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REVIEW ARTICLE

Significance of anti-angiogenic therapy in head and neck cancer—Heterogeneity of tumor endothelium

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Summary Tumor angiogenesis is necessary for solid tumor progression and metastasis. Thus, targeting tumor blood vessels is an important strategy for cancer therapy. Especially, it would give large benefit to head and neck cancer patients if ideal anti-angiogenic drug is developed. Tumor blood vessels have been shown to differ from their normal counterparts, for example, by changes in morphology. An important concept in tumor angiogenesis is that tumor endothelial cells are assumed to be genetically normal, even though these endothelial cells are structurally and functionally abnormal. To date, many anti-angiogenic drugs have been developed, but it has been also reported to cause toxic side effects. To develop ideal anti-angiogenic therapies, understanding tumor endothelial cell abnormalities is important. We have isolated tumor endothelial cells from mouse tumor xenografts and have shown that tumor endothelial cells are abnormal. Tumor endothelial cells upregulate many genes, such as epidermal growth factor receptor. Tumor endothelial cells are also more sensitive to EGF. Unexpectedly, tumor endothelial cells were cytogenetically abnormal. In marked contrast, freshly isolated normal endothelial cells were diploid. We conclude that tumor endothelial cells can acquire cytogenetic abnormalities while in the tumor microenvironment.

Here, we provide an overview of the current studies on tumor endothelial cell abnormalities.

Contents

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1. Introduction

Angiogenesis, the process of new blood vessel growth, is necessary for tumor progression and metastasis. Tumor blood vessels provide nutrition and oxygen, and get rid of waste from tumor tissue, resulting in tumor progression. Tumor vessels act as gatekeepers for tumor cells to metastasize to distant organs [1,2].

Thus, the attempt to target tumor endothelial cells with angiogenic inhibitors (anti-angiogenic therapy) has been an important strategy for cancer therapy, and many anti-angiogenic drugs have been discovered and tested to date [3].

A traditional concept in anti-angiogenic therapy has been that (i) one tumor endothelial cell supports many tumor cells. Thus, to target endothelial cells may be a much more effective strategy than targeting tumor cells. (ii) Tumor endothelial cells are the same among all tumor types. Hence, an ideal anti-angiogenic drug could be useful in treating all cancers. (iii) Tumor endothelial cells have been believed to be genetically stable until recently, so tumor endothelial cells may not acquire drug resistance unlike tumor cells (Fig. 1). However, recent studies suggest that tumor endothelial cells may be different from normal endothelial cells and also may also be heterogeneous among organs or tumor types. Contrary to the presumption that anti-angiogenic drugs are not as toxic as cytotoxic drugs, they are reported to cause severe side effects such as lethal hemoptysis [4,5] and perforation of intestines [6,7].

To develop ideal tumor anti-angiogenic therapies, understanding the biology of tumor endothelial cells is very important.

2. Phenotype of tumor blood vessels

It is well documented that tumor blood vessels differ morphologically from normal blood vessels (Fig. 2). Tumor vessels are unorganized whereas the normal vasculature shows a hierarchal branching pattern of arteries, veins and capillaries [8]. Tumor endothelial cells do not form regular monolayers and thus do not have a normal barrier function [9]. Tumor endothelial cell basement membranes have structural abnormalities including loose associations with endothelial cells, and varied thicknesses of type IV collagen layers that are usually not seen in normal endothelial cells [10]. Pericytes are present on tumor endothelial cells, but have abnormally loose associations with these cells and extend cytoplasmic processes deep into the tumor tissue [11]. These abnormalities result in leakiness of tumor blood vessels. In addition, 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 [12]. The high interstitial fluid pressure (IFP) in a tumor causes blood vessel collapse and impedes blood flow. This is one reason why tumor tissue is usually under the hypoxic condition, even though it is highly vascularized. This sometimes causes resistance to radiation therapy [13].

3. Differences between tumor endothelial cells and normal endothelial cells

The morphological abnormalities in tumor blood vessels compared to normal blood vessels raises questions as to whether there are phenotypical differences at the molecular and functional levels between tumor and normal endothelial cells. To address this question, tumor endothelial cells isolated from tumor tissue were required. However, there have not been many reports about isolation of tumor endothelial cells until recently. In fact, most studies on tumor angiogenesis have been done using normal endothelial cells such as human umbilical vein endothelial cells (HUVEC), human dermal microvascular endothelial cells (HMVEC) for a long time. To isolate tumor endothelial cells for global analysis of gene expression has been difficult because, (i) endothelial cells are usually enmeshed in a complex tissue consisting of vessel wall components, stromal cells, tumor cells; (ii) only a small fraction of cells within these tissues are endothelial cells. Besides technical difficulties, there might have been a concern about trials to isolate tumor endothelial cells themselves, because they were sometimes considered to lose their specific phenotype soon after being isolated from tumor tissue. In the first report about tumor endothelial specific markers, St. Croix et al. succeeded in isolating endothelial cells from colon carcinoma and normal colonic mucosa and compared the gene expression profiles between tumor and normal endothelial cells of a relatively low number of cells. They identified the specific genes for tumor endothelial cells and designated them as tumor endothelial markers (TEMs) using serial analysis of gene expression (SAGE). SAGE revealed there are 46 tumor endothelial markers, called TEMs [14]. Some of them are transmembrane proteins and are also conserved in mice [15,16]. Very recently, they showed that these TEMs, except TEM8, are also overexpressed during physiological angiogenesis, as well as in tumor endothelial cells. Instead, they identified 13 novel cell surface proteins as tumor endothelial markers [17]. Other studies about the gene profile of tumor endothelial using global analysis have been published recently. Buckanovich et al. identified 12 ovarian tumor vascular markers (TVMs) from vascular cells captured by laser-capture microdissection (LCM) and some TVMs correlated with the prognosis of patients. However, they commented that these markers are not strictly specific to tumor endothelial cells, because

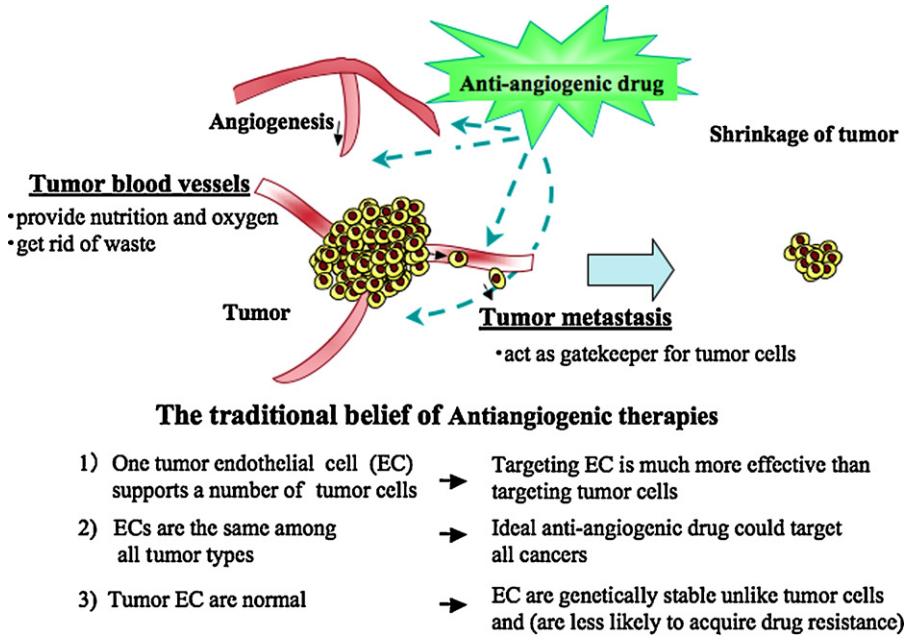

Figure 1 The traditional belief of anti-angiogenic therapy. It has been believed that anti-angiogenic therapy has several advantages traditionally. (1) To target endothelial cells may be a much more effective strategy than targeting tumor cells. (2) Tumor endothelial cells are the same among all tumor types. Thus, an ideal anti-angiogenic drug could be useful in treating all cancers. (3) Tumor endothelial cells were believed to be genetically stable until recently. Thus tumor endothelial cells may not acquire drug resistance, unlike tumor cells (modified from Hida et al., Cancer sci vol. 99 no. 3 Fig. 1).

LCM-captured cells contain not only endothelial cells but also mural cells such as pericytes or smooth muscle cells [18]. Ovarian tumor endothelial cells were also isolated with magnetic beads and 23 tumor endothelial markers were identified by DNA microarray [19]. Among the 23 markers, several genes are involved in the proangiogenic pathway. Colon carcinoma endothelial cell markers were also identified by SAGE [20,17].

However, tumor endothelial cells were not cultured in these studies and the biological phenotype in tumor endothelial cells remains to be clarified. Another study is based on cultured tumor endothelial cells. For example, human renal cell carcinoma endothelial cells did not undergo the senescence that is typical of normal endothelial cells, and were resistant to apoptotic stimuli such as serum-starvation and vincristine. They exhibited higher proliferation rates in low serum, enhanced Akt activation, and decreased expression of the tumor suppressor, PTEN [21]. Murine Lewis lung carcinoma endothelial cells were characterized by elongated morphology, and upregulated adhesion molecules such as CD31 or ICAM-1. They required a tumor-specific matrix to maintain their characteristics. Sca-1 expression was also elevated in these cells suggesting the presence of circulating endothelial progenitors (CEP) in their tumor endothelial cells [22]. We have also purified tumor endothelial cells in an attempt to better understand the effects of the tumor microenvironment on endothelial cell properties [23]. Human tumor xenograft models in nude mice were established as sources of

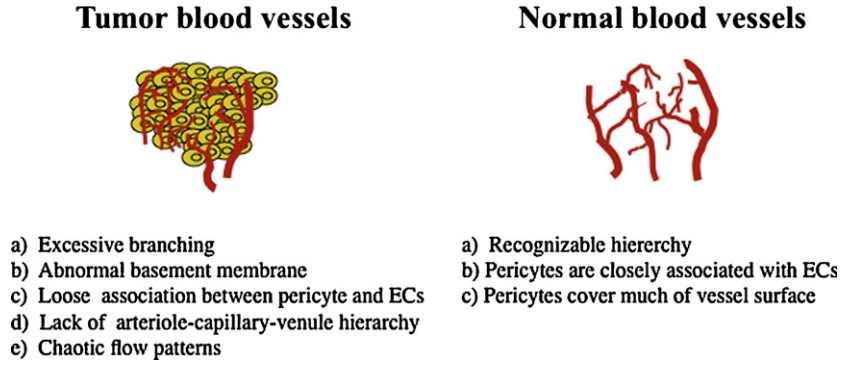

Figure 2 The differences in blood vessels between tumor and normal tissues. Tumor vessels have excessive branching. Tumor endothelial cell basement membranes have structural abnormalities including loose associations with endothelial cells, and various thicknesses of type IV collagen layers that are usually not seen in normal endothelial cells. Pericytes have abnormally loose associations with endothelial cells and extend cytoplasmic processes deep into the tumor tissue. Tumor vasculature shows lack of arteriole–capillary–venule hierarchy. Tumor vessels exhibit chaotic blood flow (modified from Hida et al., Cancer sci vol. 99 no. 3 Fig. 2).

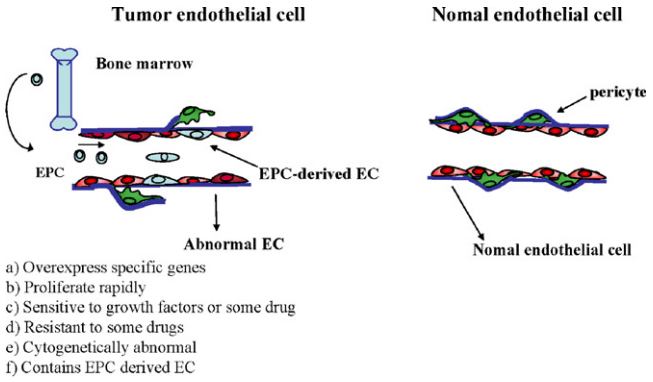

Figure 3 Tumor endothelial cells differ from normal endothelial cells. (a) Tumor endothelial cells overexpress specific genes, such as TEMs and EGFR. (b) They proliferate more rapidly and (c) are sensitive to growth factors such as bFGF, EGF and VEGF, or some drugs like EGFR inhibitors. (d) Tumor EC are resistant to apoptotic stimuli such as serum starvation or chemotherapeutic drug and (e) have cytogenetical abnormalities. (f) There are some EPC-derived endothelial cells in tumor vessels (modified from Hida et al., Cancer sci vol. 99 no. 3 Fig. 2).

mouse tumor endothelial cells. Murine tumor (melanoma and liposarcoma) endothelial cells and normal (skin and adipose) endothelial cell counterparts were isolated with high purity by combination with magnetic bead cell sorting [24]. Since it is known that heparin binding EGF like growth factor (HB-EGF) is a receptor of diphtheria toxin (DT) in human cells, but not mouse cells, and DT binds to human cells expressing HB-EGF and is toxic to them while mouse cells are resistant to DT [25], we used DT in tumor endothelial cell isolation [24]. To remove any human tumor cell contamination which might have overgrown in the endothelial cell culture, DT was added to the tumor endothelial cell subculture to kill human cells and normal endothelial cells for technical consistency. The mouse tumor endothelial cells expressed typical endothelial cell markers such as CD31, VEGF receptors and upregulated several tumor endothelial markers which have already been reported, such as TEMs [24] or Aminopeptidase N (CD13) (data not published). From these data, tumor endothelial cells retain their specificity for tumor endothelial cells (at least some) even in culture. Tumor endothelial cells grew faster, had a lower serum requirement, and were more responsive to angiogenic growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) compared to normal counterpart endothelial cells [23]. Furthermore, we have found that tumor endothelial cells express high levels of EGFR, which is not usually expressed in normal endothelial cells, such as HUVEC [26]. EGF can induce phosphorylation of tumor endothelial cell EGFR and stimulate tumor endothelial cell proliferation. EGFR tyrosine kinase inhibitors inhibit EGF-induced EGFR activation and proliferation of tumor endothelial cells. Thus, it was suggested that EGFR kinase inhibitors may target not only tumor cells, but also tumor endothelial cell EGFR. This data has clinical significance. Anti-EGFR therapy could target tumor vasculature specifically. Moreover, this therapy can be applied to any cancer in which tumor cells do not express, or express a low level of EGFR.

Taking the *in vivo* and *in vitro* studies together, there are mounting evidences that there is distinct differences between tumor and normal blood vessels and their endothelial cells in terms of biology, morphology and gene profile (Fig. 3).

4. Cytogenetical abnormalities in tumor endothelial cells

Tumor endothelial cells had relatively larger nuclei, indicating they had more DNA content than normal endothelial cells [24]. Strikingly, tumor endothelial cells were cytogenetically abnormal. Tumor endothelial cells were karyotypically aneuploid, whereas normal endothelial cells grown under the same conditions were diploid. In addition, they had structural aberrations such as non-reciprocal translocations, missing chromosomes, marker chromosomes, and double minutes by multiple colored fluorescent in situ hybridization (M-FISH) analysis [24]. Thus, tumor endothelial cells have hallmarks of chromosomal instability. To avoid possible artifacts due to culture conditions, freshly isolated, uncultured endothelial cells were analyzed by FISH. 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 [24]. After this report, we recently investigated the aneuploidy of other types of tumor endothelial cells. About 35% of oral carcinoma endothelial cells (Fig. 4A) and 54% of renal carcinoma endothelial cells (Fig. 4B) were also aneuploid even when uncultured. Significantly, the degree of aneuploidy of tumor endothelial cells almost doubled in culture in each tumor endothelial cell. On the other hand, freshly isolated, uncultured skin endothelial cells were diploid and remained diploid when cultured (Fig. 4C). These results suggest that tumor endothelial cells, unlike normal endothelial cells, have chromosomal instability.

Aneuploid tumor endothelial cells were also detected on frozen tumor sections by FISH. Tumor endothelial cells also have abnormal centrosomes.

Since tumor endothelial cells continue to proliferate in culture, it appears that these cells, like tumor cells, lack the normal cell cycle checkpoints that inhibit mitosis in response to chromosomal abnormalities. Recently, we found that tumor endothelial cells have aneuploidy in also human renal cell carcinomas as well as mouse tumor endothelial cells [27].

There are some other reports about chromosomal abnormalities in tumor endothelial cells in hematopoietic tumors

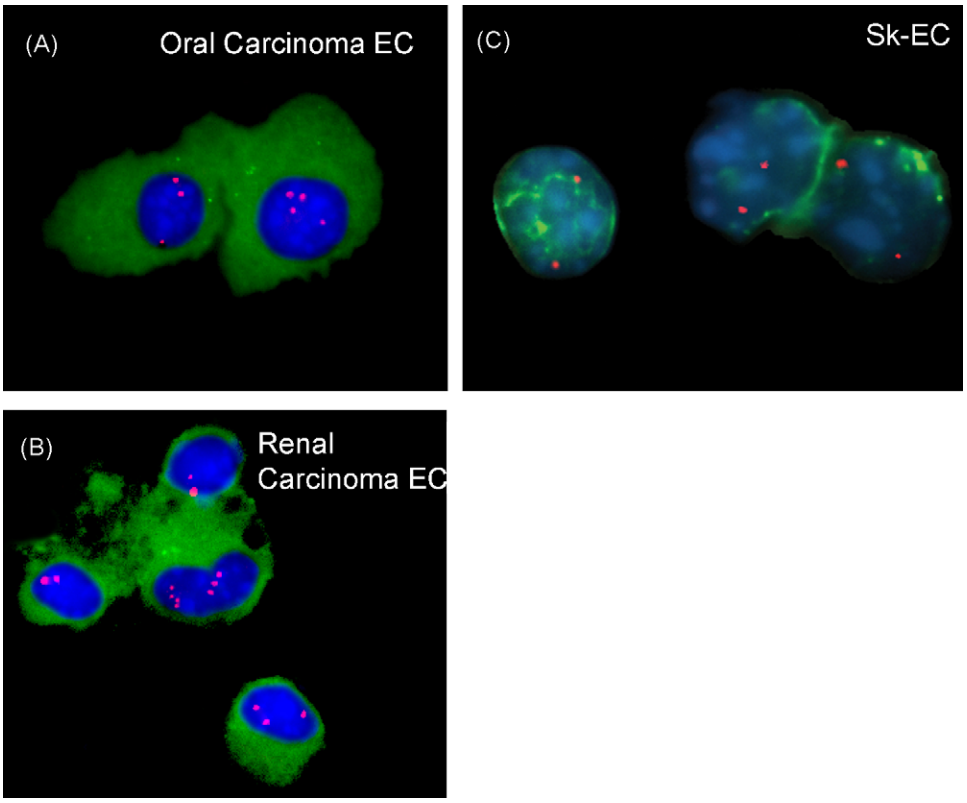

Figure 4 The cytogenetically abnormal tumor endothelial cells. Mouse oral carcinoma endothelial cells (A) and renal carcinoma endothelial cells (B) were isolated and cytospun onto glass slides, followed by immunostaining with an anti-CD31antibody and FISH with a chromosome 17 probe. Representative aneuploid endothelial cells are shown. Normal endothelial cells (skin endothelial cells) are diploid (C). Green; CD31, Red; chromosome 17, Blue; Dapi, Bar; 10 μm (modified from Hida et al., Cancer sci vol. 99 no. 3 Fig. 4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

such as leukemia [28] and lymphoma [29]. In chronic myeloid leukemia, for example, circulating endothelial cells had leukemia-specific translocations [28]. In B-cell lymphomas, 37% of endothelial cells were shown to harbor lymphoma-specific chromosomal translocations, suggesting that lymphoma and lymphoma endothelial cells may both be derived from hemangioblastic cells [29]. In addition, circulating endothelial cells in multiple myeloma had the same translocation as myeloma cells, indicating the possibility that both cells were originally from the same multipotent hemangioblast [30].

Furthermore, a recent study reported that neuroblastoma endothelial cells had a varying proportion of microvascular endothelial cells that exhibited MYCN amplification, which are typically amplified in neuroblastoma, suggesting these tumor endothelial cells are dedifferentiated from their tumor origin [31].

5. Significance of tumor endothelial cell aneuploidy

An abnormal chromosome number, aneuploidy, is a common characteristic of tumor cells. In addition, it has been proposed that aneuploidy cause tumorigenesis for nearly 100 years. However, this remains unproven since there have been controversial reports that aneuploidy is merely a benign side effect of transformation or a contributor to tumor progression, but not to tumor initiation [32]. Recently, Weaver et al. generated aneuploid cells and animals by reduction of Centromere-associated Protein-E (CENP-E). In their study, aneuploidy was shown to promote spontaneous tumorigenesis in aged animals, but at a modest frequency. However, an increased rate of aneuploidy was shown to inhibit tumorigenesis [33].

To return to the subject of tumor endothelial cells, do aneuploid tumor endothelial cells have tumorigenesity? Melanoma and liposarcoma endothelial cells were plated in soft agar to monitor anchorage-independent growth. However, these tumor endothelial cells did not form colonies in soft agar, whereas a mouse endothelial cell line (MS1) immortalized by an SV40 T antigen, formed colonies in soft agar. When injected into nude mice subcutaneously, tumor endothelial cells did not form tumors in mice, while MS1 cells did form hemangioma in mice, consistently to previous report [34] (data not shown). These data are still preliminary and many further studies should be done before concluding that aneuploid tumor endothelial cells are transformed or tumorigenic.

In any case, the aneuploidy of tumor endothelial cells is significant. Tumor endothelial cells have been considered to be genetically normal, unlike tumor cells, for a long time. However, aneuploid tumor endothelial cells may be a different matter. Tumor endothelial cells may develop drug resistance

like tumor cells, contrary to past beliefs. It has been shown previously that tumor endothelial cells in culture are more resistant to vincristine than normal endothelial cells [21]. Our studies also showed tumor endothelial cells were more resistant to 5-FU than normal endothelial cells (unpublished data).

Some anti-angiogenic drugs have been shown to lose their effectiveness over time, possibly due to acquired resistance. For example, as a mechanism of resistance to anti-angiogenic therapy, it was suggested that survival factors such as cytokines or growth factors which are rich in the tumor microenvironment, may cause epigenetic changes not only in tumor cells, but also in tumor endothelial cells [35]. For example, bFGF was reported to inhibit apoptosis signal kinase 1 (ASK1) activity, inducing chemoresistance in HUVEC [36].

Taken together, the possibility that aneuploid tumor endothelial cells are chemotherapy-resistant (or sensitive to some drugs) warrants further investigation.

6. Conclusion

As reviewed in this article, tumor endothelial cells are different from normal endothelial cells in gene profile and behavior, besides the morphological changes described previously. Furthermore, the endothelial cells even in non-hematopoietic solid tumors also have cytogenetic abnormalities, contrary to the assumption that endothelial cells in tumors are genetically stable and thus not drug-resistant. It is speculated that drug resistance could possibly develop and compromise the effectiveness of anti-angiogenic therapies. Whatever mechanism underlies tumor endothelial abnormality, it is important to understand even stroma cells can be abnormal in the tumor microenvironment. Recent studies suggest that both tumor cells and cells in the tumor microenvironment are a target for cancer therapy. Studies on tumor endothelial cell abnormalities will help to develop ideal anti-angiogenic therapies and also to understand how tumor tissues are orchestrated by various cell types.

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