High Shed Antigen Levels within Tumors: An Additional Barrier to Imunoconjugate Therapy

Yujian Zhang and Ira Pastan

Abstract
Shedding of cell surface antigens is an important biological process that is used by cells to modulate responses to signals in the extracellular environment. Because antibody-based therapies of cancer target cell surface antigens, it is important to understand more about the shedding process and how it affects tumor responses to this type of therapy. Up to now most attention has been focused on measuring the concentration of shed antigens in the blood and using these to determine the presence of a tumor and as a measure of response. The recent finding that the concentration of the tumor antigen mesothelin is extremely high within the interstitial space of tumors, where it can block antibody action, and that the concentration of shed mesothelin within the tumor is lowered by chemotherapy has important implications for the successful treatment of solid tumors by immunoconjugates and whole antibodies.

Background
Antibody-based therapies now play a major role in the treatment of cancer (1). Because there are barriers that limit the penetration of these large proteins into solid tumors, antibodies are usually given in very large amounts to try and reach all cells in the interior of the tumor (2, 3), but in some cases, large amounts of antibody cannot be given because of undesirable side effects. Antibodies or antibody fragments are also used to deliver radioisotopes, cytotoxic drugs, and protein toxins to tumors. These immunoconjugates cannot be given in as large amounts as naked antibodies because of the nonspecific side effects of the radioisotope, drug, or toxin on normal tissues. The inability to give large amounts of immunoconjugates has limited their efficacy against solid tumors (4, 5). This review discusses the role of antigen shedding in diminishing the responses to antibodies and immunoconjugates and approaches to lower the concentration of shed antigen in the tumor.

Shedding of Cell Surface Antigens
Shedding of cell surface proteins, also known as ectodomain shedding, is a process used by cells to modulate the function of surface proteins. Shedding is usually due to limited proteolysis; although phosphatidylinositol (PI)-linked proteins may also be released by activation of phospholipases. Virtually all structural and functional categories of membrane proteins have been found to be shed from cells (6, 7). These include the following:

1. Growth factors and cytokines, which are made as precursor proteins [epidermal growth factor (EGF), transforming growth factor α, HB-EGF, tumor necrosis factor (TNF)-α, Fas ligand, etc.]; many of these are involved in physiologic and pathologic processes including carcinogenesis, inflammation, cell degeneration, and apoptosis.
3. Proteins involved in adhesion and cell-cell interactions (integrins, cadherins, syndecans, mesothelin).
4. Receptors for essential nutrients (transferrin receptor, folate receptor).
5. A variety of other cell surface proteins, whose functions are not yet established. In many cases, these transmembrane proteins are processed in one or several proteolytic steps to produce the biologically active form of the protein.

Identification of specific proteases responsible for antigen shedding is a challenge because of the multitude of candidate proteases, the lack of a consensus motif for cleavage, the lack
of specific protease inhibitors, and the complex regulation of protease activity. The first protease shown to carry out a specific cleavage is tumor necrosis factor α converting enzyme (TACE) or ADAM17, which releases active TNF-α from the cell membrane (8, 9). TACE belongs to the disintegrin and metalloproteinasen (ADAM) family, which shares a metalloproteinasen domain with matrix metalloproteinasen. It is now recognized that ADAMs are major players in the shedding process (10, 11). ADAM17/TACE and ADAM10 participate in the shedding of many cytokines and receptors (12). These include many members of the TNF superfamily (TNFR1, TNFR2, CD30, and CD40), the extracellular domains of growth factors receptors (erbB2 and erbB4), cytokine receptors (IL-1RI, IL-6R α chain, IL-15R α chain, and c-kit), growth factors and cytokines (EGF, HB-EGF, and transforming growth factor α), adhesion molecules (L-selecctin, VCAM-1, and CX3CL1), as well as other important molecules (Notch, macrophage colony-stimulating factor, neurotrophin receptor, CD44, and RANK ligand). ADAM17 also regulates growth hormone signaling by releasing the extracellular domain of growth hormone receptors (13). The matrix metalloproteinasen family of proteases is also involved in the shedding process. Overall the shedding process is complex with different sheddases recognizing the same substrate protein and different substrates being hydrolyzed by the same sheddase.

Because of the importance of sheddases in various disease processes, many inhibitors have been developed and tested in the clinic. Both macromolecular inhibitors [endogenous tissue inhibitor of metalloproteinasen (14) and monoclonal antibodies (15)] and small molecules have been considered as potential therapies. But most of the inhibitors that have been developed are small molecules with either high or low selectivity. Unfortunately, the results of clinical trials have been disappointing possibly because of the lack of specificity of the inhibitors evaluated. TACE has been shown to play an important role in inflammatory processes and cancer development, and 3 TACE inhibitors [Ro 32-7315 from Roche (16), TML-1 from Wyeth (17), and GW3333 from GSK (18)] were evaluated in clinical trials and showed disappointing results despite efficacy in animal models. It is still possible that the development of more selective inhibitors of other proteases may show some clinical activity.

The importance of the shedding process in biological function implies that the proteolytic activity must be under strict regulation, and it has been classified into two processes, constitutive shedding and regulated shedding. Constitutive matrix metalloproteinasen 7 activity is responsible for the basal level of proTNF-α shedding in macrophages, and TACE cleaves proTNF-α in response to an activator (19). It has been found that shedding is frequently regulated by phorbol esters (PMA) through PKC, although PKC-independent mechanisms also exist. In addition, mitogen-activated protein kinase has a role in the shedding of many important proteins, such as HB-EGF, transforming growth factor α, TNF-α, c-Met receptor, etc., and this pathway is triggered by growth factors and cytokines. The level of intracellular calcium is also thought to have an important role, as shown in the shedding of L-selecctin (20).

The levels of sheddases (metalloproteinasen) can be regulated at three levels, transcription, proenzyme activation, and inhibition of activity. The expression level and pattern of metalloproteinasen expression are modulated by cytokines and growth factors, such as TNF-α, IL-1, and transforming growth factor β. This is carried out through signaling pathways, such as p38 mitogen-activated protein kinase, affecting transcription factor AP1 and many others (21). Single-nucleotide polymorphisms (22) and DNA methylation (23) also play a role in the regulation. Most metalloproteinasen are synthesized as zymogens, so zymogen activation is also an important regulatory step. The prodomain that keeps sheddases inactive can be removed by proprotein convertases (furin, PC7, PC6, and PACE) or other metalloproteinasen (6). Natural metalloproteinasen activity inhibitors also exist, including general inhibitors, such as α2-macroglobulin, and more specific ones such as tissue inhibitor of metalloproteinasen (24). These have an important role in modulating proteolytic activity at the protein level.

### Clinical Translational Advances

**Clinical studies.** A large number of monoclonal antibodys that react with antigens on the surface of cancer cells have been investigated in clinical trials, but to date, only the Food and Drug Administration has approved five and two of the five target the EGF receptor (1). In addition, two radiolabeled antibodies targeting CD20 are approved for non-Hodgkin’s lymphoma, an immunoconjugate of an anti-CD33 antibody with calicheamicin (gemtuzumab ozogamicin/Mylotarg) is approved for recurrent acute myelogenous leukemia, and an IL2-dipherther toxin fusion protein (Ontak) is approved for cutaneous T-cell lymphoma. The targets of all these therapeutic proteins are shed from the cancer cells and are present in the blood usually in the pg/mL or ng/mL range (25–28). Because large amounts of monoclonal antibodys are given to treat cancer, the blood levels of the antibodies are often over 100 μg/mL so that neutralization of the antibody by soluble antigen is not a significant factor. These concentrations are believed to be high enough to enable the antibody to reach all the cells within solid tumor masses.

In contrast, the amounts of immunoconjugates, which can be safely given to patients, are much lower because they exert toxic effects on normal cells, such as bone marrow suppression with antibodies carrying radioisotopes (29, 30),

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Fig. 1. A, cancer cells contain many different proteases capable of releasing cell surface antigens as well as phospholipases that can release PI-linked proteins, although such proteins may also be released by proteases. Activation of protein kinase C, various signaling pathways, and the level of intracellular calcium can modulate the activity of these proteases. Shredding can be promoted by treatment of cells with tissue plasminogen activator and inhibited by specific protease inhibitors. B, illustrates a representation of a microscopic section of a cancer showing a nest of cancer cells surrounded by a capillary supplying nutrients and capable of delivering an antibody or immunoconjugate (top left). Antigen is present on the surface of the cancer cells, and shed antigen is present at high concentrations in the ECF and much lower concentrations in the afferent capillary reflecting the average concentration in the blood. Top right, how chemotherapy decreases the number of tumor cells, disrupts their organization, and lowers shed antigen levels. Bottom left, the barriers to immunoconjugate entry, which are tight cell packing producing a so-called site barrier and shed antigen, which acts as a decoy binding immunoconjugate. Bottom right, immunoconjugate can now access more or all tumor cells when the site barrier and shed antigen is removed.
solute antigens and immunoconjugates. In addition, the rapid binding of immunoconjugates to cells in the blood can greatly reduce the amount of immunoconjugate reaching cells in lymph nodes or packed bone marrows. 

Solid tumors. As summarized above, the entry of antibodies and antibody-derived therapeutics into tumor masses and also, in some cases, into tumor-packed bone marrow, is limited by a site barrier due to the close packing of tumor cells, high interstitial pressure within tumors, and a lack of functional lymphatics. These serve as a barrier to the entry of antibodies and immunoconjugates into the interior of solid tumors. Despite this barrier, radiolabeled radioimmunoconjugates have been found to be active in lymphomas most likely because the cells are very radiation sensitive, and because the isotopes used (Y-90/I-131) are beta emitters that can kill nearby cells that have not bound the immunoconjugate by crossfire. In marked contrast, carcinomas are very radiation resistant, and it has not been possible to achieve sufficient levels of radioactivity in the tumors to produce clinical benefit.

Combinations of antibodies and chemotherapy. In an effort to improve responses, antibodies have been combined with various types of chemotherapy or radiation therapy. In some cases, the combination has produced a useful increase in patient response or survival, but in many cases, it has not. The mechanism by which combining different agents results in improved activity have not yet been clarified.

Shed antigen in tumors. Our laboratory has been involved in the development of recombinant immunotoxins, a type of immunoconjugate in which a tumor-specific Fv is fused to a bacterial toxin. The Fv binds to the cells and the immunotoxin is internalized by endocytosis enabling the toxin to reach the cytosol and kill the target cell. Using an immunotoxin (BL22) targeting CD22-expressing cells, many complete remissions were obtained in drug-resistant hairy cell leukemia. In this disease, the leukemic cells are in the blood, spleen, and bone marrow and quite accessible to the immunotoxin. There has been much less success in targeting solid tumors. For example, in a recent trial targeting mesothelin expressing cancers with immunotoxin SS1P, only minor responses were observed, yet both of these immunotoxins, BL22 and SS1P, have similar cytotoxic activities on cancer cells isolated from patients (IC50, 1-10 ng/mL). It is very likely that poor tumor entry is a major factor limiting the activity of SS1P, and several strategies are being pursued to overcome the entry barrier and improve immunotoxin uptake.

One approach is to combine immunotoxins with chemotherapy. When immunotoxin SS1P was combined with Taxol in mice with human tumor xenografts, remarkable synergy was observed with many complete tumor regressions. In contrast, when Taxol with SS1P in cell culture, only minor additive effects were observed. These findings indicated that the synergistic response was due to some special property of the solid tumor. In the initial synergy experiments, Taxol was combined with SS1P, but to be certain that synergy was a common event and not just observed with one immunotoxin and one tumor type, studies were carried out with three different immunotoxins reacting with three different target antigens on three different types of tumors using six different chemotherapeutic agents.

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are elevated in the blood of humans and mice with mesothelin expressing tumors. The levels of shed mesothelin in the tumor exceed in that by >20-fold and can act as a decoy to prevent immunocjugates and antibodies from reaching cells in the interior of solid tumors. Chemotherapy causes a dramatic decrease in mesothelin levels and allows more effective immunotherapy. We propose that shed antigen within tumors constitutes an unrecognized and important barrier to antibody-based therapies. Reduction of shed antigen by chemotherapy or possibly by protease inhibitors should enhance the efficacy of immunocjugate therapies.
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