Circulating Tumor Cell Cultures Under Pressure

In the fall of 1868, Dr. Thomas Ashworth was peering through a microscope to examine a blood sample from a deceased cancer patient when he came across a collection of cells that closely resembled tumor cells. Further study led him to conclude that “cells identical with those of the cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumours existing in the same person.” Many years later, researchers are still in hot pursuit of these tumor cells found in the blood of cancer patients, referred to in the scientific community as circulating tumor cells (CTCs). The prospect of being able to monitor and molecularly characterize a patient’s cancers through simple blood draws — less invasively and far more routinely than is possible with conventional surgical methods — remain elusive, even with significant advances in our understanding of CTC biology.

Research into CTCs has been a challenging reminder that sometimes the more we learn, the less we know. Relevant cells taken from a blood sample might number in the single digits, pushing the resolution boundaries of many molecular analytical tools. It is widely accepted that cancer is remarkably
heterogeneous, with different expression profiles found even in samples taken from different areas of a tumor. With CTCs, that issue is amplified exponentially: not only are there very few cells to analyze, but they may also have very different genetic fingerprints, and scientists must bear in mind that their origin is not clear. They may have been shed from a known tumor, a metastatic site, or even from ‘dying’ tumors that are sensitive to chemotherapy. Trying to glean information from these rare cells and then draw useful conclusions from them has been much less successful to date than anyone had hoped.

One major problem with CTC research has been the inability to reproduce findings. While this is frustrating, it shouldn’t be all that surprising: how can we expect a detailed analysis of 10 cells taken at random from a patient to give the exact same results as 10 other cells taken from another patient? Here, we review this and other challenges limiting progress in CTC research. We also discuss a new cell culturing platform that has shown potential for growing CTCs ex vivo into populations large enough to meet thresholds for molecular analysis.

The CTC Challenge

Beyond the disappointment of not being able to reproduce many findings reported in peer-reviewed CTC publications, there are many hurdles when it comes to working with these cells that could lead to inaccurate or inconclusive results.

A major problem area for working with CTCs lies in identification. Existing methods leave room for incorrectly characterizing cells as CTCs or passing over real CTCs due to limitations associated with assay sensitivity and an incomplete understanding of CTC biology. Limitations aside, the scientific consensus for recognizing a cell as a CTC demand that cells must have an intact nucleus, be positive for EpCAM and cytokeratins, and be negative for CD45, as first defined by the only FDA-approved CTC platform on the market, CellSearch from Janssen Diagnostics. That profile, however, also matches some non-cancerous epithelial cells. Recent evidence suggests that genuine CTCs can be negative for EpCAM or cytokeratins, such as aggressive subtypes that have undergone an epithelial-to-mesenchymal transition process in which EpCAM expression is lost (Thurm et al., Clin Cancer Res, 2003).

For more accurate results, some scientists now hold the view that CTC identity should be confirmed using only genomic or transcriptomic approaches to confirm that the cells in question harbor an identifying signature associated
with the primary or metastatic tumor. However, sequencing-based methods require a minimum of several hundred to thousands of cells to make accurate variant calls that can tie CTCs back to their parent tumors. When CTC numbers from peripheral blood samples are in the single digits, these types of molecular analysis have limited utility. Recent advances in single-cell isolation and sequencing technologies hold promise for addressing sample-size limitations but remain technically challenging, often requiring specialized microfluidics and genome amplification strategies that limit broad appeal and adoption among traditional cell biology labs.

Furthermore, many enrichment technologies are likely to damage the cells during processing. Microfluidic platforms and fluorescence-activated cell sorting, for instance, can inadvertently lyse cell and nuclear membranes during handling, resulting in only snippets of genomic material available for analysis. Other commonly used research tools for CTC enumeration have long processing times or workflows that are not optimized for cell handling and downstream molecular analysis. Considering these limitations, researchers have attempted to culture and expand viable CTCs in vitro to enable downstream molecular and functional analysis.

Once in culture, CTCs have been quite difficult to grow in the lab. For example, while some scientists have reported growing CTCs as organoids with cell doubling times of a few days, researchers who have attempted to independently validate those reports have found that doubling times usually take weeks or even months (Yu et al., Science, 2014). Perhaps because these cells come from such heterogeneous sources, many scientists have realized that CTC growth rates and colony formation rates are anything but uniform. Variations are seen based on the sample type, specific disease, stage of disease, cell origin, and patient physiology. Indeed, CTC growth rates may be as variable as cancer itself.

CTCs must also be collected and processed very quickly from blood samples for any chance of successfully growing them in vitro. Internal studies at Xcell Biosciences have shown that CTCs cannot be cultured more than four hours after a blood sample was taken, largely due to low cell viability of nucleated cells. For a biomedical research community accustomed to shipping frozen blood samples overnight, this is a serious limitation.
A New Method for CTC Culture

The Avatar system from Xcell Biosciences was originally designed to maintain and propagate human stem cells under culturing conditions that replicate the stem cell niche. The system was modified to cultivate a variety of primary human tumor cells, including CTCs, by replicating conditions found within the tumor microenvironment. The Avatar system incorporates a unique bioreactor that regulates settings for oxygen and atmospheric pressure levels, in addition to the usual temperature and carbon dioxide controls. Complementing the novel bioreactor are serum-free, chemically defined culture media composed of recombinant human growth factors, and a CTC-capture substrate composed of a collagen-based hydrogel.

The workflow was developed in response to mounting evidence that hypoxia and pressure are influential traits in the cellular microenvironment that contribute to the maintenance and propagation of tumor cells. When complemented with an optimal cocktail of growth factors and a collagen-rich substrate found within tumor tissues, tumor cells can be maintained and propagated ex vivo, at rates similar to those observed in the body. Other studies investigating cell morphology as well as gene and protein expression profiles have shown that cells cultured in the Avatar system more faithfully reflect in vivo biology than cells grown in traditional CO2 incubators supplemented with animal serum-based culture media.

Based on these findings, Xcell scientists conducted several research projects to understand whether this highly customized environment would make the Avatar system more amenable to growing CTCs. In one study, they used CTCs gathered from blood samples of patients with late-stage, castration-resistant prostate cancer, culturing cells with 1 percent oxygen and atmospheric pressure of 2 PSI (Lim et al, 2015). After a week, the clusters that had formed colonies were studied with a combination of immunofluorescence imaging, real-time PCR, and RNA-seq.

The analysis found that 25 percent of colonies were identified as tumor cells based on biomarker signatures associated with prostate cancer, including PSMA, EpCAM, and cytokeratins. CTC colonies based on cells from the same patient showed differential gene expression, suggesting activity from multiple tumor clones. One of the most important findings of the study was increased CXCR4-mediated signaling in the cultured cell colonies. While this pathway is consistently elevated in tumor cells taken directly from bone marrow, the same activity has not previously been found in cultured or enriched CTCs. The data suggest that cells cultured in the Avatar system reflected the true in vivo
biology. Still, it is worth noting that CTCs grow slowly even in the best conditions. After two weeks in culture, CTC colony sizes ranged from just a few dozen cells to a few thousand cells (a cell doubling time of three to four days).

Subsequent research projects have revealed more information about CTC growth using the Avatar system workflow. For example, the number of viable adherent cells cultured in the Avatar is at least 10 times higher than traditional workflows; in many cases, traditional workflows yield no adherent cells at all. Adherent cells include CTCs, macrophages, dendritic cells, and endothelial progenitor cells — all rare adherent cell types found in peripheral blood.

While many cell-sorting and enrichment protocols damage CTCs, a procedure known as leukapheresis — which involves removing nucleated cells from peripheral blood — appears to be much more successful for this cell type. Scientists at Xcell found that samples processed with leukapheresis were enriched with thousands of CTCs, which then experienced robust growth when cultured in the Avatar system. This workflow produced a colony doubling rate of 12 to 48 hours in four patient samples tested.

Finally, the Xcell team has observed that transcriptomic profiling makes it possible to characterize the heterogeneity of cultured CTC colonies. T cells, dendritic cells, fibroblasts, and endothelial cells have all been identified in these colonies, with variation that appears to be based patient dependent.

Conclusion

While using CTCs to improve care for cancer patients is a tantalizing goal, studies conducted around the world serve as continual reminders that it is remarkably difficult to work with these cells ex vivo. Research with the Avatar system suggests that more carefully constructed culture environments might improve the odds of expanding these cells, but it is by no means a panacea. The biomedical community will require many other advances in order to work with these cells consistently and to generate accurate, useful information that can benefit patients. Still, there is great hope that today, nearly 150 years after Dr. Ashworth made his initial observations, we are finally on the verge of bringing his valuable discovery into the clinic for improved patient care.
References

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