Awaiting a New Era of Cancer Immunotherapy
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Abstract
A major challenge in cancer therapy is the lack of specificity for cancer cells. Antibody-based therapies have better specificity and, thus, improved efficacy over standard chemotherapy regimens. Monoclonal antibodies (mAb) constitute one of the largest classes of drugs and have been very commercially successful. In 2009, the global market for mAbs was $36.4 billion (1). However, current strategies for antibody therapy are limited to targeting only extracellular proteins (2, 3), which are easily accessible and expressed on the outer membranes of cancer cells. Examples include anti-CD20 mAb (rituximab), anti-Her2 mAb (trastuzumab), and anti-EGF receptor mAb (cetuximab), used to treat lymphoma, some breast cancers, head and neck cancers, and colorectal carcinoma, respectively (4). These antibodies work conventionally against extracellular oncoproteins, or rarely, soluble proteins in circulation.

Researchers have not developed antibody-based drugs against intracellular cancer proteins because of the prevalent concept that intracellular locations are inaccessible to antibodies. Therefore, many intracellular cancer-specific therapeutic targets, such as cancer and/or testis antigens or mutated antigens (Ras mutations) with excellent tumor specificity, have not been pursued for antibody therapy. To expand the repertoire of possible cancer targets, we recently showed that intracellular proteins (PRL-3, EGFP, and mT) could be used as antigens to challenge and stimulate the immune system to produce antibodies and achieve a similar effect as observed in antibody therapy. We subsequently showed that the 3 distinct intracellular proteins could be targeted with their respective antibodies, one could anticipate that intracellular antigens could also be used to trigger the host immune system to produce antibodies and achieve a similar effect as observed in antibody therapy. We subsequently showed that the 3 intracellular proteins (PRL-3, EGFP, and mT) could be used as antigens to challenge and stimulate the immune system to produce antibodies. After successfully showing how tumors expressing the 3 distinct intracellular proteins could be targeted with their respective antibodies, one could anticipate that intracellular antigens could also be used to trigger the host immune system to produce antibodies and achieve a similar effect as observed in antibody therapy. We subsequently showed that the 3 intracellular proteins (PRL-3, EGFP, and mT) could be used as antigens to challenge and stimulate the immune system to produce antibodies.

Our unconventional concept of targeting intracellular oncoproteins with antibody therapies or vaccines presents a more specific strategy of targeting internal cellular proteins than using small-molecule inhibitors. These immunotherapies would render many intracellular targets not only for cancer treatments but also for therapeutics in human infectious diseases.

Antibody Therapy Could Target Intracellular Oncoproteins
Actually, a substantial body of evidence suggests that antibodies are able to penetrate human cells to target intracellular proteins (12). In 1978, Alarcon-Segovia and colleagues described a human immunoglobulin G autoantibody to ribonucleoproteins that could enter viable human lymphocytes using Fc receptors and react with its nuclear antigen (13).

References

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mAbs against human ribosomal P proteins have been shown to be able to penetrate living cells to cause apoptosis of Jurkat T cells in culture (14), and exogenous Hsp27 antibody may induce apoptosis in retinal neuronal cells (15). An elevated level of autoantibodies can bind to their respective intracellular antigens and cause apoptosis leading to self-destruction, as has been observed in different autoimmune diseases. These antibodies have been found to penetrate living epithelial cells (16) and induce glomerular proliferation and proteinuria in vivo (17).

This naturally occurring pathogenic role of autoantibodies indicates that antibody targeting of intracellular oncoproteins can potentially cause cancer cells to self-destruct as well. If one could disregard the long-standing dogma that antibodies react with their respective antigens exclusively in the extracellular compartment, one would immediately appreciate a vast new array of intracellular oncoproteins as possible cancer therapeutic targets. New prognostic markers or “druggable” targets are urgently needed to provide more therapeutic options to oncologists in developing strategies for individual patients with cancer. To investigate whether antibody therapy could be used to target intracellular cancer proteins, we set up separate experiments targeting PRL-3, EGFP, and mT oncoprotein. PRL-3, the overexpression of which was reported by Saha and colleagues to be highly correlated with colorectal cancer metastasis (7), was first identified by our group in 1998 (6), and continues to be actively studied (9). PRL-3 upregulation has been shown to be correlated with invasive behavior and poor clinical outcomes of numerous types of advanced human metastatic cancers, including colorectal, gastric, breast, cervical, and lung cancer (8, 9, 18). PRL-3 protein levels have been shown to be elevated in about 22.3% of more than 1000 cancer samples examined (19). Second, to investigate generalizing this approach to other intracellular proteins, the popular EGFP reporter was used. EGFP is an intracellular protein localized in the nucleocytoplasmic region and is not expressed in host tissues. Therefore, it represents an artificial “cancer-specific” intracellular protein when overexpressed in cancer cells. Third, we examined another mouse tumor model using mouse mammary tumor virus (MMTV)–PymT transgenic (TG) mice, which carry the mT intracellular DNA viral protein, under the transcriptional control of the MMTV promoter–enhancer (10). EGFP and mT are 2 intracellular proteins used to elucidate a general phenomenon that an antibody can target intracellular proteins within cancer cells. To increase the clinical relevance of our animal models, we used immunocompetent C57BL/6 mice grafted with syngeneic tumors and MMTV-PymT TG mice that develop spontaneous breast cancer at the age of 2 to 3 months. These TG mice have been widely used as excellent spontaneous tumor models for decades by the cancer research community (10, 11). The tumor sizes were markedly reduced in the “treated” group, and Kaplan–Meier analysis showed significantly improved overall survival in “treated” mice (5).

However, not all intracellular oncoproteins are viable targets for antibody therapy. Genes that are specifically upregulated during tumor formation, but poorly expressed or not expressed in host tissues, are particularly promising as tumor-specific targets. Desirable anticancer therapeutic agents should specifically target cancer cells while sparing normal tissue (20). It should be emphasized that the PRL-3 antibody therapy has few detectable side effects in our animal models, as PRL-3 expression in normal tissues is not ubiquitous. In contrast, PRL-2 (a phosphatase with 75.4% homology with PRL-3) is ubiquitously expressed in most of the mouse tissues. As expected, PRL-2 antibody therapy to PRL-2–expressing cancers was unsuccessful (20), likely due to the side effects of anti-PRL-2 antibodies causing normal PRL-2–expressing tissues to be targeted by the PRL-2 antibodies. Thus, the choice of a good therapeutic target should be tumor specific to avoid harming host normal tissues.

Our preclinical data implicate a wide variety of future new candidates, including both extra- and intracellular oncoproteins to be considered as targets for mAb-based therapy. Indeed, antibody therapies have targeted not only proteins but also carbohydrates (21). To translate these preclinical findings to clinical settings, we have engineered a PRL-3–humanized antibody to target cancer cells expressing PRL-3 phosphatase (20). Efforts should be focused on 3 PRL-3–associated lethal malignancies, such as lung cancer, liver cancer, and acute myeloid leukemia, which often relapse within short timeframes. Because these cancers are aggressive, therapeutic effects will be observed quickly, the outcomes between “treated” and “untreated” patients will be more clearly defined, and terminal patients suffering from these cancers will be more easily recruited into clinical trials. Hopefully, clinical success in future trials may make an impact on targeting other intracellular oncoproteins.

Possible Mechanisms for Antibody Targeting of Intracellular Antigens

Although the molecular mechanisms behind our findings remain elusive and need to be deciphered, a number of possible mechanisms of antibody action can be envisaged (Fig. 1). First (Fig. 1A), the antibody may potentially enter PRL-3–expressing cells to target intracellular PRL-3 and neutralize its function. We observed that about 10% of PRL-3–expressing cancer cells could take up antibodies in culture, and this uptake was enhanced 6-fold upon serum starvation (22). Serum starvation is used to arrest cells at G1 and G0 phases (23). It is possible that particular stages (perhaps G1–G0) of the cell cycle contribute to the ability of cells to take up the antibodies. In vivo, cancer cells are under hypoxic stress and serum deprivation, conditions that might enhance cancer cells to take up antibodies. It was reported that an antibody can be taken into live human mononuclear cells through surface Fc receptor (13). We found that the phenomenon of antibody uptake was abolished if endocytosis was blocked by NH4Cl or when cells were incubated on ice (C.W. Hong and Q. Zeng; unpublished data), suggesting that most likely, these antibodies bind to some cell-surface components, followed by endocytosis or pinocytosis. Once in the endocytic compartments, the antibodies could be released into the cytosol, triggering cancer cell apoptosis. Second (Fig. 1B), some of the intracellular antigens may be externalized and displayed on the surface of cancer cells by unconventional secretion (24), enabling the antibodies to bind and trigger immune responses, such as antibody-
dependent cellular cytotoxicity, leading to cancer cell destruction. Third (Fig. 1C), proteolytic fragments of intracellular targets may be presented by MHC class I molecules to attract CTLs to mediate lysis of cancer cells. In addition, a small fraction of intracellular antigens is released because of necrosis or cancer cell lysis, producing an antigen–antibody complex within the tumor and stimulating local inflammatory response to attract immune cells in targeting neighboring viable cancer cells within the tumor. The most likely mechanism could be a combination of several modes, possibly also including complement-mediated events that are actually involved in achieving the final therapeutic consequence of antibodies against intracellular oncoproteins. These possible underlying mechanisms are discussed in a Perspective article in *Science Translational Medicine* (25). Currently, how antibodies destroy tumor cells *in vivo* is still poorly understood. This situation is similar to the clinical use of trastuzumab, which has been used for more than 2 decades in treating Her2/neu-positive breast cancers without full understanding of its mechanism (26). Using an *in vitro* cell-culture system to represent the *in vivo* system may be unrealistic, because the traditional *in vitro* cell-culture system is oversimplified. There are limitations of using a single cell type grown in an incubator, which typically uses medium supplemented with 10% FBS without involvement of the immune system. The *in vitro* system is also unlikely to actually mimic *in vivo* complexities of multiple types of cells and organs, coordinating in the native blood circulation and lymphatic system. The central finding of this study is that antibodies to intracellular protein can exert therapeutic effects. Because the therapeutic effects of antibodies to intracellular proteins are substantial and reproducible, the incomplete understanding of the underlying mechanisms should not hinder future research in this new class of potential therapeutics.

**Intracellular Proteins Could Be Used for Cancer Vaccine**

Vaccines have generally been associated with infectious diseases. When a foreign virus or bacterium enters the body and is recognized by the adaptive immune system, immune cells start producing antibodies. The immune system is also important in fighting cancerous cells. The main premise of cancer vaccination is stimulating the patient's immune system to attack and reject malignant tumor cells. The patient's immune system is thus trained to recognize tumor cells as targets to be destroyed. Cancer cells can carry both self-antigens and cancer-associated antigens. The cancer-associated antigens mark the cancer cells as foreign, causing B cells and killer T cells to attack them. However, if the vaccine targets are also expressed in healthy cells, antibody and killer T cells might destroy them and cause autoimmunity. As such, inducing host immunity against cancer-specific antigens will maximize our immune system's ability to specifically target cancer cells, expressing that antigen without harming normal tissue.

Because the antibodies can somehow recognize their intracellular antigens, it is expected that the intracellular antigens could induce antibody production in the host immune system to prevent tumor formation, an ideal route for treatment. To investigate this possibility, we next explored the use of 3 purified proteins, PRL-3, EGFP, and mT, as therapeutic vaccines to inhibit cancer formation in animal models. We
injected a small amount (14 μg) of mT antigen to stimulate the immune system to produce mT antibodies in young female PymT TG mice. Similar to results with antibody-“treated” mice, median survival was 5 weeks longer in the “immunized” mice (19.5 weeks vs. 14.5 weeks), and they developed fewer tumors. Both antibody therapies and antigen-induced antibody (vaccination) therapies against intracellular proteins can specifically arrest tumor progression in vivo. Therefore, hosts can be trained to produce antibodies for their own anticancer therapies by vaccinating cancer-bearing animals with antigens derived from the same "tumor-specific" proteins. Preventive antigen-induced vaccination may be particularly useful in genetically based familial cancers. Furthermore, we hypothesize that one potential advantage of using intracellular self-antigens is that intracellular proteins may have a better chance of provoking an immune response than extracellular self-antigens, because immune cells targeting extracellular self-antigens are generally eliminated during development and maturation. Because most cellular proteins are intracellular, including intracellular targets for the immunotherapies will significantly expand the availability of therapeutic strategies.

Although the idea of using "oncoproteins" for vaccination may be disconcerting to some, it is worth noting that the purified antigens are unlikely to behave as oncoproteins in isolation because they have lost their specific intracellular spatiotemporal localizations. As such, they are unable to interact with their native neighborhood partners to elicit pathway-specific responses to drive tumorigenesis. Nonetheless, a more conservative approach would be the use of specific peptide antigens (partial oncoproteins) to achieve similar therapeutic and/or preventive vaccinations. Epitope-based peptide (or fragment) vaccination will be more specific and less cross-reactive with homologous proteins, hence minimizing side effects. Because existing conventional clinical antibody therapy is costly, vaccination may be more useful, economical, and effective as a means of inducing high titers of antigen-induced antibodies. This concept of "cancer vaccination" is challenging, but promising. If successful, vaccines would revolutionize cancer therapy.

Future Perspectives

Our study provides a proof-of-concept for targeting intracellular oncoproteins using antibody therapy and vaccination, and this can be translated into new treatments for patients in many potential ways. One could envisage that antibodies against hepatitis B virus (HBV) proteins, such as the HBV X-protein localized in the nucleus of infected cells (27), could be used as therapeutic intracellular targets for hepatocellular carcinoma (HCC). As most HCC are associated with HBV infections, antibodies targeting virus-specific proteins will specifically destroy virally infected cells but leave normal cells unharmed. Similarly, when a self-antigen is overexpressed in cancer cells in comparison with normal tissues, because of the higher expression levels of target antigen in cancer cells, those cancer cells will be more responsive to the antibody therapies (with the normal tissues, more self-tolerance) when antibody treatment is dosed properly. As such, breast cancer caused by overexpression of the estrogen receptor (28) can be treated with antibodies against estrogen receptor or with vaccination using an estrogen receptor fragment to prevent cancer progression and spreading. This strategy is particularly useful for targeting estrogen receptor–positive breast cancers, regardless of the expression of Her2 or other proteins.

A potential scheme in future cancer treatment could be to remove and/or biopsy the primary tumor and use immunohistochemistry or Western blot (or to collect blood and/or urine samples and use ELISA or PCR) to identify at least one tumor-expressing antigen and administer either antibodies against that specific tumor marker or a therapeutic vaccine that stimulates antibodies against it. Cancer represents a tremendous burden on patients, their families, and society, but existing antibody therapy for cancer treatment is very costly. We hope that our research will pave the way for cancer vaccination to become a mainstream cancer treatment that is both effective and affordable for patients with cancer. For patients with a strong family history of cancer, immunization of young susceptible family members with an antigen associated with the familial cancer could prime the immune system against tumor cells expressing that antigen and may prevent cancer before it develops. If the myriad of previously unexplored candidate target proteins are investigated, a new era of cancer therapies may soon become a reality.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C.W. Hong, Q. Zeng
Development of methodology: C.W. Hong, Q. Zeng
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.W. Hong
Writing, review, and/or revision of the manuscript: C.W. Hong, Q. Zeng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.W. Hong

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