High Shred Antigen Levels within Tumors: An Additional Barrier to Immunoconjugate Therapy

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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 metalloproteinase (ADAM) family, which shares a metalloproteinase domain with matrix metalloproteinases. 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 TNFR super family (TNFR1, TNFR2, CD30, and CD40), the extracellular domains of growth factors receptors (erbB2 and erbB4), cytokine receptors (IL-1RII, 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-selectin, 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 metalloproteinase 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 metalloproteinases (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), TMI-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 metalloproteinase 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-selectin (20).

The levels of sheddases (metalloproteinases) can be regulated at three levels, transcription, proenzyme activation, and inhibition of activity. The expression level and pattern of metalloproteinase 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 metalloproteinases are synthesized aszymogens, 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 metalloproteinases (6). Natural metalloproteinase activity inhibitors also exist, including general inhibitors, such as α2-macroglobulin, and more specific ones such as tissue inhibitor of metalloproteinases (24). These have an important role in modulating proteolytic activity at the protein level.

### Clinical Translational Advances

**Clinical studies.** A large number of monoclonal antibodies 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 immunonjugate of an anti-CD33 antibody with calicheamicin (gemtuzumab ozogamicin/Mylotarg) is approved for recurrent acute myelogenous leukemia, and an IL2-diphtheria 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 antibodies 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 immunonjugates, 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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**Table 1.** Synergy was observed with three different tumors, three immunotoxins, and six chemotherapies

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Target</th>
<th>Immunotoxin</th>
<th>Chemotherapy</th>
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<tr>
<td>A431/K5</td>
<td>Mesothelin</td>
<td>SS1P</td>
<td>Taxol</td>
</tr>
<tr>
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<td>CA46</td>
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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. Shedding 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.
liver thrombosis with Mylotarg (31), and liver toxicity with Ontak (32). In addition, Campath itself causes bone marrow suppression so that the maximum tolerated dose of Campath is much less than that of other antibodies now in clinical use (33). Because of these low doses, soluble antigen levels can be high enough to interfere with the action of immunocompounds. In addition, the rapid binding of immunocompounds to cells in the blood can greatly reduce the amount of immunocompound reaching cells in lymph nodes or packed bone marrows (34, 35).

**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 (2, 36). These serve as a barrier to the entry of antibodies and immunocompounds into the interior of solid tumors. Despite this barrier, radioimmunocompounds 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 β emitters that can kill nearby cells that have not bound the immunocompound by crossfire (37). 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 (38). 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 (39, 40).

**Shed antigen in tumors.** Our laboratory has been involved in the development of recombinant immunotoxins, a type of immunocompound 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 (41). Using an immunotoxin (BL22) targeting CD22-expressing cells, many complete remissions were obtained in drug-resistant hairy cell leukemia (34). In this disease, the leukemic cells are in the blood, spleen, and bone marrow and quite accessible to the 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 (Table 1). In all cases, synergy was observed indicating the response is quite general. Only when the tumor was drug resistant was synergy not observed (47). Because it is known that entry of antibodies and immunocompounds into solid tumors is poor (2), the effect of chemotherapy on immunotoxin uptake by the tumors was measured, and no increase in total immunotoxin uptake was detected, indicating another mechanism was needed to explain synergy (46).

It has been found that mesothelin, like many tumor antigens, is shed into the blood and that mesothelin levels in the blood are elevated in many patients with mesothelioma and ovarian cancer (48, 49). Because the tumor is the source of mesothelin, we hypothesized that the levels of shed mesothelin within the tumor were much higher than in the blood and could act as a decay and block immunotoxin action more effectively than antigen in the blood. Using a method developed by Wiig et al. (50) to measure albumin present in the extracellular fluid (ECF) of tumors, the levels of shed mesothelin in the ECF of tumors was measured and found to be extremely high, up to 100 nmol/L, greatly exceeding the amount of immunotoxin in the tumor (up to 10 nmol/L). In addition, soluble mesothelin was being produced and released into the blood very rapidly (47). Recently, investigations in our laboratory showed high levels of two other shed antigens in tumors: CD22 in the ECF of CA46 lymphomas and transferrin receptor in KB tumors. These results indicate that soluble antigen in the tumor ECF can present an additional barrier to the entry and activity of immunocompounds.

**Control of antigen in ECF.** The levels of mesothelin within the ECF of KB tumors were measured before and after Taxol treatment, and were found to decrease dramatically over the 2- to 5-day period after treatment as tumor cells underwent apoptosis (47). Presumably, this decrease in shed antigen is due to the slowing of synthesis by dying and dead tumor cells and the increased transfer of antigen from the ECF into the blood. These changes combine to allow the immunotoxin to reach more cells within the tumor accounting for the synergistic interaction of immunotoxins with chemotherapy. Another way to decrease the release of shed antigen is to use specific protease inhibitors (51, 52). Figure 1 illustrates some of the important steps involved in the pathway of antigen shedding and how shed antigen and the tight packing of tumor cells can act as a barrier to the entry of antibodies and immunocompounds into solid tumors.

**Summary**

Antigen shedding is a common biological event and tumor cells shed many antigens into the blood. Mesothelin levels

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1 Y. Zhang, R.J. Kreitman, and I. Pastan, unpublished data.
References


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