Functional Studies on Viable Circulating Tumor Cells

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BACKGROUND: Research on circulating tumor cells (CTCs) as new biomarkers has received great attention over the past decade. In particular, the capture and analysis of CTCs as “liquid biopsies” provides the possibility to avoid invasive tissue biopsies, with obvious implications in cancer diagnostics.

CONTENT: The focus of this review is to describe and discuss how functional studies on viable CTCs can enlarge the spectrum of applications of liquid biopsies, with emphasis on breast, prostate, colon, and lung cancer as the major tumor entities in industrialized countries. The low number of CTCs in the peripheral blood of most cancer patients makes challenging the in vitro culture of CTCs. Epithelial tumor cells are difficult to culture, even when starting with millions of tumor cells. Recently, several groups have achieved important advances in the in vitro and in vivo expansion of CTCs from cancer patients at very advanced stages with higher amounts of CTCs. Here, we present current technologies to enrich and detect viable human CTCs, including positive and negative enrichment strategies that are based on antigen expression and physical properties of CTCs. We also discuss published data about functional studies on CTCs that use in vitro and in vivo models.

SUMMARY: Functional analyses on CTCs offer the possibility to identify the biological properties of metastatic cells, including the identification of metastasis-initiating cells. Moreover, CTC-derived cell lines and xenografts might reveal new therapeutic targets and can be used for drug screening.

Over the past decade, numerous studies have shown that circulating tumor cell (CTC)4 counts have prognostic value in various tumor entities (1, 2). Most studies have dealt with the most frequent epithelial tumors such as breast, prostate, or lung cancer; these tumor types are also the focus of this review. Five years ago, we introduced the capture and analysis of CTCs as “liquid biopsies” (3), and this concept has received tremendous attention. The possibility to avoid invasive tissue biopsies and obtain similar or even increased information by a simple blood test has enormous implications in cancer diagnostics.

Despite the development of numerous new CTC capture devices (1) and a wealth of clinical studies demonstrating strong correlations between CTC counts and clinical outcomes in large multicenter cohort studies with defined therapies (4–6), the information on the functional properties of CTCs is still very limited, because CTCs occur at very low concentrations in the peripheral blood of cancer patients (1). A prerequisite for functional analyses was therefore the recent advances in our ability to culture CTCs in vitro or expand the CTC pool in vivo by use of xenografts.

Here, we describe how to enrich and detect viable human CTCs and discuss functional studies by use of in vitro and in vivo models. Besides unraveling the biology of metastasis in cancer patients, these studies can provide important clinical information in the future, including the testing of antimetastatic drugs for personalized treatment of cancer patients.

Strategies for Enrichment and Detection of Viable CTCs

An enrichment step that increases the concentration of CTCs by several logs enables easier detection of single CTCs. To keep tumor cells in a viable state during the enrichment and detection of functional CTCs requires (a) performing the blood draw in tubes without fixatives, (b) rapid (<24 h) shipment of the blood samples to the laboratory, (c) rapid isolation and detection of CTCs without any cell fixation or permeabilization, (d) under appropriate culture conditions. We will discuss the potential and limitations of current CTC assays in their ability to capture viable CTCs for subsequent analyses.
ENRICHMENT OF LIVE CTCs

Protein expression–based technologies. Cell surface proteins could be used for an antibody-based enrichment step to attach CTCs [positive selection by use of the epithelial cell adhesion molecule (EpCAM), the cell surface marker that is most frequently used for positive enrichment of epithelial CTCs]. In light of the capacity of tumor cells to undergo epithelial-to-mesenchymal transition, the capture of CTCs may not rely solely on EpCAM expression, but may require additional targets [e.g., epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1)] (7, 8). Obviously, intracellular proteins cannot be used, as the cells must remain intact. Different technologies have been used for positive selection, including immunomagnetic separation or flow cytometry–based cell sorting (7). If immunomagnetic separation is used, it is important that the beads do not impair cell viability. The size of the beads may also be a limitation if flow cell sorting follows immunoseparation. High-speed flow sorting is required to achieve rapid isolation of CTCs and avoid loss of cell viability.

The drawback of positive selection is that, to select the appropriate antibodies, an assumption has to be made about cell surface expression of capture antigens. Tumor heterogeneity is a hallmark of cancer; thus, it is very difficult, if not impossible, to predict the spectrum of suitable capture antigens expressed on CTCs in an individual patient. To circumvent this problem, it might be attractive to use the opposite strategy: depletion of leukocytes by negative selection, by use of CD45, which is expressed on healthy hematopoietic cells but not on carcinomas or other solid tumors. Negative selection usually results in a lower purity of CTCs than positive selection, but this is not a serious drawback for subsequent functional studies, because the contaminating leukocytes usually do not disturb either cell cultures or xenograft assays. Examples of negative selection technologies are the immunomagnetic depletion of leukocytes or the use of tetrameric complexes of antibodies that react with erythrocytes and leukocytes, resulting in rosettes that are removed by Ficoll gradient centrifugation (9–11).

Physical property–based technologies. Another way to enrich CTCs is to sort them by physical properties that are supposed to be different from those of leukocytes: indeed, tumor cells were initially thought to be bigger and less deformable than leukocytes. However, recent CTC studies indicate that small CTCs exist and might even contribute to metastatic progression (12).

Zheng et al. reported on a novel 3-dimensional (3D) microfilter device that can enrich viable CTCs (13). This device consists of 2 layers of Parylene membrane with pores and a gap precisely defined with photolithography. The positions of the pores are shifted between the top and bottom membranes. The bottom membrane supports captured cells, minimizes the stress concentration on the cell membrane, and sustains cell viability during filtration. Viable cell capture of this device was investigated with scanning electron microscopy, confocal microscopy, and immunofluorescent staining by use of model systems of cultured tumor cells spiked in blood or saline.

More recently, Zhou et al. presented a separable bi-layer (SB) microfilter for viable size-based CTC capture (14). Unlike other single-layer CTC microfilters, the precise gap between the 2 layers and the architecture of pore alignment resulted in a drastic reduction in mechanical stress on CTCs. With multiple cancer cell lines spiked in healthy donor blood, the SB microfilter demonstrated high capture efficiency (78%–83%), high retention of cell viability (71%–74%), high tumor cell enrichment (1.7–2 × 10³), and widespread ability to establish cultures postcapture (100% of cell lines tested). In a metastatic mouse model, SB microfilters successfully enriched viable mouse CTCs from 0.4–0.6 mL whole blood and allowed the establishment of in vitro cultures for further genetic and functional analysis.

Harouaka et al. designed a flexible microspring array device for the enrichment of viable CTCs, independent of antigen expression (15). Unlike previous microfiltration devices, flexible structures at the microscale minimize cell damage, to preserve viability while maximizing throughput and allow rapid enrichment directly from whole blood with no need for sample preprocessing. CTCs and CTC microclusters were enriched from clinical samples obtained from breast, lung, and colorectal cancer patients. The device enriched tumor cells with 90% capture efficiency, >10⁴ times enrichment, and >80% viability from 7.5-mL whole blood samples in <10 min on a 0.5-cm² device.

Independent of the enrichment methods, for subsequent functional assays, it is vital that cells are neither fixed nor permeabilized and that they can be removed easily from the device without any damage.

CTC function–based technologies. Functional properties of CTCs can be also used for their enrichment. The first assay was described approximately 10 years ago and is based on the ability of tumor cells to invade a collagen matrix in vitro. This assay has been used for enumeration and molecular characterization of CTCs in ovarian and prostate cancer (16, 17), but to our knowledge, its efficiency to culture CTCs remains to be demonstrated.

More recently, King and colleagues explored the mechanisms by which tumor cells adhere to blood vessels for the capture of CTCs (18, 19). They developed microscale flow devices that consist of a surface functionalized with E-selectin glycoprotein in addition to antibodies against epithelial markers. This device has been
successfully used to capture cancer cells from the blood of metastatic cancer patients. Captured cells are maintained for ≤15 days in culture after isolation. The same group described immobilized surfactant–nanotube complexes that support selectin-mediated capture of viable CTCs in the absence of EpCAM capture antibodies (20), which allows EpCAM-independent capture of CTCs that have undergone epithelial-to-mesenchymal transition.

DETECTION OF VIABLE CTCs
Numerous studies have indicated that cancer patients can harbor both apoptotic and viable CTCs (1). Because only viable CTCs can contribute to metastatic progression, it is important to identify this subset of CTCs. One approach is to introduce markers of apoptosis (M30−Bcl-2−) into immunologic CTC assays such as the CellSearch system (21, 22). Surprisingly, in patients with high numbers of CTCs, higher rates of CTC apoptosis were associated with worse prognosis, whereas higher CTC Bcl-2 concentrations correlated with better outcomes (22). Thus, the clinical relevance of introducing apoptotic markers into CTC assays is still under investigation.

An alternative approach is to use a functional assay of CTC viability. To our knowledge, the only existing functional test that has been used on hundreds of patients with different tumor types is EPISPOT, which allows the detection of viable clinically relevant CTCs enriched by leukocyte depletion (23, 24). This technology enables the detection of EpCAM+ and EpCAM− CTCs. Cells are cultured for a short time on a membrane coated with antibodies that capture the secreted/released/shed tumor-associated proteins, which are subsequently detected by secondary antibodies labeled with fluorochromes. For breast cancer, cytokeratin 19 (CK19), HER2, cathepsin D, and MUC-1 have been used as marker proteins, and the associated clinical data have shown that patients with CK19-releasing cells had an unfavorable outcome (24, 25). For prostate cancer, prostate-specific antigen has been used for CTC detection and fibroblast growth factor 2 as stem cell growth factor for further characterization (26). For colon cancer, CK19 has been used for CTC detection (23), and our analysis showed that a considerable portion of viable CTCs detectable by EPISPOT are trapped in the liver as the first filter organ in colon cancer patients. Our clinical data demonstrated that patients with localized colon cancer and high numbers of CTCs have an unfavorable outcome. By adding drugs in the EPISPOT assay, the number and intensity of the immunospots can be decreased or inhibited when tumor cells are sensitive to these drugs, demonstrating their efficiency during short-time CTC culture (27). This assay might be then considered an “oncogram” and might help to improve the clinical management of individual cancer patients.

In Vitro Culture of CTCs: Establishment of Cell Lines

The low number of CTCs in the peripheral blood of most cancer patients makes challenging the in vitro culture of CTCs. For decades, it has been challenging to develop primary cell cultures or cell lines from primary tumors or metastases of carcinoma patients, even when starting with millions of tumor cells. Recently, several groups have developed appropriate culture conditions for CTCs obtained from cancer patients at very advanced stages with higher numbers of CTCs. Next, we discuss these developments.

COLON CANCER
Cayrefourcq et al. have provided the experimental proof that CTCs isolated from the blood of a colon cancer patient are able to give rise to a permanent cell line (9). To our knowledge, no other group has published the establishment of a permanent cell CTC line or even transient CTC cultures from patients with colon cancer. One reason is that the frequency of CTCs is lower in peripheral blood of colon cancer patients compared with breast or prostate cancer patients, making it even more difficult to find and grow CTCs in colon cancer. Besides the establishment of a permanent cell line, we have also demonstrated that this cell line has tumorigenic properties in immunodeficient mice.

Besides its capacity to expand ex vivo for >2 years, the CTC-MCC-41 line showed (a) epithelial properties with stem cell–like characteristics, (b) intermediate epithelial/mesenchymal phenotype, (c) potential to quickly induce in vitro angiogenesis, (d) osteomimetic signature, and (e) tumorigenic properties in SCID mice. Importantly, this CTC line shares the main features of the original primary tumor and lymph node metastasis of the colon cancer patient.

PROSTATE CANCER
Prostate cancer is very difficult to expand in cell culture, as documented by the few cell lines available in biobanks. Gao et al. used a 3D organoid system to develop long-term culture of prostate cancer from biopsy specimens (28). The first 7 fully characterized organoid lines recapitulate the molecular diversity of prostate cancer subtypes, including TMPRSS2-ERG fusion, SPOP mutation, SPINK1 overexpression, and CHD1 loss. The authors established a CTC line from a metastatic prostate cancer patient; the report did not provide any information on the success rate of establishing the CTC line, such as how many times the culture conditions failed to yield primary CTC cultures or a cell line. Moreover, the authors have not yet demonstrated the tumorigenic properties of their cell line in a xenograft model.
BREAST CANCER
Zhang et al. reported for the first time the establishment of primary cultures from CTCs of breast cancer patients who were in advanced stage and presented with brain metastases (7). However, the CTCs did not yield permanent cell lines but only transient cell cultures. In EpCAM− CTCs, Zhang et al. identified a potential signature of brain metastasis comprising “brain metastasis selected markers” (BMSMs), HER2+/EGFR+/HPSE+/Notch1+. CTC lines expressing the BMSM signature were highly invasive and capable of generating brain and lung metastases when xenografted in nude mice.

The next publication on breast cancer CTC cultures reported on oligoclonal CTC cultures (sustained in vitro for >6 months) from CTCs isolated from 6 patients with metastatic luminal-subtype breast cancer (29). Three of 5 CTC lines tested were tumorigenic in mice. Genome sequencing of the CTC lines revealed preexisting mutations in PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α) and newly acquired mutations in ESR1 (estrogen receptor 1), PIK3CA, and FGFR2 (fibroblast growth factor receptor 2), among others. Drug sensitivity testing of CTC lines with multiple mutations revealed potential new therapeutic targets.

LUNG CANCER
Zhang et al. developed a novel in situ capture and culture methodology for ex vivo expansion of CTCs with a 3D coculture model, simulating a tumor microenvironment to support tumor development (30). They expanded CTCs isolated from 14 of 19 early-stage lung cancer patients. Expanded lung CTCs carried mutations of TP53 (tumor protein p53) identical to those observed in the matched primary tumors. Next-generation sequencing revealed additional matched mutations between primary tumor and CTCs of cancer-related genes.

In Vivo Expansion of CTCs: Xenografts from Freshly Isolated CTCs
In this section, we discuss the studies in which no cell cultures or cell lines were established, but enriched CTC fractions of blood from patients with breast, prostate, and lung cancer were directly injected into immunodeficient mice. Cell culture before xenografting might affect the composition of CTCs; however, so far no direct comparisons between xenografts developed from freshly isolated vs cultured CTCs have been reported.

BREAST CANCER
Baccelli et al. developed a xenograft assay and used it to show that primary human luminal breast cancer CTCs contain cells that give rise to bone, lung, and liver metastases in mice (11). These CTC subsets expressed EpCAM, CD44, CD47, and MET. In a small cohort of patients with metastases, the number of EpCAM+ CD44+CD47+MET+ CTCs, but not all EpCAM+ CTCs, correlated with lower overall survival and increased number of metastatic sites. This report might imply a special subset of CTCs with potential metastasis-initiating activity; however, it should be noted that successful xenografting was observed only in advanced-stage patients with high CTC counts. Indeed, the injection of <1000 CellSearch-evaluated CTCs did not lead to metastatic growth of human tumor cells within 15 months after transplantation (n = 106 patients). In contrast, 6 recipient mice receiving ≥1109 CTCs developed multiple bone, lung, and liver metastases within 6–12 months after transplantation of CTCs from 3 patients.

In a small pilot study on 2 breast cancer patients, Rossi et al. confirmed that CTCs have the potential to grow in immunodeficient (NOD/SCID) mice (31). In contrast to Baccelli et al. (11), who injected CTCs into the tibia, Rossi et al. injected CTCs subcutaneously. Thus, CTCs sustained their migratory capacity, and both routes of injection seem to work for the establishment of CTC xenografts in breast cancer.

LUNG CANCER
Most cases of small cell lung cancer (SCLC) are inoperable, and biopsies to investigate SCLC biology are rarely obtainable. Previous studies have shown that SCLC patients have the highest CTC counts among all solid tumor patients, providing the best conditions for developing functional models (32). Hodgkinson et al. showed that CTCs from patients with either chemoresistant or chemorefractory SCLC are tumorigenic in immunocompromised mice, and the resulting CTC-derived explants (CDXs) mirrored the donor patient’s response to platinum and etoposide chemotherapy (10). Genomic analysis of isolated CTCs revealed considerable similarity to the corresponding CDX. Most marked differences were observed between CDXs from patients with different clinical outcomes.

PROSTATE CANCER
Rossi et al. isolated EpCAM+ CTCs from metastatic prostate cancer patients (n = 6), and the xenograft assay was developed in NOD/SCID mice (31). Human tumor cells were found in murine peripheral blood, bone marrow, and spleen, which demonstrated that the EpCAM-positive fraction of CTCs retains migratory capacity.
Conclusions

Recent advances in cell culture technologies have opened a new avenue for CTC research, with obvious clinical implications. It is now possible to develop primary cell cultures from CTCs, and in some instances, even permanent cell lines have been established. In addition to in vitro studies, several groups have been able to graft tumors after injection into immunodeficient mice, and the first drug testing analyses suggest that these models might be useful predictors of the response of cancer patients (Fig. 1) (33).

It needs to be emphasized that functional analyses of CTCs are still at an early proof-of-principle stage. Most reports are based on patients with advanced metastatic stages and high CTC counts. Nevertheless, the development of cell lines or xenografts from hundreds of CTCs at the beginning is a major achievement, considering the enormous challenge to establish cell lines from even millions of primary tumor cells. This can be explained by the assumption that viable CTCs might already be selected for better survival and growth properties, which is consistent with published reports demonstrating that tumor cell dissemination and survival of disseminated tumor cells and CTCs is not a random process (34, 35) and that CTC cell lines and xenografts express cancer stem cell properties (9, 11). These studies nourish the hope that in-depth functional analysis of CTCs might lead to the identification of metastasis-initiator cells. However, to achieve this important goal, it is crucial to extend the current studies to patients at earlier stages and correlate the findings with the development of metastatic relapse.

Recently, the liquid biopsy concept has been extended to circulating cell-free tumor DNA (ctDNA)
The capture of viable CTCs from millions of blood cells is certainly more time consuming than the harvest of cell-free circulating DNA from blood plasma or serum samples. However, functional analyses of whole tumor cells offer the possibility to identify the biological properties of metastatic cells through in-depth in vitro and vivo analyses, which opens new avenues for basic and translational research that are far beyond sequencing of fragmented DNA present in blood plasma.

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