Evaluation of Interstitial Protein Delivery in Multicellular Layers Model

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The limited efficacy of anticancer protein drugs is related to their poor distribution in tumor tissue. We examined interstitial delivery of four model proteins of different molecular size and bioaffinity in multicellular layers (MCL) of human cancer cells. Model proteins were tumor necrosis factor-related apoptosis-including ligand (TRAIL), cetuximab, RNase A, and IgG. MCLs were cultured in Transwell inserts, exposed to drugs, then cryo-sectioned for image acquisition using fluorescence microscopy (fluorescent dye-labeled TRAIL, RNase A, IgG) or immunohistochemistry (cetuximab). TRAIL and cetuximab showed partial penetration into MCLs, whereas RNase A and IgG showed insignificant penetration. At 10-fold higher dose, a significant increase in penetration was observed for IgG only, while cetuximab showed an intense accumulation limited to the front layers. PEGylated TRAIL and RNase A formulated in a heparin-Pluronic (HP) nanogel showed significantly improved penetration attributable to increased stability and extracellular matrix binding, respectively. IgG penetration was significantly enhanced with paclitaxel pretreatment as a penetration enhancer. The present study suggests that MCL culture may be useful in evaluation of protein delivery in the tumor interstitium. Four model proteins showed limited interstitial penetration in MCL cultures. Bioaffinity, rather than molecular size, seems to have a positive effect on tissue penetration, although high binding affinity may lead to sequestration in the front cell layers. Polymer conjugation and nanoformulation, such as PEGylation and HP nanogel, or use of penetration enhancers are potential strategies to increase interstitial delivery of anticancer protein drugs.

Key words: Protein delivery, Multicellular layers, TRAIL, PEGylation, Heparin-Pluronic nanogels, Cetuximab

INTRODUCTION

Over the past decade, proteins and peptides including monoclonal antibodies and growth factors have emerged as effective therapeutic agents for various human diseases. Although monoclonal antibodies have gained the most attention as a successful example of targeted protein therapeutics in oncology, only four monoclonal antibodies (trastuzumab, cetuximab, panitumumab, and bevacizumab) have been approved for solid tumors among the 21 marketed therapeutic monoclonal antibodies (Beckman et al., 2007; Weiner et al., 2010), reflecting the limited biodistribution of antibodies into solid tumors and the difficulty in achieving an effective drug concentration in tumor tissues. Adequate delivery of therapeutic agents to tumor cells is limited due to abnormal vasculature with irregular blood flow, variable intervascular distances, complex and tortuous branching patterns, and absence of functional lymphatics. In addition, some unique
characteristics of solid tumors, such as abundance of extracellular matrix (ECM) and increased interstitial fluid pressure (IFP), can impede the movement of molecules within the tumor tissue, limiting tissue delivery of drugs and contributing to drug resistance (Baker et al., 2008).

A number of strategies can be used to overcome the limited diffusion of antibody such as optimization of molecular size, valence, charge, and affinity. Derivatives of smaller antibody fragments including diabodies, minibodies, Fab fragments, single chain Fv domains, and single chain antibodies have been developed to improve diffusion efficiency (Beckman et al., 2007). These fragments range in size from 15 to 60 KDa and generally offer more rapid and homogeneous penetration in tumor tissues; however, the concomitant decrease in tumor retention and antibody-dependent cell mediated cytotoxicity (ADCC) functionality should be compensated by other means (Yan et al., 2008, 2009).

Besides the issue of tissue penetration (penetration across biological membranes and matrix), proteins and peptides with demonstrated activity on the molecular level often fail to produce sufficient efficacy when applied in vivo because of their unsatisfactory pharmacokinetic profiles. These include: (1) fast elimination from the systemic circulation, (2) immunogenicity, and (3) inadequate stability (Torchilin and Lukyanov, 2003; Lu et al., 2006; Na et al., 2008). Many efforts have been made to overcome these problems, such as formulating proteins with various nanoparticulate drug carriers: liposome (Mastrobattista et al., 2002; Hao et al., 2005; Lee et al., 2011), biodegradable nanospheres (Na et al., 2008), electrostatic complexes (Liao et al., 2005), and hydrogel nanoparticles (nanogels) (Vinogradov et al., 2002). In these cases, the issue of tissue penetration should be revisited to assure sufficient efficacy because of the increased size and physicochemical properties of carrier.

Several methods are available to study drug distribution within the microenvironment of tumors either in experimental animals or in cell culture models in vitro (Tredan et al., 2007). Window chambers and fluorescent microscopy are applied to assess directly drug penetration in growing tumors of the living animal (Dreher et al., 2006). Cryosections of tumors are also for direct quantification of fluorescent drugs (e.g., doxorubicin). Although in vivo assessment has the advantage of representing the clinical environment, in vitro techniques offer the advantage of providing data in the absence of complicating factors (Minchinton and Tannock, 2006). As in vitro assessment model, multicellular spheroids (MCS) and multicellular layers culture (MCL) have been used to examine the penetration of drugs (low molecular chemical compounds) through solid tissue. These models mimic the in vivo environment of solid tumor avascular micro-regions such as 3-dimensional architecture, hypoxia, cell density, abundance of extracellular matrix (ECM), and cell-to-cell or cell-to-ECM communication. Studies using MCL have confirmed the tissue penetration kinetics of several drugs and various factors affecting their penetration (Tredan et al., 2007). The correlation with in vivo data, shown with gemcitabine (Huxham et al., 2004) and taxane (Kyle et al., 2007) has demonstrated the utility of this in vitro assay.

In order to evaluate the penetration of proteins in MCLs of human colorectal cancer HT-29 cells, we used several macromolecules: these proteins differed in size and degree of affinity for cancer cells and included tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, 66 KDa), cetuximab (Erbitux® 145 KDa), RNase A (14 KDa), and IgG (Goat, 150 KDa). As an anticancer agent, TRAIL is capable of selectively inducing apoptosis in cancer cells (Johnstone et al., 2008; Kruyt, 2008). Cetuximab (145 KDa) is a chimeric monoclonal antibody that binds the human epidermal growth factor receptor (EGFR), resulting in the inhibition of EGFR-driven signal pathway of human cancer cells. (Delbaldo et al., 2005; Wild, 2006). We employed RNase A as a model protein of small size without cellular affinity and IgG (150 KDa), which is similar in size to cetuximab with no specific bioaffinity, in order to compare it with cetuximab in terms of MCL penetration. We also used biochemical methods to improve of protein delivery such as PE Gylation, self-assembled heparin-Pluronic (HP) nanogels or penetration enhancer. Therefore, in the present study, we were to evaluate whether MCL can be successfully used for examining the penetration of macro-molecular protein drugs, when proteins unformulated or formulated through interstitium of solid tumors.

MATERIALS AND METHODS

Drugs and reagents
A Transwell insert® (0.4 µm microporous membrane) was purchased from Corning Costar. Penicillin/streptomycin solution was purchased from Sigma-Aldrich. RPMI-1640 medium was purchased from Gibco BRL, and fetal bovine serum (FBS), Tris Buffered Saline (TBS) and Dulbecco’s phosphate-buffered saline (PBS) was purchased from WelGENE. Cetuximab (Erbitux®, Fig. 1B) was purchased from Merck and doxorubicin (DOX, Fig. 1E) was donated by Dong-A Pharmaceuticals. Antibodies (Alexa 488 and Alexa 594 conjugated goat anti-rabbit Immunoglobulin G, Fig. 1D) were