Reshaping the Immunosuppressive Tumor Microenvironment: The Fusion Protein Strategy

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Abstract

Advanced tumor cells often create immunosuppressive tumor microenvironment to block immune system's attack in defense. The conversion of the environment from immunosuppressive to immune active holds hopes for effective cancer immunotherapies. Fusion proteins coupled with appropriate delivery approaches represent a promising strategy for this conversion. In the last decade, a variety of fusion cytokine proteins (GPI-anchored IL-2/IL-12, RGD/Fc, MULT1E/FasTI, MULT1E/IL-12, and IL-12/FasTI) have been created and tested for their anti-cancer activities in a series of *in vitro* and *in vivo* studies. The efforts are going on to develop effective method to deliver the fusion proteins specifically into tumors.

Keywords: Cancer; Immunotherapy; Fusion protein; Tumor microenvironment

Introduction

Recent progresses in tumor immunology have not only enhanced our understanding of the immune system's role in tumor development but also opened new windows for developing new therapies for cancer treatment [1]. A three "E" theory (elimination, equilibrium, and escape) has been posted to explain how tumor cells escape from immunosurveillance through a process called immune editing. In phase I, cancer cells in their early development are recognized and destroyed by the immune system. Major anticancer players in this phase include natural killer (NK) cells, cytolytic T lymphocytes (CTLs), NKT cells, CD4+ helper T cells, M1 macrophages together with two major cytokines: interleukin-12(IL-12) and interferon-γ (IFN-γ). In phase II, some cancer cells acquire mutations that allow them to resist immune destruction, but their proliferation and spread are still limited by immune responses. In phase III, further mutations in the surviving or advanced tumor cells lead to the capacity for immortal tumor growth and metastasis. In this phase, although some immune cells, such as CTLs or helper T cells, still exist, their function is largely inhibited; IL-12 production is also greatly suppressed. The immune activity shifts from anti-to pro-tumor growth due to the inhibitory cytokine production by tumor cells: TGF-β, IL-10, CCL-22, CXCL-12, COX02, PGE2, etc., and the significant recruitments of regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSC) to the tumor site [2,3]. In addition, tumor cells also produce cytokines, such as tumor necrotic factor-α (TNFα), IL-1,IL-6, CCL2, COX-2 which promotes chronic inflammation and vascular endothelial growth factor (VEGF) which promotes angiogenesis. Both processes lead to significant tumor growth [4,5].

As information about tumor immunosurveillance and tumor immune editing is accumulating, various strategies have been attempted with the goal of developing effective

cancer immune therapies. These include adoptive transfer of monoclonal antibodies [6-8], T cells, or chimeric antigen receptor (CAR) engineered T cells [9, 10] and cancer vaccines, including whole-cell vaccine [11], tumor antigen vaccine [12], or dendritic cell mediated cancer vaccines [13]. Although many of these immunotherapeutic approaches showed promising results in animal models and are relatively safe compared to conventional cancer treatment regimens, their clinical efficacies are mostly disappointing due to the lack of strategies to systematically tackle the immunosuppressive tumor microenvironment [1].

Therefore, how to reshape the tumor microenvironment to favor antitumor immune responses or how to break the immune tolerance created by advanced tumor cells is becoming critical in order to develop effective cancer immune therapies. In the last decade, our focus has been on the development of fusing cytokine proteins, which when delivered into tumors will be able to revive the immune cells activity and control tumor growth.

Rationale

Three groups of proteins, IL-12, stress proteins and Fas play critical role in the fighting between the immune system and tumor cells. We, therefore, have been focusing on these three proteins in our efforts to develop multifunctional proteins for cancer immunotherapy.

IL-12: Observations of a relationship between cancer regression and infection date back to the 18th century and was confirmed clinically by Dr. Coley [14]. Although Coley's toxin is still controversial, it is claimed that IL-12 is the key cytokine responsible for the observed cancer regression [15]. Recent studies agree that IL-12 plays a central role in antitumor immune surveillance and the elimination of IL-12 production is one of the key factors affecting the anti-to pro-tumor transition in tumor microenvironment [16]. Severe toxicity of systemic IL-12 impedes the application of this effective therapy [17]. Therefore, a locally high level expression of IL-12 within the metastatic tumor microenvironment in the form of bifunctional proteins (IL-12/FasTI and MULT1E/IL-12) will effectively engage the IL-12R pathways of various immune cells and significantly improve their anticancer function without the systemic side effects.

Stress protein: Most tumor cells at their early development stage express *stress* proteins, such as MHC class1 chain related protein A and B(MICA, MICB), unique long 16 binding protein (ULBP), and ribonucleic acid export 1 like transcript (RAET1) for human or UL16-binding protein-like transcript 1 (MULT1), retinoic acid early transcript 1 (Rae1), histocompatibility 60 (H60) for mouse. These *stress* proteins are ligands to activating receptor NK group 2 (NKG2) of receptors as member D(NKG2D) on NK cells, CTLs, and other immune killer cells and this way of the engagement of NKG2D path way is the most effective way to activate the immune cells [18]. Unfortunately, *advanced* tumor cells tend to devise strategies to down-regulate or shed off these proteins to avoid immune cells' killing [19]. Labeling of advanced cancer cells with these stress proteins in the form of bifunctional proteins (MULT1E/FasTI, MULT1E/IL-12, and MICA/FasTI) will effectively engage the NKG2D pathway to activate the immune cells.

Fas: Fas is a transmembrane cell surface death receptor. The intracellular portion of Fas contains a death domain that is essential for transducing the apoptotic signal [20]. Several of our fusion proteins combined the transmembrane and intracellular domains of Fas with either of the extracellular domain of MULT1 or IL-12 as bifunctional proteins. The engagement of MULT1 portion of the protein to NKG2D or IL-12 portion of the protein to IL-12R will not only activate the corresponding immune killer cells, but also send apoptotic signals through the Fas portion of the proteins to kill tumor cells.

GPI-anchored IL-2/IL-12

While cancer immunotherapy with IL-2 and/or other cytokines has proved effective in activating immune responses against tumor cells, the major obstacle with the use of these cytokines in cancer patients is their severe side effects when delivered systemically at high doses [21, 22]. In an effort to overcome this problem, a fusion protein containing human IL-2 and a glycoinositol phospholipid (GPI) anchor sequence of decay accelerating factor (DAF) was generated. When expressed by transfected cells, these fusion proteins were presented on the cell surface in the GPI anchored form as demonstrated by fluorescent activated cell soring (FACS) and enzyme-linked immune sorbent assay (ELISA) analyses. This GPI-anchored IL-2 is highly functional as indicated by significantly increased T cell infiltration in tumor masses. Immunohistochemical analysis of tumor cells isolated from experimental tumors indicated that a local high level of IL-2 was achieved by GPI anchored IL-2. More importantly, when injected into mice intravenously, the growth of mouse B16F0 melanoma cells that were engineered to express this fusion protein was significantly inhibited. In contrast, the inhibition of secreted IL-2 on

tumor growth was not observable in this study [23]. We then expended the idea and created GPI-anchored IL-12. *In vitro* and *in vivo* studies showed that GPI anchored IL-12 is functional as indicated by increased T lymphocyte infiltration in tumors and has anti-tumor activity. More importantly, a synergistic anti-tumor effect was observed when GPI anchored IL-12 and GPI anchored IL-2 were co-delivered [24].

RGD/mFc

Targeting tumor vasculature represents an interesting approach for the treatment of solid tumors. The αvβ3 integrins have been found to be specifically associated with angiogenesis in tumors. By using bacteria phage display technology, Ruoslahti [25] found that a group of peptides containing the RGD (Arg-Gly-Asp) motif have high binding affinity to the αvβ3 integrins in tumors. A fusion protein containing the RGD sequence and the Fc fragment of mouse IgG was designed in order to target the Fc portion of IgG to the tumor vasculature to elicit an anti-angiogenesis immune response. *In vivo* angiogenesis and tumor studies demonstrated that fusion protein RGD/mFc inhibited tumor angiogenes and tumor growth and improved overall survival [26].

MULT1E/FasTI

Tumor cells evade immunosurveillance by elements of the innate immune system, such as NK cells, by down regulating or "shedding" certain cell surface molecules like MULT1 that can activate NK cells through NKG2D; they also avoid Fas mediated apoptosis by down regulating its expression. We designed and evaluated the antitumor activity of a fusion protein, MULT1E/FasTI, consisting of the extracellular domain of MULT1 and the transmembrane and intracellular domains of Fas*.* The fusion construct (pMULT1E/FasTI) was transfected into the mouse pulmonary carcinoma cell line TC-1; and there by stable cell clones expressing the fusion protein were established. *In vitro* cell culture studies demonstrated that the binding of the NKG2D/Fc, a recombinant protein of mouse NK cell receptor, to MULT1E/FasTI expressed on tumor cells was able to elicit apoptosis as assayed by Annexin V-FITC staining and caspase-3 ELISA. The fusion protein was also able to bind to NKG2D and activate NKG2D expressing cells, such as NK cells. *In vivo* subcutaneous tumor studies demonstrated that tumor cells expressing MULT1E/Fas TI grew significantly slower than cells without the protein. Pulmonary metastasis studies showed that most of the mice completely rejected tumor cells expressing MULT1E/FasTI. [27] We then tried to use an adenoviral gene delivery system to deliver this

fusion protein and demonstrated that adenoviral vector can efficiently deliver the MULT1E/FasTI fusion protein into TC-1 cells both *in vitro* and *in vivo* as assayed by RT-PCR, FACS analysis, caspase 3 activity and decreased *in vivo* tumor growth [28].

MULT1E/IL-12

NK cells have the potential to be effective killers of tumor cells. They are governed by inhibitory and activating receptors like NKG2D, whose ligands are normally up regulated in cells that are stressed, like cancer cells. *Advanced* cancer cells, however, have ways to reduce these ligands' expression, leaving them less detectable by NK cells. Along with these receptors, NK cells also require activating cytokines, like IL-12. We proposed and created a fusion protein combining the extracellular domain of MULT1 and mouse IL-12 with a hypothesis that when expressed by tumor cells, the protein will activate NK and other killer cells using the NKG2D receptor, and deliver mIL-12 to the NK cells where it can inter act with the IL-12R and enhance cytotoxicity. The fusion protein, when expressed by engineered tumor cells, indeed activated NK cells *in vitro* as assayed by increased production of INF-γ and cytotoxicity and significantly reduced tumor growth *in vivo* [29]. We then expanded the concept of developing a novel bifunctional fusion protein for enhanced NK cell activation to human killer cells. This time, MULT1E portion of the fusion protein was replaced with a human stress protein MICA. It is hypothesized that when expressed by tumor cells, the protein will activate human NK and other killer cells using the NKG2D receptor, and deliver IL-12 to the NK cells where it can inter act with the IL-12R and enhance cytotoxicity. The fusion protein, when expressed by engineered tumor cells, indeed activated NK 92 cells as measured by an increase in IFN-γ production and an increase in cytotoxicity of tumor cells. The fusion protein was also able to increase the proliferation of human peripheral blood mononuclear cells (PBMCs) and augment their production of IFN-γ [unpublished data].

IL-12/FasTI

Whereas cancer immune therapy with cytokines in recent researches demonstrated them to be effective in activating immune response against tumor cells, one major obstacle with the use of these cytokines is their severe side effects when delivered systemically at high doses. Another challenge is that *advanced* tumor cells often evade immunosurveillance of the immune system as well as of the Fas-mediated apoptosis by various

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mechanisms. We designed and evaluated the antitumor activity of another fusion protein, mIL-12/FasTI, consisting of mouse IL-12 and the transmembrane and intracellular domains of mouse Fas. The fusion construct (pmIL-12/FasTI) was transfected into mouse lung carcinoma cell line TC-1. Stable cell clones expressing the fusion protein were established as assayed by RT-PCR and immunohistochemistry. ELISA and cell proliferation analyses demonstrated that NK cells were effectively activated by the fusion protein with increased IFN-γ production and cytotoxicity. Enhanced caspase 3 activity of the clones when co-cultured with NK cells indicated that apoptosis was induced through Fas/FasL signaling pathway [30].
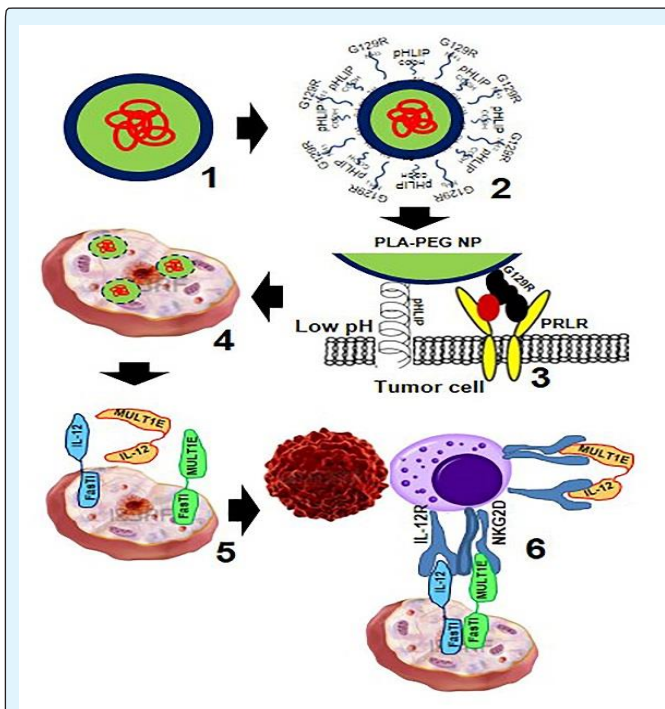
Fusion Protein Delivery into Tumor Cells

Figure 1: Immune reactivation and apoptosis induction in breast cancer using nano-technology. 1. Plasmid DNA loaded PLA-PEG-NP; 2. G129R/pHLIP conjugated, plasmid DNA loaded NPs; 3. NP binds to breast cancer cell via PRLR and pHLIP; 4. Plasmid DNAs delivered into tumor cells; 5. Bifunctional proteins expressed; 6. Immune cell activated & tumor cells killed (Tumor cells not expressing the bifunctional proteins will also be killed by activated immune cells).

Although the above mentioned individual fusion cytokine proteins all demonstrated their enhanced anti

cancer activities, challenge remains how to effectively deliver multiple fusion gene constructs simultaneously and specifically to tumors. Nanotechnology has been shown to be able to deliver various molecules, including small drug molecules, peptides, protein-based drugs, and nucleic acids into cells [31-33]. Plasmid DNA can be efficiently encapsulated into polylactide-co-polyethyleneglycol (PLA-PEG) nanoparticles (NPs) by controlling processing/formulation parameters [34-40]. GenexolPM, composed of biodegradable and biocompatible PLA-PEG, has been approved in Korea for breast cancer therapy and in clinical trials in the US for multiple types of cancer treatments including breast cancer [41,42]. Using breast cancer as a model, we propose to use PLA-PEG as the core to load with plasmid DNAs of multiple bifunctional fusion gene constructs to develop a unique NP based, breast cancer specific gene delivery system (Figure 1).

Conclusion

Immunotherapy is considered a dream treatment for metastatic cancers due to immune system's specificity and effectiveness. The potential impact of cancer immunotherapy and the importance of understanding and modulating the complex interplay among cell types and signaling pathways in the tumor microenvironment have been well recognized by the research field and the public. However, the currently available cancer immunotherapeutic approaches are generally inefficient and by an large ineffective due to lack of systematic strategies targeting at the immune suppressive tumor microenvironment, a hall mark of metastatic cancer. We are confident that an effective delivery of multi-functional fusion cytokine proteins into tumors will provide cancer patients with a new hope.

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