Review Article

Significance of circulating tumor cells in lung cancer: a narrative review

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Background and Objective: In cancer patients, circulating tumor cells (CTCs) are employed as "Liquid Biopsy" for tumor detection, prognosis and assessment of the response to therapy. CTCs are responsible for tumor dissemination but the mechanisms involved in intravasation, survival in the circulation and extravasation at secondary sites to establish metastases are not fully characterized. In lung cancer patients, CTCs are present in very high numbers in small cell lung cancer (SCLC) that is found disseminated in most patients upon first presentation and has a dismal prognosis. This review aims at the discussion of recent work on metastatic SCLC and novel insights into the process of dissemination derived from the access to a panel of unique SCLC CTC lines.

Methods: PubMed and Euro PMC were searched from January 1st, 2015 to September 23th, 2022 using the following key words: "SCLC", "NSCLC", "CTC" and "Angiogenesis" and supplemented by data from our own work.

Key Content and Findings: Experimental and clinical data indicate that the intravasation of single, apoptotic or clustered CTCs occur via leaky neoangiogenetic vessels in the tumor core and not via crossing of the adjacent tumor stroma after EMT. Furthermore, in lung cancer only EpCAM-positive CTCs have been found to have prognostic impact. All our established SCLC CTC lines form spontaneously EpCAM-positive large and chemoresistant spheroids (tumorospheres) that may become trapped in microvessels *in vivo* and are suggested to extravasate by physical force. The rate-limiting step of the shedding of CTCs is most likely the presence of irregular and leaky tumor vessels or in case of SCLC, also via vessels formed by vasculogenic mimicry. Therefore, lower microvessel densities (MVD) in NSCLC can explain the relative rarity of CTCs in NSCLC versus SCLC.

Conclusions: The detection of CTCs lacks standardized techniques, is difficult in non-metastatic patients and important cell biological mechanisms of dissemination need still to be resolved, especially in respect to the actual metastasis-inducing cells. Expression of VEGF and the MVD are key prognostic indicators for tumors and ultimately, enumeration of CTCs seems to reflect neoangiogenetic vascular supply of tumors and prognosis.

Keywords: Lung cancer; non-small cell lung cancer (NSCLC); small cell lung cancer (SCLC); circulating tumor cells; angiogenesis; metastasis

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Introduction

Although great progress has been made in the treatment of cancer patients, metastatic disease is frequently not amenable to any further therapy (1,2). Metastasis takes place in several steps comprising cancer cell intravasation, migration, extravasation and establishment of secondary lesions (*Figure 1*). Because the course of tumor metastasis is difficult to follow in patients, the sequence of steps resulting in metastases has not been elucidated in detail so far (3). Primary tumors shed cells into the circulation, termed circulating tumor cells (CTCs), which migrate and spread to distant sites to establish metastases (4). Therefore, metastasis represent the growth of distinct cancer cells that are present in parent tumors (5). Discrete genetic clones eventually forming metastases can be generated in tumors early, preceding the diagnosis of cancer (6). CTCs were suggested as potential founders of metastatic lesions more than 100 years ago (7). These cells are found in most advanced malignancies, although their numbers are relatively low in the circulation except for small cell lung cancer (SCLC) and inflammatory breast cancer (IBC) (8,9). Counting and molecular characterization of CTCs were expected to allow for early detection of tumor dissemination and for personalized therapy according to tumor markers and chemosensitivity (10). The techniques to identify CTCs represent a type of "Liquid Biopsy" that can be repeated easily to follow the course of the disease in contrast to invasive biopsies (10,11). However, the latest approaches to CTCs have concentrated largely on the different methods of enrichment of these rare cells and correlation to prognosis and response to therapy although basic questions such as the mechanisms of the release of tumor cells, their fate in the circulation, the characteristics of metastasis-inducing CTCs and their ways of extravasation are not fully characterized (*Figure 1*). This review concentrates on the role of CTCs in SCLC and non-small cell lung cancer (NSCLC) as well as the involvement of the angiogenic switch in shedding of CTCs. SCLC and NSCLC represent two extremes of a high and low count of CTCs, respectively. We present the following article in accordance with the Narrative Review reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-712/rc).

Methods

This narrative literature review used publications cited in PubMed (https://pubmed.ncbi.nlm.nih.gov/) and EuroPMC (https://europepmc.org/), as well as own work, to identify studies and literature reviews covering SCLC using specific terms (*Table 1*). Studies published in peer reviewed journals were used. Specific terms used include small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), circulating tumor cells (CTCs) and angiogenesis.

Detection and enrichment of CTCs

Tumors shed cancer cells but CTCs are rare even in patients with malignancies (12,13). The numbers of CTCs range from a few cells to a few hundred CTCs per 10 mL blood with exception of SCLC and Inflammatory Breast Cancer (IBC) (8,9). The daily shedding of 1–4 million CTCs per gram of tumor tissue has been reported for a breast cancer xenograft model that may not truly reflect actual human tumors (14,15). As cancer cells are heterogeneous, CTCs may have large variations in the expression of surface biomarkers (16). Therefore, strategies have been developed for the enrichment and detection of CTCs using techniques either based on cell surface markers or physical characteristics of the cancer cells (17). Despite of a large number of methods described, approval of the FDA is limited to the CellSearch© system, that detects CTCs according to their expression of EpCAM using 7.5 mL of blood (18). The CellSearch© system employs on immunomagnetic enrichment of EpCAM-positive tumor cells and confirmation of their characteristics by a demonstration of the presence of cytokeratins, a positive DAPI nuclear staining and negativity for the leucocyte marker CD45 (18,19). A poorer prognosis has been predicted for patients exhibiting CellSearch© CTC counts of more than 5, 5 and 3 CTCs/7.5 ml blood for breast, pancreatic and colon cancer, respectively. The CTC marker EpCAM is a transmembrane glycoprotein that is expressed by the majority of breast, colorectal, and prostate cancers but is absent in blood cells (16). Alternative CTC antigens such as keratin 19, tumor-specific antigen 9, and progastrin-releasing peptides have been reported (20). The detection of CTCs is challenging due to their extremely low frequency in the circulation. Even in metastatic disease, the CellSearch© system failed to find CTCs in ~20% of prostate, ~25% of breast, and ~50% of colon cancer patients (21). Furthermore, the CellSearch© detection system dismisses EpCAM-negative CTC subpopulations of putative significance for tumor dissemination.

Marker-independent CTC detection techniques rely on physical characteristics of CTCs such as cell

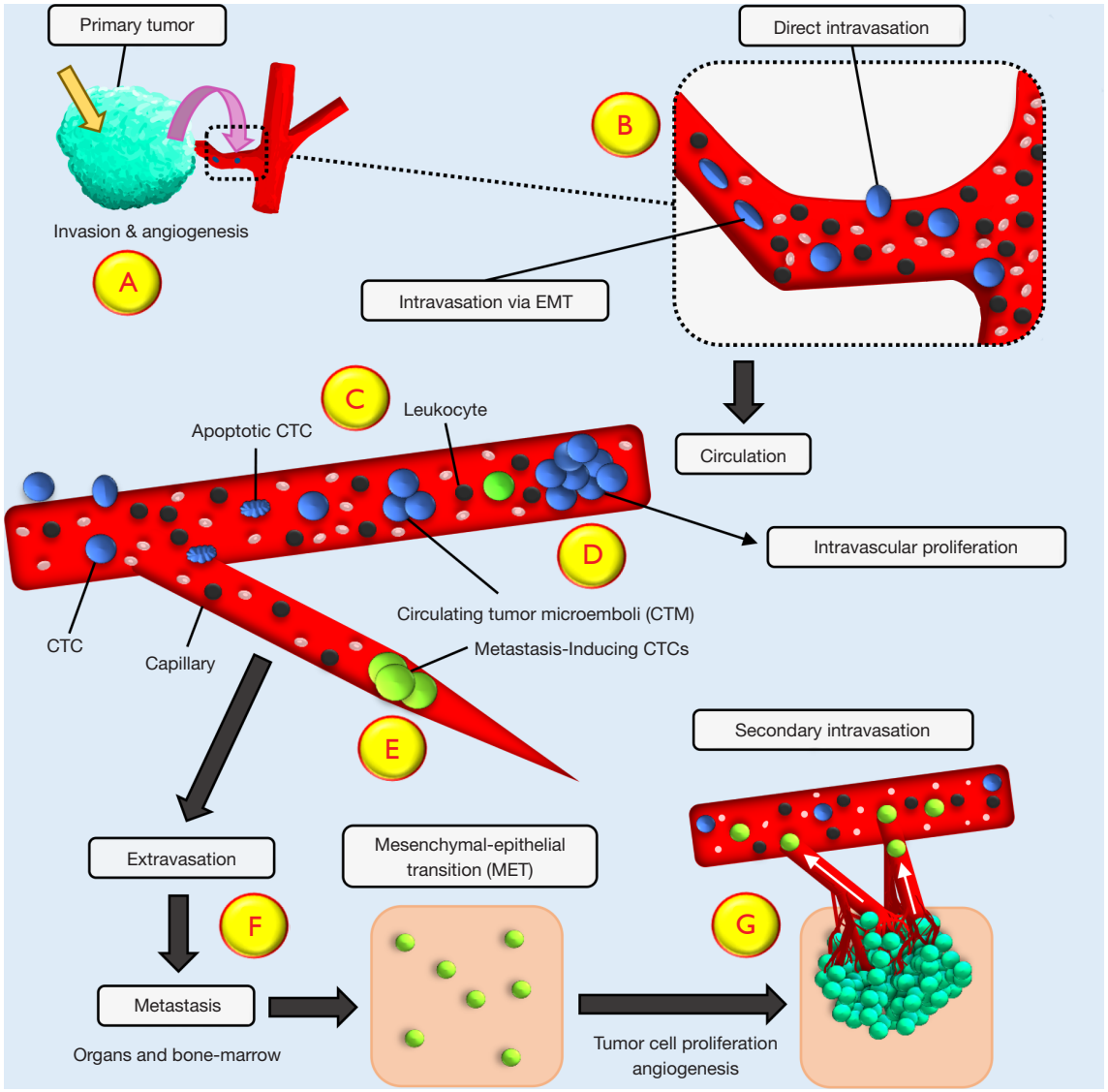

Figure 1 Schematic representation of CTC-mediated metastasis. (A) Depicts the primary tumor and intravasation of CTCs in the tumor core (yellow arrow) or intravasation via EMT into coalescing vessels (violet arrow). In detail, (B) shows the direct trans-endothelial intravasation and elongated mesenchymal type CTCs having entered vessels after EMT. (C) represents the dissemination of CTCs in the circulation, either as single intact or apoptotic CTCs or in form of small cell clusters (D) with some cells in the proliferative state. Some cells or clusters become trapped in capillaries (E) and extravasate either by endothelial transmigration or due to physical force exerted by proliferation of CTC clusters. Within secondary organs CTCs induce metastatic lesions (F), in case of EMT CTCs after a reversal of this phenotype by MET. The invaded cells may convert to silent DTCs or grow as metastases that in turn may release a second wave of CTCs following neoangiogenesis (G). CTC, circulating tumor cell; EMT, epithelial-mesenchymal transition; DTCs, disseminated tumor cells.

size, deformability, electric charge, flow properties and others (22). The relatively small size differences between leukocytes (6.2–9.4 µm), leukemic blood cells (8.9–15.3 µm) and solid tumor cells (11.7–23.8 µm) complicate the separation of CTCs (23). These variant approaches detect larger numbers of CTCs of potential aggressive and invasive properties compared to the CellSearch© system (17,24,25). Therefore, these methods recognize CTCs with low or negative EpCAM expression in contrast to the CellSearch© (9). Size-dependent CTC enrichments

Table 1 Selection strategy for this narrative review

Specification	Items
Date of search	September 26th, 2022
Timeframe	January 1st, 2015 to September 23rd, 2022
Databases and other sources searched	PubMed, Euro PMC and own work
Search terms used	"SCLC", "NSCLC", "CTC" and "Angiogenesis"
Inclusion and exclusion criteria	Original Investigations and Reviews in English language were included
Selection process	Selected by all authors

comprise "Isolation by the size of epithelial tumor cells" (ISET®), diverse filtration devices, such as Metacell, ScreenCellCyto and the Parsortix™ system, among others. A host of recent reviews summarize the wide range of techniques to assess CTCs in various malignant diseases (26-28). However, in contrast to cell-free DNA (cfDNA; circulating DNA fragments released by tumor cells), enriched CTCs constitute viable tumor cells that can be tested for DNA, RNA and protein expression (29). Additionally, CTC analysis at single-cell resolution has been reported to offer valuable insights into tumor heterogeneity (28). Due to clinical and technical restrictions, the precision of CTC detection methods by repetitive sampling has been rarely confirmed.

CTCs should become trapped in capillaries because their size is larger than the diameter of microvessels (30). However, the small SCLC CTCs may pass into capillaries and clusters consisting of several tumor cells were reported to migrate as single-file chain-like cell assembly (31,32). In addition to single cell CTCs, Circulating Tumor Microemboli (CTM)/Clustered Circulating Tumor Cells (CTC) can be detected in cancer patients (33). These CTMs were suggested to stem from the intravasation of tumor cells that have migrated as cluster and have entered the circulation via "leaky" and irregular tumor vessels that are a characteristic of highly angiogenic tumors (8,33).

Breast cancer cells monitored through cancer progression in an experimental model showed that the majority of CTC clusters have undergone hypoxia, while single CTCs are mostly normoxic (34). Inhibition of VEGF results in tumor shrinkage, but elevates intratumoral hypoxia, with increased release of CTC clusters shedding and formation of metastases. Tumor hypoxia and defective angiogenesis are key factors affecting cancer progression (35). In a tumor, hypoxia in the core and poorly vascularized regions generally upregulate cell-cell junction proteins of cancer cells, promoting intravasation of CTCs clusters rather than individual CTCs. In breast cancer, clusters of heterogenous tumor cells have been reported to hold a markedly increased metastatic potential (36). Tumor cell clusters are rapidly cleared from the circulation and their high metastatic potential is possibly associated with their trapping in microvessels (31,37). Tumor colonies were primarily released in a murine model by breast cancer xenografts rather than generated by aggregation in the circulation (36). These CTC clusters comprise 2 to 50 tumor cells, fibroblasts, endothelial cells and leukocytes with improved survival in the circulation in addition to the higher metastatic potential (36). Arrest of cancer cells in microvessels may lead to the formation of firm attachments, such directing CTCs to specific organs (*Figure 1*) (38). Liver and brain tissues containing a high number of small capillaries are a frequent target for metastatic seeding (39). Some CTCs interact with platelets to form a shell around them most likely protecting them from shear forces in the circulation (26,33,40-42). CTCs survive in the circulation for very short times, typically less than 24 h. Driemel *et al.* reported that that the expression of EpCAM by CTCs correlates positively with high proliferative activity (43). A contradictory investigation found low expression of the proliferative marker Ki-67 in CTCs, indicating a dormant state (44). Elimination of CTCs from circulation seem to be effected by immune cell attacks, shear stress, anoikis and the absence of growth factors (45,46). A fraction of CTCs is capable of colonizing distant organs and to persist as disseminated tumor cells (DTCs) that in rare cases progress to establish metastases. The fate of DTCs in regard to survival, growth and resistance to therapy is dependent on the tumor microenvironment.

The numbers of CTCs and DTCs are highly correlated, although the frequency of DTCs was mostly higher (46). Blood analyses seem to represent only a 'snapshot' of tumor cell dissemination (47). The major difficulties for an early tumor detection is the rarity of CTCs at the nonmetastatic stage and the low blood volume (<10 mL) available for routine tests (24,25,48). Since CTCs are absent in many metastatic patients and only a very small fraction of CTCs has metastatic potential, CTC counting alone is insufficient in staging and prognosis of the malignant diseases. Biomarkers of metastasis-inducing CTC subsets that assess homing and colonization potential

need yet to be identified (49-51).

CTCs and tumor dissemination

Tumor cell dissemination comprises invasion through the basement membrane during malignant progression, intravasation into blood, survival in the circulation defending immune cells and shear stress, arrest in capillaries of organs and extravasation followed by formation of micrometastases and secondary lesions (*Figure 1*) (52-54). It has been calculated that no more than 0.01% of CTCs are capable of setting metastases (45). Evidence for a metastatic potential of CTCs has been derived from breast cancer xenografts and enriched CTC populations from SCLC that are tumorigenic in immunocompromised mice (8,49).

The small fraction of CTCs with metastases-inducing properties has been explained by destruction of CTCs in the circulation by shear stress and immunological attack as well as a slow rate of extravasation at a secondary organ (5,55). In respect to shear stress, CTCs survive the size-dependent enrichment by the Parsortix system that relies on the pumping of blood through a chip with 6–10 µm restrictions at a pressure that is at least comparable to the diastolic blood pressure (99 mbar) (13,56). At least in case of SCLC with excessive numbers of SCLC the CTCs seem to be less immunogenic despite a host of mutations induced by tobacco consumption (57). A report has described the frequent extravasation of CTCs that eventually remain inactive in the target tissue (39). Furthermore, with exception of SCLC, IBC and heavily metastasized patients, the actual CTC count is low due to a low rate of shedding. The presence of CTCs alone does not prove metastasis, since most CTCs are rapidly removed from blood and extravasation at distant sites ends unproductively in most instances. Cellular immobilization in capillaries frequently results in cell death, dormancy as DTC and very rarely in clinically detectable metastases (5).

The identification of the fraction of rare CTCs which have the potential to initiate metastases is difficult. From data of 38,715 breast cancer patients a tumor doubling time of 1.7±0.9 months was calculated between detection of a primary tumor to overt metastasis (14). For T1B breast cancer cases, a metastatic efficiency of 1 metastasis formed per 60 million CTCs was estimated. Thus, the primary tumor would have to be removed at a size of 3 mm, corresponding to 9±6 CTC/L to prevent dissemination. Firstly, the tumor will not be detected clinically and, secondly, the sensitivity of CTC detection would have to be improved manifold and combined with techniques reducing false positive results. In conclusion, it will not be possible in most cases to find and characterize the primary metastasis-inducing CTCs that exist long before the tumor is detected in clinics. The high numbers of CTCs in metastasized patients may likewise stem from the primary tumor and all secondary lesions.

Mechanisms of the intravasation of CTCs

Intravasation via EMT

Solid tumor cells are supposed to intravasate via passive or active approaches (58,59). The frequently used term "shedding" for this release of tumor cells into the circulation is an indicator of the lack of detailed knowledge. Most CTCs may be released by mechanical forces due to tumor growth or surgery (60). In one proposed model of intravasation, cells at the invasive front of tumors undergo epithelial-mesenchymal transition (EMT) and enter nearby blood vessels (*Figure 1*). The mesenchymal phenotype is distinguished by spindle-shaped cells with higher expression of vimentin and downregulation of cell adhesion molecules that may actively cross the tumor stroma and enter blood vessels (35,61,62). Thus, EMT constitutes a cell regulatory program which enables selected epithelial cells to acquire invasive potency and resistance to cell death for dissemination (58,63-66). For single cells and small clusters, a mesenchymal spindle-cell morphology facilitates the migration through stromal tissue and in the periphery cells after EMT seem to be more tolerant to shear stress by the expression of vimentin and α-actin (39). EMT of cancer cells seem to be triggered by cytokines released from tumor-infiltrating immune cells (67). Tumor cell intravasation mediated by macrophages appear in breast cancer in the absence of angiogenesis (68).

The linear intravasation model states that some cancer cells mutate and develop the preconditions for invasiveness (69,70). Accordingly, EMT may constitute a bottleneck for recruiting CTCs at the invasive front of tumors delaying the dissemination after some time of tumor development (61,71,72). Accordingly, dissemination of cancer cells via EMT occurs later in tumor development and would require a reversal of EMT via mesenchymal-epithelial transition (MET) after extravasation since metastases of epithelial tumors are EpCAM-positive again (4,73). The support for the EMT-mediated intravasation comes mainly from *in vitro* studies and experimental animal models but the

in vivo data favoring this model are scarce and a partial alteration in the expression of mesenchymal markers does not indicate a true phenotypic switch (71,74). Fractions of EpCAM-negative CTCs that had undergone (partial) EMT have been claimed to possess increased metastatic potential and chemoresistance as well as greater prognostic value. CTC detection methods relying on epithelial EpCAM would miss this potentially critical CTC subpopulation (18).

EMT is seldom detected in tumor pathological preparations, although a high number of these invading cancer cells should be present, and it remains debatable whether this *in vitro* model has an *in vivo* counterpart (71,73,75,76). That cells bearing a host of random gene mutations intravasate, survive immune cell attacks, extravasate at distal sites and eventually recapitulate an intact epithelial phenotype seems unlikely (58,73). Moreover, many changes in protein expression associated with EMT are detectable in most non-metastatic benign tumors (77). The significance of EpCAM-positive EpCAM-low CTCs has been investigated by correlating CellSearch© and ISET detection with the prognosis of cancer patients (78,79). Approximately 70% of prostate cancer patients and 64% of breast cancer patients had in total ≥5 EpCAM-positive and/or EpCAM-low CTCs and castration-resistant prostate cancer patients with ≥5 EpCAM-positive CTC had shorter overall survival versus those with <5 EpCAM-positive CTC. Observations in NSCLC, breast and prostate cancer demonstrated that both $EpCAM^+$ and $EpCAM^-$ CTC populations are frequently present. However, in patients with metastatic NSCLC and prostate cancer $EpCAM^+$, CK^+ CTC, and not the $EpCAM^-$, CK^+ are correlated with a poor outcome (80). In conclusion, results of these experiments indicate that the presence of $EpCAM^{low}$ CTC had no relation with overall survival.

Intravasation via core tumor vessels

Furthermore, clinical and experimental data suggest early tumor dissemination shortly after neoangiogenesis and intravasation of tumor cells in the tumor core region (81). CTCs directly intravasate through fenestrated and irregular tumor vessels and, therefore, the metastasis-initiating cells may establish secondary lesions a long time before the clinical detection of cancer (*Figure 1*). This model of cancer cell intravasation fits better to the intravasation of larger CTC clusters and apoptotic CTCs. Furthermore, this eliminates the necessity for a complex EMT/MET phenotypic change and the movement of groups of CTCs as single files through tissue. Intravasation of CTCs via EMT after active crossing of tumor stroma is expected to result in fully viable and not apoptotic cells. Marker-independent CTC enrichment techniques demonstrate a variety of CTC EMT partial phenotypes with minor upregulation in vimentin and downregulation in E-cadherin without a true cell-type transformation (82,83). Furthermore, the number of CTCs that have intravasated seems to be correlated to the microvessel density of the tumor but not to the tumor mass.

Intravasation in the tumor core occurs early and high numbers of CTCs may disseminate into the blood circulation via passive shedding (81,84-86). Single DTCs exist in the lymph nodes or bone marrow of women with a history of early-stage breast cancer that show no clinical of metastasis or tumor recurrence (81). Peaks of the release of CTCs seem to correspond to the time angiogenic switch during the early, preinvasive stage of tumor growth (87). Intravasation of CTCs is facilitated by the irregular and leaky blood vessels formed during the tumor neoangiogenetic process (38,88). Programmed necrosis (necroptosis) of endothelial cell induced by tumor cells may be involved in this process (89). Leaky vessels in the primary tumor facilitate the collective migration and intravasation of CTC clusters with less resistance compared to an EMT-mediated migration in solid tumor stroma.

Intravasation cannot be fully simulated *in vitro* and is rarely observable *in vivo* but can be modeled in appropriate animal experiments (68). Deryugina and Kiosses investigated the structure of intravasated cells within fibrosarcomas in mice ears and chick embryos and localized intravasation almost exclusively in the tumor core and intratumoral vasculature (81,90,91). The great majority of intravasation events was found within the new and immature blood vessels in the tumor core, completely independent from any putative intravasation of the invasive front. It was concluded that possible vasculotropic cancer cells invading the tumor-adjacent stroma and intravasate into coalescing blood vessels are of minor significance for cancer dissemination. This type of intravasation forwards the generation of metastases, immediately after the first neoangiogenic vessels are formed (92). These blood vessels are typically aberrant, marked by irregular branching, distorted structure, intermittent blood flow, leakiness, and abnormal levels of endothelial cell death (88). The size of such pores of neoangiogenetic tumor vessels varies from 100 nm to 2 μm depending on the tumor entity (60,93).

The quantification of human cancer growth rates in the

past showed that metastasis must be initiated a long time before the primary tumor diagnosis because metastases were too large to be initiated at a late stage of tumor history (14,85,94). Tumor volume doubling times of approximately 60–200 days are up to two times faster for metastatic lesions (85,95). For breast cancer, a median time from resection to distant metastasis of 35 and 20 months has been calculated for patients with T1 and T3 tumors, respectively, instead of a presupposed time span of 12 years (96,97). Early release of CTCs fits better to the observed time course of tumor development by setting the start of tumor dissemination to several years before actual primary tumor diagnosis. Furthermore, the clonal diversity of DTCs which may require prolonged time to develop again indicates an early dissemination of CTCs. Accordingly, the occurrence of several large brain metastases in connection with a very small primary tumor is quite frequently reported (98). Furthermore, cancers of unknown primary sites account to up of 5–10% of diagnoses (99).

Experimental patient-derived xenograft (PDX) models

CTCs enriched by negative selection have been grown in immunocompromised mice as CTC-derived xenografts (CDX) (100). CDXs derived from different patients shared genomic alterations, but showed marked heterogeneity within tumors and between distinct PDX (101,102). As drawbacks, SCLC PDX are not administered orthotopically, develop under murine background and lack interaction with human immune effector cells (103). Our own experiments have demonstrated tumorigenicity of the first SCLC permanent CTC lines (BHGc7, 10 and 16) in NOD-SCID mice but high variability of marker expression and chemosensitivity of recultivated PDX suspensions in dependence of time to tumor formation and specific localization of the PDX (unpublished observation).

The scarcity of actual metastasis-inducing CTCs has hindered a characterization of these CTCs *in vitro*, except in short term expansion and as PDX (19). However, SCLC may display an excessive number of CTCs in advanced stage and this allowed us to establish 8 permanent CTC lines *in vitro* which could be employed to characterize several aspects of CTCs (104-108). Provided that the calculations giving the frequency of metastasis-inducing CTCs as 0.01% are valid then extremely high CTC counts are necessary to have a few of such cancer cells present. This condition seems to be fulfilled only for extensively metastasized patients and, especially for SCLC. Our SCLC CTC cell lines have been established from 10 ml blood of patients with extended disease containing several hundreds to thousands of CTCs in total. A recent overview of the published attempts to culture CTCs of different tumor entities has been published by Shimada *et al.* (109). It was concluded that negative selection of CTCs, hypoxia, 3D growth conditions are necessary for the long-term culture of CTC although no definite methods are described. Furthermore, CTCs were assigned hybrid epithelial/mesenchymal phenotypes and characteristics of stem cell-like properties. Yu *et al.* previously suggested that CTC perish through senescence after a few cell divisions in monolayer cultures and growth in suspension and hypoxia are required as implemented for stem cell cultures (51).

In most cases, CTC were kept in short-term cultures, whereas our panel of SCLC CTC cell lines has now been cultivated from 1–4 years so far. These cell lines keep their characteristics in respect to morphology, growth rate and phenotype, SCLC markers, chemosensitivity and expression of proteins. The lines were established in 2D culture under normoxic conditions without enrichment since normal blood cells perish in cell culture and cancer cells are left. Hypoxic conditions and 3D culture seems not to be appropriate for cells having existed in the circulation but may be suitable for DTCs. The SCLC CTC panel does not express stem cell antigens or characteristics and all of these cells exhibit high levels of EpCAM (110). These SCLC CTC cells are unique in spontaneous generation of large tumorospheres containing resistant quiescent and hypoxic cells in their interior (*Figure 2*) (104). The permanent growth of these pure and tumorigenic SCLC CTC populations indicates their representation of stable metastasis-inducing cancer cell lines.

Extravasation

The molecular mechanisms that regulate extravasation are poorly characterized (111). Coumans *et al.* have developed a model for primary breast cancer to estimate the size of the tumor and corresponding CTC numbers before formation of the first metastases (14). The CTC number for breast cancer before surgery has been calculated as 0.03 CTC/mL (14). The CTC mean intravasation rate of a 8 mm tumor is 280 CTC/h · g tumor indicating a requirement of approximately 60 million CTCs for the establishment of a single metastases formed. Although a metastatic efficiency rate has not been finally determined in humans, a fraction

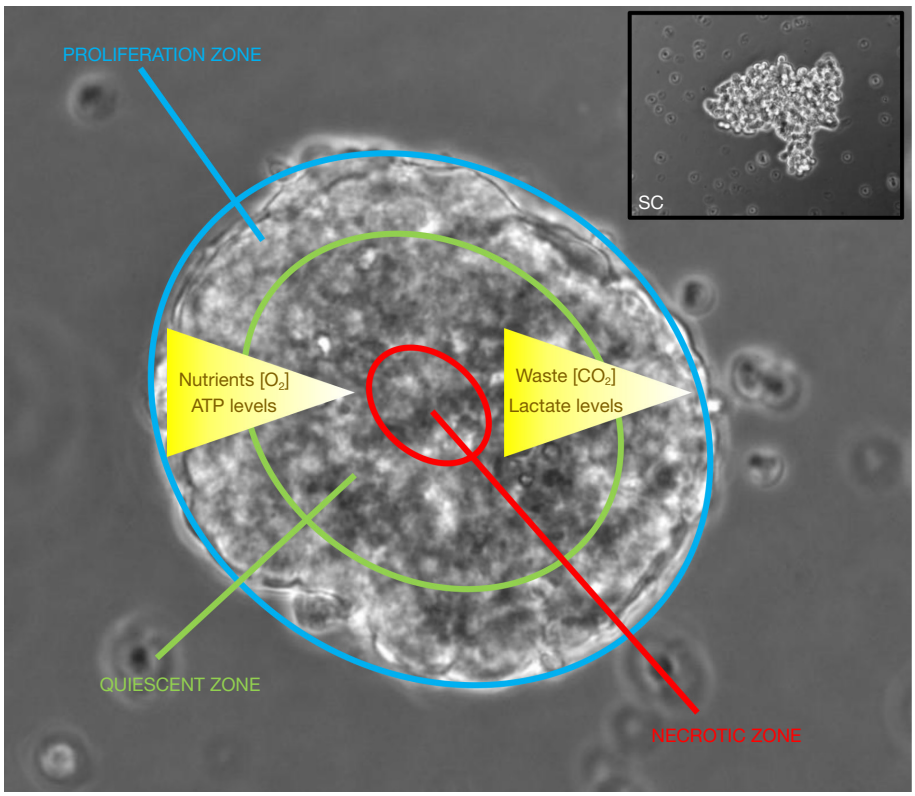

Figure 2 SCLC CTCs as single cells (SC; insert) or in form of a large cluster termed tumorosphere. The SCLC CTC lines assemble spontaneous into tumorospheres with diameters exceeding 500 µm. The gradient of nutrients, oxygen and ATP decrease from the periphery to the core, whereas cellular waste and lactate accumulates in the interior of the spheroid. Accordingly, the outer proliferative zone overlays a zone with quiescent cells and finally a necrotic core (104). This structure impedes the access of chemotherapeutics and the quiescent and hypoxic cells are resistant to cytotoxic drugs and irradiation, respectively. ATP, adenosine triphosphate; SCLC, small cell lung cancer; CTCs, circulating tumor cells.

of under 0.01% of the CTCs has been suggested to set secondary lesions, most likely due to cell death in the circulation and non-productive extravasation.

The extravasation of CTCs is a complex process, that is initiated with the adhesion of CTCs to the endothelium of blood vessel and progresses with transendothelial migration to establish secondary lesions in distant organs (*Figure 1*). CTCs may pass through the endothelial junctions forcefully one at a time (112). An alternative mechanism, angiopellosis, consists of active remodeling of vascular ECs to form a sheet around cancer cells and active expulsion of the CTCs out of the circulation (113). After becoming trapped in capillaries, CTCs first form weak adhesions to the endothelium, involving several pairs of cell ligands and receptors, comprising selectins, cadherins, integrins, and immunoglobulins (114). First contact of CTCs to the endothelium is mediated by neuronal cadherin (N-cadherin) that is expressed by both cancer cells and endothelial cells (115). In addition, the flow rate and shear force trigger remodeling of the ECs and promote extravasation (116). Tumor cells trapped in the pulmonary capillaries initially show luminal proliferation but intravascular colonies eventually rupture the capillary wall and allow for the invasion of the cancer cells into host tissue (117,118).

Detection of early metastatic disease and prognosis

Blood-borne dissemination of cancer cells seems to take place early in tumor development, which may commend CTCs as marker for early detection. CTCs are scarce in non-metastatic patients and, therefore, detection for CTCs at early stages is not sensitive enough, especially limited by the larger amounts of blood required (119). Whereas

few CTC-like cells were detected in healthy donors and for benign diseases a large range of CTC occurs in cancer patients (120). CTCs may be missed by sampling small blood volumes or released intermittently and avoid detection (98). The CTC detection cutoff in nonmetastatic patients has been set at ≥1 CTC/7.5 mL of blood for the CellSearch© system, in contrast to the cutoff in the metastatic setting with ≥5 CTC/7.5 mL of blood. The closest association between CTC count and tumor size is found for T4d inflammatory breast cancers. In a multicenter analysis of 3,173 patients with localized breast cancer using CellSearch© the percentage of patients with at least one CTC was 20%, while at least five CTCs were detected in half of the patients with metastatic disease (121).

For the CellSearch© system, the prognostic CTC limit for colon, breast and prostate cancer patients has been reported as 3–5 CTCs/7.5 mL blood suggesting a limited shedding of tumor cells (18). In case of COPD, detection of premalignant CTCs has been reported (122). However, the frequency of a malignant progression in COPD patients was approximately 1 in 200 patients and a large number of people need to be screened with considerable effort to detect a few high risk patients (123). Ilie *et al.* reported that among 168 COPD patients followed annually with spiral CT scans, all 5 positive patients developed lung cancer during follow-up (122). The application of "Isolation by Size of Epithelial Tumor Cells" (ISET) for selection of CTCs to differentiate benign from malignant lung lesions using a cutoff of 25 cells detected CTCs in 90% of patients with malignant and 5% of patients with benign disease (124,125). The ISET method proved insensitive to predict the subsequent development of cancers, as only 2 of the 13 lung cancers and 0 of the 13 extrapulmonary cancers found within 2 years had positive CTC counts. So far, the search for CTCs has not been demonstrated to be useful in cancer screening. The pilot study in patients with COPD generated public attention, but the results of the later validation study yielded negative results (122,126).

Clearly, the CTCs circulating in cancer patients differ from the bulk of primary tumor cells and as such may represent a poor surrogate population assessing response to therapy (127). In 35% of colorectal cancer cases, metastases shared common traits with the original tumors, whereas 65% were derived from independent subclones (128). The SCLC CTCs proved to be chemosensitive in form of single cells but the tumors which have relapsed after primary treatment exhibit a general drug resistance (129,130). Therefore, successful reduction of the CTCs correlates not necessarily with response of the resident lesions. Since metastases are formed by less than 0.01% of the CTCs, single-cell analysis to identify the true metastasis-inducing subpopulation will be demanding. In addition, processes directing cell adhesion and protein-protein interaction for physical manners of resistance may be difficult to be identified at the level of the transcriptome. One solution may be the *in vitro* expansion of viable CTCs and their genetic and phenotypic characterization (104).

CTCs are also reported to be detectable in patients with early-stage cancers and may have prognostic significance (131-133). One or more CTCs are found in 20% of node-negative and 24% of stage 1–3 breast cancer patients (48,134,135). Baseline CTC counts were not correlated with tumor size for different cancer types and a proliferation index from 1 to 80% of CTCs has been described by Ki67 staining (136). CTCs numbers were reported to possess prognostic significance in several tumor types (18,19). Assessment of CTCs suggests a correlation of high CTC counts with shorter disease-free survival and a poorer prognosis as well as a response marker to chemotherapy upon a drop in CTC counts (19,24,69,119). Correlation of high CTC counts with a poor prognosis has been described for various types of cancers, including colorectal, breast, lung and pancreatic cancer (12,24). The presence of ≥3 CTCs per 7.5 mL of peripheral blood indicated a shorter of progression-free survival (PFS) (137). Hou *et al.* found that patients with SCLC and a CTC count of ≥50 CTCs/7.5 mL prior to chemotherapy had a poorer clinical outcome in terms of OS than those with a CTC count <50 CTC/7.5 mL (HR =2.5) (33). A European multicenter study in 550 patients with NSCLC reported ≥2 CTCs in 27% patients and 13% with ≥5 per 7.5 mL of blood using the CellSearch© system (85). These CTC counts were associated with reduced PFS (≥2 CTCs: HR =1.7, ≥5 CTCs: HR =2.2) and OS (≥2 CTCs: HR =2.2, ≥5 CTCs: HR =2.8), in good agreement with other studies (138,139).

CTCs counts may constitute a surrogate marker of the response to chemotherapy showing a rapid decline in CTC counts with up to a 50% reduction (140). In a meta-analysis comprising 2,239 breast cancer patients, the CTC count before neoadjuvant chemotherapy had a negative impact on patient survival, as patients with one to five or more CTCs hold a HR of death of 1.1–6.25, respectively (141). However, the role of CTC counts in diagnostic and treatment guidance remain to be defined (142). For instance, in metastatic breast cancer,

clinical practice guidelines do not recommend currently the use of CTCs for the selection of treatments (143).

Angiogenesis and CTCs

Several mouse models of tumorigenesis revealed an angiogenic switch that is initiated during the early stages of tumor development, indicating that the regulation of angiogenesis is a potentially rate-limiting step in the development of many solid tumors (35). Tumor vessels formed in response to upregulation of VEGF are fenestrated and leaky as well as accompanied by a disorganized or loose basement membrane (144). These conditions typically lead to high interstitial pressures, escalated tissue hypoxia and further release of additional VEGF. The endothelial fenestrations formed are of dimensions ranging from 200 to 2,000 nm (145). The efficacy of the drug delivery into cancer cells is mostly dependent on angiogenesis. All these changes promote the enhanced permeability and retention effect (EPR) effect of the cancer cells (146).

Tumor neoangiogenesis forms immature blood vessels that facilitate the intravasation of CTCs (147). Shed tumor cells built cell aggregates in blood vessels due to intravascular proliferation (148). The ability for intravasation of mammary carcinoma cells showed no correlation with the size of the primary tumor (68). Generally, the microvessel count of tumors and density grade correlate with distant metastases. In breast cancer, a prognostic indicator for survival is the degree of angiogenesis (149,150). Breast cancer cells exhibiting increased levels of angiogenic growth factors show higher aggressiveness and an elevated risk of the invasive breast cancer (35). The microvessel density (MVD) of surgical samples of invasive breast cancer may be a predictor of metastasis or relapse. Assessment of the microvessel densities of breast carcinomas of 49 patients (30 with metastases and 19 without) demonstrated that for each increase of 10 microvessels a 1.17-fold increase in the risk of distant metastasis has been observed (150).

VEGF is the most important angiogenesis-provoking cytokines and is overexpressed in solid cancers. In metastatic breast cancer, patients with serum-VEGF levels (sVEGF) ≥367 pg/mL and ≥5 CTCs had the shortest overall survival (OS) compared to patients with lower sVEGF and non-elevated CTCs (151). VEGF-B promotes metastasis through the remodeling of tumor vessels to pseudo-normalized vasculatures of high leakiness and knockdown of this mediator resulted in increased perivascular cell coverage and reduced pulmonary metastasis of human melanomas (152). Despite retarded growth of primary tumors, VEGF-B markedly promotes metastasis. High levels of VEGF-B in patients with lung squamous cell carcinoma and melanoma correlated with poor survival (152).

Intravasation requires interactions between tumor cells, perivascular cells and possibly inflammatory cells (153,154). Microvessels are barriers for the invasion and tumor cells secrete mediators that restructure the vessels to become more permissive for intravasation through the reduction of perivascular cell coverage of the vessels. VEGF-B promotes cancer metastasis at the level of intravasation animals bearing VEGF-B-positive tumors have high CTCs counts (152). VEGF-B–induced cancer cell intravasation is accompanied by tissue hypoxia and attraction of inflammatory macrophages. Our group has shown that SCLC CTCs recruit macrophages (155). Similarly, angiopoietin-2 increases vascular permeability by damaging pericytes through the induction of superoxide. Analysis of intravasation in distinct areas of primary tumors demonstrated that the majority (>90%) of tumor cell shedding events were localized to the interior angiogenic core depending on damaged vessels (90).

SCLC and CTCs

SCLC is a neuroendocrine tumor that has a 2-year survival rate below 10% due to metastatic spread in most patients at first presentation (156). SCLC shows high sensitivity to first-line chemotherapy consisting of platinum drugs/etoposide regimens but relapses within approximately one year and responds poorly to further treatment. This tumor is distinguished by extreme numbers of CTCs with a mean count of 400 CTCs/7.5 mL blood and peak values of up to several thousand cells/7.5 mL blood (8). For SCLC at advanced metastatic state, a CTC count of 1,589±5,565 (mean ± SD) in 7.5 mL of blood was also published (157).

High blood perfusion of the lung, invasive growth of the tumors and inflammation characterized by the recruitment of macrophages seems to contribute to the shedding of CTCs (158). The SCLC CTCs are small (approximately 8 μm) compared to other tumors and, thus, can more easily migrate through capillaries and recirculate. The excessive numbers of CTCs in advanced SCLC patients are expected to include a number of actual metastasis-inducing cells and, therefore, we could establish a panel of 8 permanent SCLC CTC *in vitro* (110,159). The cancer stem cell hypothesis assumes that a subpopulation of rare stem cells survive

the initial chemotherapy and are responsible for tumor recurrences after elimination of the bulk tumor tissue (160). However, SCLC CTCs lack stem cell markers, such as CD133, CD44, ABCG2 and the expression of vimentin causing a hybrid EMT phenotype indicates no real cell-type transformation (83,161). The *in vitro* expanded SCLC CTCs form spontaneously large multicellular aggregates, termed tumorospheres, that contain quiescent and hypoxic cells in the inner layers and are much more chemoresistant compared to the same cells in form of single cell suspensions (104). SCLC tumors are typically found in smokers with high tobacco consumption and the cancer cells exhibit numerous mutations (162). In this context, the controlled transformation to a mesenchymal phenotype and its reversal following extravasation is an unlikely mechanism (73). Our results with the SCLC CTCs cell lines have shown the production of angiogenic factors, like CHI3L1 and VEGF and proteolytic enzymes (MMPs, cathepsins) that enables them to disseminate without mesenchymal switch (163). In case of SCLC CTCs, small cell aggregates may become trapped in capillaries, especially of liver and brain, and extravasate by physical pressure force upon reaching larger sizes (164).

SCLC patients (37/38) have rare CTC subpopulations co-expressing vascular endothelial-cadherin (VE-cadherin) and cytokeratins consistent with vasculogenic mimicry (VM), a process whereby tumor cells form endothelial-like vessels (165). Higher levels of VM in limited stage SCLC patients were reported to be associated with a poorer overall survival possibly due to increased access of CTCs to the circulation. However, using VE-cadherin as a VM biomarker its expression in SCLC cytokeratin (CK)-positive CTCs was assessed using the ISET method that may enrich SCLC cells with less specificity.

NSCLC and CTCs

Among lung cancer patients, NSCLC blood samples enclose very low numbers of CTCs in contrast to SCLCs. In a study of Li *et al.* blood samples of NSCLC patients were obtained on the first day of treatment and during chemotherapy or targeted therapy cycles for CTCs detection. Of 100 patients, 48 were identified to be CTC-positive at baseline defined as one or more CTCs per 7.5 mL (166). A higher CTC-positive rate was found for stage IV NSCLC patients compared to stage III patients (69% *vs.* 40%). CTC clusters, defined as aggregates with ≥2 CTCs, were significantly correlated with disease control rate. Patients were divided into those with low (<4 CTCs, LL) and high CTC counts (≥4 CTCs, HL) yielding significant shorter OS and PFS in high count group. A number of CTC ≥50 were detected using ISET in pre-surgery blood samples from 102/208 (49%) patients undergoing curative tumor removal for NSCLC with significantly shorter DFS and OS (167). The large number of "CTCs" in this case was associated with the ISET technique that isolates more cells than the CellSearch© system but of unclear significance. In a study by Ichimura *et al.* patients undergoing planned surgery for lung cancer were recruited (168). Samples were collected at recruitment, before treatment and approximately 3 months later. CTC numbers counted at recruitment were 1.4±0.4, 1.8±1.2, 1.3±0.6 and 7.4±5.1 (mean ± SE) in clinical stages I, II, III and IV, respectively. At baseline, 51 (62.96%) patients were positive for high definition (HD)-CTCs with a median of 2.20 (mean 26.21±15.64) (169). The baseline CTC count exhibited no correlation with patient characteristics. In a review by Qian *et al.* cutoff values for prognosis of 10 studies were 5 CTCs for CellSearch© and 15–25 for other methods (170). Similarly, Kejik *et al.* cited limits of 5, 5, 1-2 and 8 CTCs for a total of more than 200 patients (171). The CTC count was approximately twice as high for NSCLC patients as for patients with benign lung diseases carrying nonmalignant "CTCs" (pneumonia, pulmonary tuberculosis, bronchiectasis, or pneumothorax) or healthy subjects (172). However, due to a number of highly sensitive techniques to detect CTCs, a few CTCs appearing sporadically in the circulation of NSCLC patients proved obviously sufficient to infer prognostic and predictive statements. However, despite of 15 years of research on CTCs in NSCLC this test did not enter into routine clinical guidance for the choice of therapeutic options.

Conclusions

Despite a wide range of techniques available to count and identify CTCs their clinical applications in diagnosis and guidance for therapy are still limited. In principle, the various methods to enrich and detect CTCs are tedious and time-consuming and in conjunction with the lack of a standardization of the counts and characteristics of these cells show limited potential in the clinical setting. Absence of CTCs even in metastatic patients, detection in late phases of the tumor development after dissemination has occurred and poor resemblance of the bulk tumors in assessment of response and prognosis put the costs, effort and manual input into question. Especially, the use of CTCs for tumor

screening and early detection seems not possible due to few positive results of debatable significance. Furthermore, patients with zero CTC detected at a given time point may have been registered as false negative cases (173). Repeated blood draws to clarify the temporal distribution of CTCs in patients are not tolerable for the patients. However, in contrast to studies on circulating nucleic acids, exosomes, or other blood biomarkers, the analysis of CTCs can define their cellular genetics, epigenetics, transcriptomics, and protein profile. However, due to the host of non-validated methods and increased work load, the use of CTCs numbers as prognostic and predictive biomarkers lags behind the utilization of cell-free DNA (cf-DNA) in plasma in daily practice of oncologists (157,174).

Research on CTCs is hindered by the rarity and heterogeneity of these cancer cells and the low number of short-or long-term cultured specimen. Therefore, assignation of their biologic significance and their participation in all steps of dissemination is unclear and poorly characterized. CTCs appear early in tumor neoangiogenesis years before the primary tumor or metastases are detected clinically in patients by symptoms, biomarkers and imaging. In respect to the intravasation of cancer cells into the circulation, transformation to a mesenchymal phenotype (EMT) seems to be dispensable as tumor cells can easily pass into leaky newformed vessels in cores already at small tumor sizes. Accordingly, the inverse process of EMT, namely MET, for extravasation is not required (73). The assumption that CTCs are representative of the bulk tumors is questionable. For example, in metastatic SCLC the CTCs are chemosensitive but responses of the bulk tumor to chemotherapy are poor leading ultimately to a dismal prognosis (104). Of course, CTCs are easily accessible to high concentrations of chemotherapeutics in the circulation, in contrast to poorly vascularized tumors or spontaneously formed tumorospheres. Ultimately, the CTC count seems to be linked to the prognosis of tumor patients via the extent of neoangiogenesis.

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