).

Leukemia and lymphoma endothelial cells have been reported to have some chromosomal abnormalities. In chronic myeloid leukemia, for example, endothelial cells derived from bone marrow and peripheral blood had leukemia-specific translocations (11). Recently, endothelial cells in B-cell lymphomas were shown to harbor lymphoma-specific chromosomal translocations, suggesting that lymphoma and lymphoma endothelial cells may both

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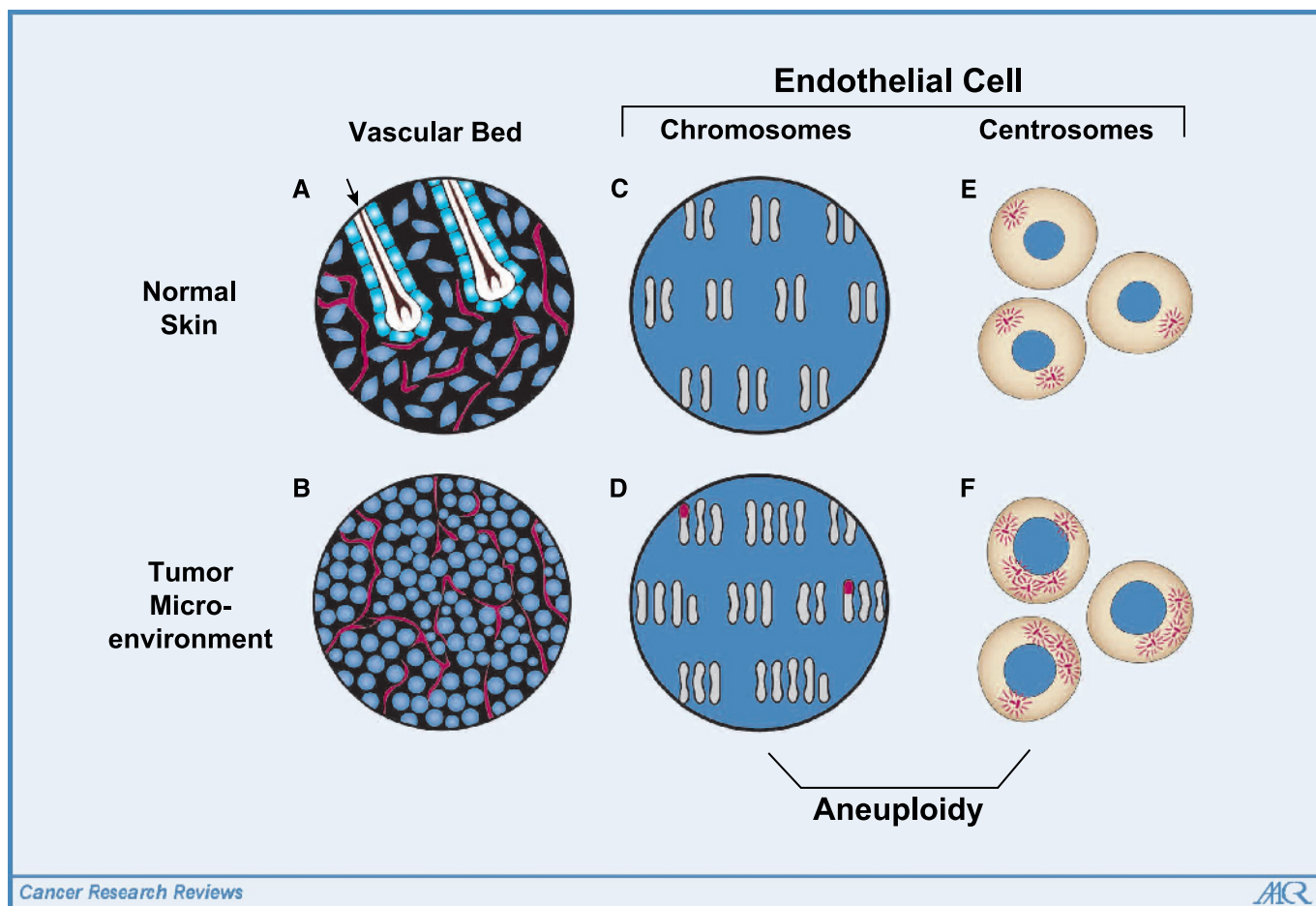


Figure 1. Schematic of tumor endothelial cell cyto-genetic abnormalities. Endothelial cells were isolated from mouse skin (A) and a melanoma (B). In normal skin, there are hair follicles with associated epithelial cells (arrow), blood vessels (red), and fibroblasts (blue). In the melanoma tumor microenvironment, there are tumor and stromal cells (blue) and blood vessels (red). Tumor blood vessels are thinner and more disorganized than skin blood vessels. Normal endothelial cells are diploid (2N; C), whereas tumor endothelial cells have multiple chromosomes (>2N), translocations (red), and deletions (D). Normal endothelial cells have one centrosome in G₁ and two polarized centrosomes in G₂ (E). Tumor endothelial cells, on the other hand, have multiple ($n = 3-5$) centrosomes (F) which leads to chromosome missegregation.

be derived from hemangioblastic cells (12). Together, the SAGE, cell culture, morphology, and cytogenetic analyses show that tumor endothelial cells differ from normal endothelial cells in many aspects. It is becoming quite clear that the tumor vasculature is much more complex and unpredictable than initially perceived.

Tumor Endothelial Cell Aneuploidy

We have purified tumor endothelial cells in an attempt to better understand the effects of the tumor microenvironment on endothelial cell properties. To do so, xenograft models of human tumors growing in nude mice were established as sources of mouse tumor endothelial cells (13). Tumor (melanoma and liposarcoma) endothelial cells and normal mouse endothelial cell (skin and adipose) counterparts were isolated with 99% purity by combination of magnetic bead cell sorting and a strategy of killing human tumor cells with diphtheria toxin (mouse cells are diphtheria toxin resistant; ref. 13). The mouse tumor endothelial cells expressed typical endothelial cell markers such as CD31, vascular endothelial growth factor (VEGF) receptors, Tie receptors, neuropilins, and TEM1, TEM5, TEM7, and TEM8. The tumor endothelial cells

retained endothelial cell properties at least up to passage 20 and the cells could be maintained in culture for at least 50 passages. Tumor endothelial cells grew faster, had a lower serum requirement, and were more responsive to epidermal growth factor (EGF) and fibroblast growth factor than normal counterpart endothelial cells.

Tumor endothelial cells had relatively larger nuclei, indicating a DNA content that was different from normal endothelial cells. Strikingly, tumor endothelial cells were cytogenetically abnormal. Karyotype analysis indicated that tumor endothelial cells had multiple chromosomes characteristic of aneuploidy, whereas normal endothelial cells grown under the same conditions were strictly diploid. Multiple-colored fluorescent *in situ* hybridization (M-FISH) analysis (in collaboration with Dr. Cynthia Morton, Brigham and Women's Hospital, Boston, MA) showed that tumor endothelial cells were characterized by structural aberrations such as nonreciprocal translocations, missing chromosomes, marker chromosomes, and double minutes. Individual tumor endothelial cells had different cytogenetic profiles indicating that tumor endothelial cells were heterogeneous and not clonal. Thus, tumor endothelial cells have hallmarks of chromosomal instability.

To avoid possible artifacts due to culture conditions, FISH analysis was carried out on endothelial cells that were freshly isolated and that had never been cultured. CD31 staining was used to confirm endothelial cell identity. About 16% of liposarcoma endothelial cells and 34% of melanoma endothelial cells were aneuploid by FISH using a mouse chromosome 17 probe. Significantly, the degree of aneuploidy of tumor endothelial cells reached over 80% in culture by passage 6. On the other hand, freshly isolated skin and adipose endothelial cells were diploid and remained diploid when cultured even up to passage 20. These results suggest that tumor endothelial cells, unlike normal endothelial cells, are inherently unstable. FISH on frozen tumor sections also showed a degree of aneuploidy, although these experiments were more difficult to interpret because unlike freshly isolated endothelial cell (95% pure), CD31-positive cells were only a minority of the cells in a section.

Tumor endothelial cells have abnormal centrosomes as well. The normal function of centrosomes is to establish cell polarity and to properly segregate chromosomes. Defects in centrosome function with loss of polarity and with chromosome missegregation have been detected in aggressive human malignant tumors. For example, both invasive and preinvasive carcinomas have abnormal centrosomes (14), which contribute to aneuploidy (15). A normal cell has one centrosome per cell at G₁, whereas an aneuploid tumor cell may have multiple centrosomes. We found that tumor endothelial cells have between one and five centrosomes and thus mimic tumor cells. Because the tumor endothelial cells continue to proliferate in culture, it seems that these cells, like tumor cells, lack the normal cell cycle checkpoints that inhibit mitosis in response to centrosome abnormalities. In addition, our studies show a correlation between the number of tumor endothelial cells with extra chromosomes (16-34%) and those with multiple centrosomes (26-32%) suggesting a potential causal relationship. As cells continue to proliferate in the presence of multiple centrosomes, aneuploidy may arise due to the missegregation of chromosomes. Figure 1 shows a schematic of chromosome and centrosome abnormalities in tumor endothelial cells.

Significance of Tumor Endothelial Cell Aneuploidy

The aneuploidy of tumor endothelial cells is significant. Tumor endothelial cells have long been considered normal diploid cells that, unlike tumor cells, do not mutate and do not develop drug resistance. However, the aneuploid tumor endothelial cell may be a different matter. These cells are inherently genetically unstable as shown by the large increase in aneuploidy when cultured. Acquired drug resistance as a consequence of aneuploidy is a possibility. It has been shown previously that tumor endothelial cells in culture are more resistant to vincristine than normal endothelial cells (9). Some antiangiogenic drugs have been shown to lose their effectiveness over time possibility due to acquired resistance. For example, Kerbel et al. have suggested that resistance can develop to many types of angiogenesis inhibitors, including those that act directly on endothelial cells (16). A novel possibility based on our results, but yet untested, is that tumor blood vessel endothelial cell become increasingly aneuploid, unstable, heterogeneous, and resistant to antiangiogenesis therapy.

On the other hand, tumor endothelial cells may be more sensitive than normal endothelial cells to certain drugs. For

example, we have found that EGF receptor (EGFR) is not expressed in normal endothelial cells but that in contrast, tumor endothelial cells express high levels of EGFR protein.¹ EGF and other ligands of the EGF family can induce phosphorylation of tumor endothelial cell EGFR and stimulate tumor endothelial cell proliferation. Normal endothelial cells are not affected by these ligands, consistent with the lack of EGFR expression in these cells. EGFR kinase inhibitors that have been assumed to inhibit tumor cell EGFR activity in patients may also target tumor endothelial cell EGFR, adding another dimension, possibly clinically relevant, to EGFR kinase inhibitor activities. Interestingly, a recent report indicates that suppressing EGFR and erbB2 reverses aneuploidy in tumor cell lines (17). In another example of tumor endothelial cell markers, St. Croix et al. have shown that tumor endothelial cells up-regulate TEMs (7). Our own studies confirm that TEM7 is specifically up-regulated in tumor endothelial cells (13). These cell surface TEMs are being tested as potential targets that could be useful for diagnosis and therapy in cancer treatment.

Possible Mechanisms of Tumor Aneuploidy

The mechanisms that result in tumor endothelial cell aneuploidy are not yet understood. Unraveling this mystery would be significant in understanding how endothelial cells become cytogenetically abnormal and unstable, and possibly allow insights into how to reverse this process. Possible mechanisms include the following: (a) The tumor microenvironment containing tumor and stromal cells may produce factors causing genetic instability such as growth factors and cytokines. For example, VEGF and EGFR ligands expressed in tumor and stromal cells are antiapoptotic survival factors that activate the Akt survival signal transduction pathway. Activation of this signal pathway is known to cause genetic instability (18). (b) Loss of tumor suppressor and checkpoint activity might result in aneuploidy. During tumorigenesis, mitotic diploid cells can acquire genetic mutations resulting in aneuploidy. These cells undergo apoptosis in response to gatekeeper genes such as *p53* (18). A number of studies have shown a strong correlation between loss or mutational inactivation of *p53* and chromosomal instability and aneuploidy. Loss or mutation of *p53* induces abnormal amplification of centrosomes, leading to an increased frequency of defective mitoses organized by multiple spindle poles. This results in missegregation of chromosomes into daughter cells. (c) Oncogenes may play a role in inducing aneuploidy. For example, overexpression of cMyc in nonadherent murine pro-B lymphocytic cells induced structural and genomic instability within 48 hours including gene amplification, chromosomal breakage and deletions, increased aneuploidy and polyploidization (19). (d) Transdifferentiation of tumor cells into endothelial cells is another possible mechanism (11, 12). However, this mechanism is less likely in our xenograft model of human tumor because we did not detect human genomic material in the mouse tumor endothelial cells that we had isolated, using mouse specific FISH and multicolor FISH probes. (e) Stem cells derived from mature tissue such as bone marrow can be aneuploid and have the potential to fuse with mature cells. EPC, a subset of stem cells derived from bone marrow, can be incorporated into tumor blood vessels (20). Thus, it is possible that some of the

¹ D. Amin and M. Klagsbrun, in preparation.

aneuploid tumor endothelial cells are EPC derived. Our preliminary results show that tumor endothelial cells up-regulate Sca-1, a stem cell marker suggesting that bone marrow-derived endothelial cells migrate into tumors.²

Summary

In summary, endothelial cells in solid tumors have previously been shown to have morphologic differences compared with normal blood vessels and to have altered gene and protein expression patterns. Our results indicate possibly for the first time, that the endothelial cells in solid tumor blood vessels have

cytogenetic differences as well and display a degree of aneuploidy as evidenced by extra chromosomes and multiple centrosomes (Fig. 1). However, these results were obtained using xenograft models. In the future, it is imperative to determine whether aneuploidy occurs in human cancer patient tumor endothelial cells. The effectiveness of antiangiogenesis therapy is based on the assumption that endothelial cells in tumors are normal diploid and thus not drug resistant. However, if these cells were aneuploid and unstable, it is speculated that drug resistance could possibly develop and compromise the effectiveness of antiangiogenesis therapies.

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² Y. Hida and M. Klagsbrun, unpublished data.

References

- Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003;9:653–60.
- Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964–7.
- Folkman J, Kerbel R. Role of angiogenesis in tumor growth and metastasis. Clinical translation of angiogenesis inhibitors. *Semin Oncol* 2002;29:15–8.
- Jain RK. Molecular regulation of vessel maturation. *Nat Med* 2003;9:685–93.
- McDonald DM, Baluk P. Significance of blood vessel leakiness in cancer. *Cancer Res* 2002;62:5381–5.
- Pasqualini R, Arap W, McDonald DM. Probing the structural and molecular diversity of tumor vasculature. *Trends Mol Med* 2002;8:563–71.
- St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. *Science* 2000; 289:1197–202.
- Nanda A, Buckhaults P, Seaman S, et al. Identification of a binding partner for the endothelial cell surface proteins TEM7 and TEM7R. *Cancer Res* 2004;64:8507–11.
- Bussolati B, Deambrosio I, Russo S, Deregibus MC, Camussi G. Altered angiogenesis and survival in human tumor-derived endothelial cells. *FASEB J* 2003; 17:1159–61.
- Allport JR, Weissleder R. Murine Lewis lung carcinoma-derived endothelium expresses markers of endothelial activation and requires tumor-specific extracellular matrix *in vitro*. *Neoplasia*, 2003;5:205–17.
- Gunsilius E. Evidence from a leukemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. *Adv Exp Med Biol* 2003;522: 17–24.
- Streubel B, Chott A, Huber D, et al. Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas. *N Engl J Med* 2004; 351:250–9.
- Hida K, Hida Y, Amin DN, et al. Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res* 2004;64:8249–55.
- Duensing S, Munger K. Human papillomaviruses and centrosome duplication errors: modeling the origins of genomic instability. *Oncogene* 2002;21: 6241–8.
- Storchova Z, Pellman D. From polyploidy to aneuploidy, genome instability and cancer. *Nat Rev Mol Cell Biol* 2004;5:45–54.
- Kerbel RS, Yu J, Tran J, et al. Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. *Cancer Metastasis Rev* 2001;20: 79–86.
- Pack SD, Alper OM, Stromberg K, et al. Simultaneous suppression of epidermal growth factor receptor and c-erbB-2 reverses aneuploidy and malignant phenotype of a human ovarian carcinoma cell line. *Cancer Res* 2004;64:789–94.
- Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004;10: 789–99.
- Fest T, Mougey V, Dalstein V, et al. c-MYC overexpression in Ba/F3 cells simultaneously elicits genomic instability and apoptosis. *Oncogene* 2002;1: 2981–90.
- Rafi S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med* 2003;9:702–12.