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Challenges in pre-clinical testing of anti-cancer drugs in cell culture and in animal models

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Abstract

Experiments with cultures of human tumor cell lines, xenografts of human tumors into immunodeficient mice, and mouse models of human cancer are important tools in the development and testing of anti-cancer drugs. Tumors are complex structures composed of genetically and phenotypically heterogeneous cancer cells that interact in a reciprocal manner with the stromal microenvironment and the immune system. Modeling the complexity of human cancers in cell culture and in mouse models for preclinical testing is a challenge that has not yet been met although tremendous advances have been made. A combined approach of cell culture and mouse models of human cancer is most likely to predict the efficacy of novel anti-cancer treatments in human clinical trials.

Keywords

cancer model; cell culture; mouse; xenograft; preclinical

Cell culture and animal studies are critical steps in the determining the efficacy, pharmacodynamics, and mechanism of action of novel anti-cancer drugs. Tumors are composed of a heterogeneous population of cells with a high degree of genetic instability and phenotypic variability. The interaction between tumor cells and the host environment leads to activation of fibroblasts and endothelial cells and recruitment of inflammatory and immune cells. The modified microenvironment in turn selects and modifies the genetic and phenotypic nature of the tumor. This reciprocal interaction between tumor cells and the microenvironment is essential for tumor progression and metastasis [1]. The host environment itself, viewed from a population perspective, is genetically diverse and subject to different environmental stimuli. Thus, the same tumor will evolve and behave differently in different patients. Modeling this in cell culture and in animal models to test the efficacy of candidate drugs that work across tumor variants and in a broad population is a daunting task, and a single approach is unlikely to suffice. Here, we will briefly review and comment on

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the utility and challenges of cell culture and mouse models in pre-clinical testing of anti-cancer drugs.

Tissue culture

The use of tumor cell lines allows investigators to test compounds under highly controlled and reproducible conditions. The availability of well characterized cell lines enables parallel screening assays in which the effect of a drug candidate on proliferation of multiple tumor cell lines can be determined. This is the basis for the NCI-60 Human Tumor Cell Line Screen offered through the Developmental Therapeutics Program of the National Cancer Institute [2, 3]. The cell lines incorporated in this panel represent tumors from nine different origins, namely brain, breast, colon, hemopoietic cells, kidney, lung, melanocytes, ovary and prostate. The end-point measurements of the cell line screen are growth rate and cytotoxicity, but the combined information from the cell lines also provides information about the mechanism of action of drugs [3]. Although the use of cell lines in simple two dimensional cell culture systems allows for high throughput screening, such cells and culture conditions do not approximate the complexity of tumors in patients. The cell lines are highly selected and do not have the genetic and phenotypic heterogeneity of neoplastic cells in tumors. Furthermore, propagation of cells in cell culture frequently results in accumulation of additional genetic and epigenetic changes. For example, recent analyses of DNA methylation profiles of cell lines and primary tumors revealed higher levels of methylation in tumor cell lines accompanied by decreased gene expression compared with primary tumors [4, 5]. Use of primary cells may alleviate these problems. However, difficulties in obtaining sufficient amounts of cancer cells fully reflecting intratumoral diversity represent a significant challenge to such approach.

Tumor cells interact closely with the extracellular matrix and this affects cell polarity, nuclear organization and gene expression [6]. This is consistent with the observations that cells grown in three dimensional (3D) cultures have different growth characteristics and responses to chemotherapeutic drugs compared with cells grown in two dimensional culture systems [7, 8]. Tumor cells interact not only with each other and the extracellular matrix, but also with other host cells including endothelial cells, fibroblasts and immune and inflammatory cells. Recent studies aim to add some of these components, in particular extracellular matrix, fibroblasts, and endothelial cells, creating 3D/organoid cell culture models that mimic human tumors more closely and, hopefully, have a more predictive response to drug candidates [8, 9]. However, it is highly unlikely that the complexity of tumors and their interactions with the host can be completely recapitulated in cell culture.

Xenograft models

Xenograft models are established by injection or implantation of human tumor cells or primary tumor fragments into immunodeficient mice. Commonly used immunodeficient mice include nude mice (*Foxn1*^{nu}), severe combined immunodeficiency (*scid*) mice (*Prkdc*^{scid}), RAG1 and RAG2-deficient mice, and NOD-*scid* and NOD-*Rag1*$^{-/-}$ mice [10]. Nude mice were the first genetically immunodeficient mouse model used for xenotransplantation [11]. They lack a thymus and normal T cell development. However, the T cell deficiency is not complete and these mice have increased NK cell activity. As a result, the success rate of xenografts is relatively low. Only about 20 to 40% of tumor cell lines grow in nude mice. The *Prkdc*^{scid} mice carry a mutation in a component of the DNA-dependent protein kinase complex that is necessary for the DNA recombination events during B and T cell development. These mice lack B and T cells, but have an intact innate immune system. The DNA-dependent protein kinase is also involved in DNA repair, and *Prkdc*^{scid} mice have increased susceptibility to radiation injury [10]. RAG1 and RAG2 are

required for gene segment recombination in the generation of T cell receptors and immunoglobulin, and *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ mice lack B and T cells. Like *scid* mice, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ mice have an intact innate immune system, but they are radioresistant [10]. The level of innate immune system activity is lower in mice crossed onto the non-obese diabetic (NOD) background. As a result, NOD-*scid* and NOD-*Rag1*$^{-/-}$ mice are more receptive to xenografts. Because diabetes mellitus in NOD mice is T cell dependent, NOD-*scid* and NOD-*Rag1*$^{-/-}$ mice do not develop diabetes. A further increase in the acceptance rate of human cells and tissues is achieved by crossing T and B cell-deficient mice with mice in which the common cytokine receptor chain IL2RG is knocked out. The IL2RG chain is part of the receptor complexes for IL2, IL4, IL7, IL9, IL15, and IL21, and signaling through these receptors is required for normal development of NK cells [12, 13]. The absence of NK cells contributes to the high success rate of xenografts in *Il2rg*$^{-/-}$ mice.

Mice with different degrees of immunodeficiency not only vary in the acceptance rate of xenografts, but the engrafted tumors may also differ in their biological behavior and response to drugs. Tumors implanted in *scid* and *Rag2*$^{-/-}$ mice had a different response to cancer treatments compared with tumors implanted subcutaneously in nude mice [14]. As discussed in more detail below, the innate and adaptive immune systems are activated by therapy-induced cancer cell death, and the immune response can contribute to the efficacy of anti-cancer therapy. Differences in the response to chemotherapy of human tumors in immunodeficient mice may reflect a role of the remaining intact components of the immune system in these mice. It is also important to consider the genetic background of the immunodeficient mouse strains. The *Foxn1*^{nu} mutation has been crossed onto genetically different mouse strains and these strains vary significantly in the rate of tumor growth and response to chemotherapeutics [15, 16].

Subcutaneous xenograft models are extensively used because of the ease of handling and measurement of tumor growth, but the correlation between the results from xenografts and human clinical studies is generally poor [17]. There are several reasons for this. As mentioned above, tumor cell lines that have been cultured for long periods are highly selected and differ from primary tumors in patterns of DNA methylation and gene expression [4, 5]. Furthermore, only a few cell lines have been found suitable for xenograft experiments and this is another selection step. An alternative approach is to implant fragments of primary tumors removed from human patients subcutaneously. These xenografts retain the DNA methylation pattern of the original tumor and may more faithfully mimic the response of human tumors to cancer drugs. Another concern with the subcutaneous xenograft is that this model does not allow for the normal complex interactions between tumor cells and the microenvironment to develop. An alternative approach is to inject or implant tumor cells or tumor tissue orthotopically, i.e. in the same mouse organ or tissue from which the human tumor cells were derived. Orthotopically implanted tumors tend to metastasize more frequently than ectopic (subcutaneous) tumors [18]. The tumor microenvironment can also affect the response of tumors to chemotherapeutic agents. This was demonstrated in a syngeneic tumor model with mouse CT-26 colon carcinoma cells and a human breast cancer cell xenograft injected into nude mice [19, 20]. However, orthotopic injection or implantation is technically more demanding and time consuming. Moreover, the tumors are not easily accessible in live animals, making it difficult to monitor tumor growth. Following subcutaneous injection, palpable tumors develop typically after one to four months, and tumor growth and size serve as the endpoints in most studies. Tumor growth can be detected after implantation into an internal organ by transfection of tumor cells with green fluorescent protein, luciferase or another label that allows in vivo imaging of the tumor cells [21]. Perhaps the most fundamental concern with xenotransplantation is that the recipient mice lack a functional immune system. The role of the immune system in preventing tumor development and progression has been controversial

probably reflecting differences in the immunogenicity of tumors and differences in the type of immune response. However, studies over the past decade have clearly demonstrated an important role of the innate and adaptive immune system in preventing the outgrowth of tumor cells and in selection of tumor cells that escape the immune response [22, 23]. In xenograft models, the selection of phenotypic and functional variants by the immune system does not occur. The immune response can also play an important role in the effect of cancer therapies. Chemotherapy and radiotherapy was less effective in nude mice than wild-type mice with established syngeneic tumors [24]. Further experiments indicated that this could be attributed at least in part to activation of dendritic cells by high mobility group 1 (HMGB1) protein from dying cells via Toll-like receptor 4 (TLR4). In xenotransplant models in nude mice, the effect of treatment of human tumor cells with cyclophosphamide is mediated in part by activation of innate immune cells by HMGB1 [25]. Depletion of neutrophils and NK cells abrogated the effect of the drug treatment. One may therefore expect different responses to the same drug treatment of xenograft models in different immunodeficient mice. Other studies have identified roles for both innate and adaptive immune mechanisms in the effect of chemotherapeutic drugs including CD8 T cells, TCRgamma-delta T cells, IL1beta, IL17, IFNB and IFNG [26-28].

Recently, several approaches have been developed aimed to provide humanized microenvironment for engrafted human cancer cells. For example, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice were used successfully for highly efficient engraftment of human hematopoietic stem cells [29]. Furthermore, normal human cells and cancer cells can be orthotopically transplanted together with human stromal cells (reviewed in [30]). In this system, transplantation of normal human cells with ex vivo introduced genetic alterations may allow studies of early stages of carcinogenesis. Unfortunately, even in these systems, some challenges remain, such as potential mismatch between human and mouse ligand and receptors, artificial introduction of genetic alterations and absence of an adaptive immune system.

Autochthonous mouse models of cancer

An ideal mouse model of human cancer should have common molecular mechanisms with human disease, have similar pathology of developed neoplasms, show neoplastic progression which recapitulates human cancer, including its metastatic changes, and have therapeutic and host immune responses that resemble those in human patients. As discussed above, xenografts are human cells grown in an immunodeficient mouse, and have significant limitations which are difficult to overcome. An alternative to xenograft-based approaches are mouse models of cancer based on its spontaneous development, induction by chemical, irradiation, hormonal or genetic approaches, or transplantation of syngeneic cells. One of the classical examples of chemical carcinogenesis is induction of skin tumors by a two-step treatment protocol of topical application of 7,12-dimethyl-benz[a]-anthracene (DMBA) followed by repeated application of 12-O-tetradecanoylphorbol-13 acetate (TPA) [31]. The skin tumors progress from papillomas to invasive squamous cell carcinomas. Unfortunately, initiating mutations in case of chemically induced tumors, as well as in case of the majority of other non-genetic approaches, remain unknown. While some tumors, such as squamous cell carcinomas of the skin are morphologically similar to those in humans, others, such as spontaneous mammary carcinomas and many myelodysplastic processes, have little in common with the pathology of human cancers.

One of the most commonly used current approaches for modeling human cancer involves generation of genetically engineered (also known as genetically modified) mice (GEM). These approaches are results of rapid advances in mouse and human genomics, as well as of development of sophisticated tools for manipulation of the mouse genome during past 30

years. The first tumors in GEM were created by transgenic overexpression of oncogenes [32]. Such models have significant limitations because they result in random and frequently multiple integrations of transgene copies into the genome. Furthermore, transgenes commonly have mosaic pattern of expression. These shortcomings have been addressed by the development of the 2007 Nobel award-winning technology for homologous recombination in embryonic stem cells [33]. This allowed accurate modification of endogenous genes, leading for example to the creation of mice with deletion of tumor suppressor genes [34]. However, the creation of mice with congenital gene inactivation, in which all somatic cells have introduced genetic defect, resulted in hereditary models of cancer. This is unlike human cancers which are sporadic in the vast majority of cases. Furthermore, such mice frequently developed rapidly growing hematopoietic tumors thereby precluding studies of slower forming epithelial neoplasms. Subsequently, several approaches for controlled gene expression have been introduced, such as Cre-*loxP* mediated recombination, tetracycline- and tamoxifen- dependent gene expression (reviewed in [30] and [35]). Such approaches allow conditional gene expression in a temporal, spatial and/or lineage-restricted manner. Importantly, introduction of genetic lesions similar to those in humans have resulted in formation of neoplasms closely resembling those in humans according to both morphological and gene expression profile features, as well as to the extent of disease progression, including metastases [30, 35, 36]. For example, Cre-*loxP* mediated conditional inactivation of tumor suppressor genes *p53* and *Rb* in the ovarian surface epithelium of adult mice has resulted in formation of metastatic high grade serous adenocarcinomas closely similar to human epithelial ovarian cancers of the same type [34]. Recently, an extensive integrated genomic analysis of 489 human high grade serous ovarian adenocarcinomas has shown that such tumors carry *p53* mutations and alterations of the Rb pathway in 96% and 67% cases, respectively [37]. Likewise, adenocarcinomas initiated by conditional activation *K-ras* gene in the lung are similar to human pulmonary adenocarcinomas frequently carrying the same mutation [38].

The advantages of mouse cancer models are that the tumors are initiated and undergo selection in physiologically natural conditions including stromal microenvironment and the immune system. Disadvantages are the length of time to develop tumors and, in some cases, individual variability in the time, number of tumors and progression to malignancy, which necessitates more animals per treatment group to achieve sufficient statistical power. It is also important to note that there are differences in the sensitivity among mouse strains to tumor induction using various approaches [31]. Thus changing the genetic background can affect the phenotype of the mouse [39]. Since GEM are frequently produced on a mixed genetic background, the backcrosses onto a commonly used background may take a significant time and expenses, even in case of using speed congenics [40]. Last, but not least, the use of many GEM is limited by patents and intellectual property rights [41].

Thus, while GEM hold great potential for understanding of cancer pathogenesis, their use in preclinical testing remains limited so far. Establishment of predictive value of such models for the efficacy of drugs in human clinical trials is among the most urgent tasks. Recently, several attempts have been made to address limitations of GEM by development of non-germline genetically engineered mouse models that are based on transplantation of genetically modified cells into syngeneic hosts [30]. Recombinant viruses such as adenovirus expressing Cre-recombinase have been used to create models of lung and ovarian cancer in which the cancer arises from a small number of virus-infected cells [42, 43]. The introduction of additional genetic modifications can create tumors that are more likely to metastasize. In spite of these advances, the use of GEM in preclinical testing is limited for various reasons. Like the chemically-induced mouse tumor models, the time for the development of tumors is often long and variable. The breeding of GEM can be complicated and the maintenance of a sufficiently large breeding colony is expensive, while

changing the genetic background can affect the phenotype of the mouse [39]. The predictive value of GEM for the efficacy of drugs in human clinical trials is currently uncertain.

Endpoints for preclinical testing in mouse tumor models

Commonly used outcome parameters for preclinical testing of cancer drugs are tumor size and growth rate [18]. This can be readily determined in skin tumors or subcutaneously implanted tumors, but requires in vivo imaging techniques for tumors localized in internal organs [21]. Alternatively, the number of tumors and tumor size can be assessed at necropsy. Other endpoints can give information about the mechanism of action of drugs such as histopathology combined with immunohistochemistry and morphometry to quantify angiogenesis, characterize the infiltration by immune and inflammatory cells and to measure the presence of apoptotic cells. Furthermore, rapid advances in high-throughput technologies and systems biology approaches, make it feasible to perform genome-wide assessments of changes in signaling networks based on both on RNA and protein levels [44]. Although labor intensive, it is likely that the use of multiple robust outcome criteria will enhance the utility and predictive value of mouse models of cancer [18, 45].

Conclusion

There is no single cell culture or in vivo cancer model that faithfully predicts the efficacy of anticancer drugs in human clinical trials. Cell culture approaches offer the advantage of human-derived cell lines or tissue fragments from primary tumors, but cannot mimic the complexity of the reciprocal interaction between the growing tumor and the co-evolving microenvironment. Xenografts in immunodeficient mice have limited added value over cell culture models as the lack of an intact immune system and insufficient interactions between the human tumor cells and mouse stromal cells do not recapitulate human cancers. The use of sophisticated genetically modified mouse models can recapitulate human cancer pathogenesis more closely and is likely to become increasingly utilized in preclinical testing, particularly in conjunction with non-germline syngeneic models An approach of cell culture testing of a panel of human cell lines or primary tumor tissues combined with in vivo testing in a genetically modified model that closely approximates the targeted human cancer has the greatest chance of success in predicting the efficacy of novel treatments in human cancers. This approach requires a multidisciplinary team effort of cell and molecular biologists, pharmacologists, pathologists, bioinformaticians, and clinical oncologists.

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