Improved Circulating Half-Life and Efficacy of an Antibody-Interleukin 2 Immunocytokine Based on Reduced Intracellular Proteolysis

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ABSTRACT

Purpose: Previous studies showed that reducing the interaction of antibody-interleukin 2 immunocytokines with Fc receptors improved their circulating half-life in mice and increased their antitumor activity. We sought to modify sequences that would increase half-life but retain the ability to activate Fc receptor-mediated effector functions.

Experimental Design: Modified immunocytokines were assessed in vitro for effector function and protease sensitivity and in vivo for pharmacokinetic and antitumor activities in an syngeneic tumor regression model.

Results: Single amino acid changes in the junction sequence between the antibody and interleukin-2 components had dramatic effects on circulating half-life and antitumor activity. This effect was independent of Fc receptor binding to either Fcγ receptors or the Fc protection receptor, but was associated with changes in susceptibility to intracellular proteases.

Conclusions: We have identified modifications that dramatically improve the circulating half-life of immunocytokines based on increased resistance to intracellular degradation and thus have demonstrated how these molecules can be recycled in and out of an intracellular compartment. Use of these improved immunocytokines with highly favorable pharmacokinetic properties and retained effector functions should lead to more effective treatment of epithelial cancers.

INTRODUCTION

In our previous studies we showed that IL-22 fused to the COOH terminus of an antibody decreases its serum half-life in mice and that this is attributable to an increased affinity for FcR (1). To increase the efficacy of IL-2 immunocytokines, we changed the H-chain isotype to those with low affinity for FcR, and indeed, serum half-life was dramatically improved. For human clinical studies it might be advantageous to retain effector functions that are lost by the use of either the Cγ2 or the Cγ4 H-chain isotype. Therefore, we aimed to test whether it is possible to maintain FcR binding (and effector function) and still have a favorable pharmacokinetic profile.

Because retention of the FcR binding site would promote entry into cells bearing this receptor, we thought it might be possible to try to promote recycling of the immunocytokine back into the circulation. This process is known to occur naturally with antibodies through intracellular binding to the FcRnp at the acidic pH of endosomal vesicles and release back outside the cell at physiological pH (2, 3). FcRnp is closely related to MHC class I molecules and requires heterodimerization with β2-microglobulin for functional binding and protection of IgG.

This report describes how simple mutations in the region joining the COOH terminus of the human Fc with the mature NH2 terminus of IL-2 can dramatically change the circulating half-life of an immunocytokine molecule without the loss of antibody effector functions and can improve efficacy in mouse tumor models. We also examine the mechanism through which these mutations promote a sustained half-life, presumably by promoting recycling of the antibody-based fusion protein from an intracellular to intravascular compartment.

MATERIALS AND METHODS

Expression and Purification of Mutated IL-2 Immunocytokines. The expression vector for immunocytokines was described previously (4). In the human γ-1 heavy chain gene, the XbaI restriction site located 280 bp upstream of the translation stop codon was destroyed by introduction of a silent mutation (TCC to TCA). Another silent mutation (TCT to TCC) was introduced to the Ser codon three residues upstream of the COOH-terminal lysine of the heavy chain to create the sequence TCC CCG GGT AAA, which contained a new XbaI site (5). The IL-2 cDNA was constructed by chemical synthesis and contained a new and unique PvuII restriction site (6). Both the XbaI and PvuII sites were unique in the expression vector and facilitated mutagenesis of the junction of the CH3 exon and the IL-2 DNA. Substitutions, insertions, or deletions in this junction were achieved by replacing the XbaI-PvuII fragment in the immunocytokine expression vector with oligonucleotide duplexes encoding the desired mutations. The variable regions of the heavy and light chains were derived from the humanized KS antibody (4), which recognize the human EpCAM antigen (7). Another set of molecules was generated by replacing the KS V regions with those of the anti-GD2 antibody, 14.18 (8).

To obtain stably transfected clones, we introduced plasmid DNA into the mouse myeloma NS/0 cells by electroporation.
Stably transfected clones were selected by growth in the presence of 100 nM methotrexate, which was introduced 2 days post-transfection. For purification, the fusion proteins were first bound on protein A-Sepharose and then eluted in a sodium phosphate buffer [100 mM NaH2PO4 (pH 3), 150 mM NaCl]. The eluate was then immediately neutralized with 0.1 N NaOH. Purified proteins were diafiltered into PBS and stored at −20°C until use.

**Pharmacokinetic Studies.** The fusion proteins were tested for their pharmacokinetic behavior after i.v. injection into BALB/c (Taconic Farms, Germantown, NY) or transgenic β2-microglobulin knockout mice (Jackson Research Labs, Bar Harbor, ME). Protein concentrations of dosing solutions were determined by absorbance measurements, and 25 μg of each protein were injected into groups of four mice. At various times after dosing, blood was collected from mice by retro-orbital bleeding in heparinized Eppendorf microcentrifuge tubes. After centrifugation to remove cells, the sera were stored frozen until tested by ELISA. To determine the concentration of the intact antibody-IL-2 fusion protein, an IL-2-detection ELISA was used. The ELISA consists of a capture step with goat antihuman IgG (Jackson ImmunoResearch) and a detection step with an antibody directed against IL-2 (PharMingen, San Diego, CA) as described (1). To determine the total concentration of injected protein (intact immunocytokine and cleaved antibody), a human IgG ELISA was used as described previously (1).

For data analyses, kinetics of the initial (distribution) phase were characterized because this is the phase during which the majority of each protein is removed from circulation, and the initial half-life ($t_{1/2a}$) has a dominant influence on total systemic exposure. To assess total exposure, the area under the concentration vs. time curve was measured up to 8 h ($AUC_{0-8}$) for each protein because 8 h provided the latest time point with consistently reliable concentration data. For the individual kinetic profiles, concentration data from all 10 proteins were normalized to the original KS-IL2 molecule because this was the protein for which the ELISA was optimized.

**FcR Binding and Effector Activity Assays.** Binding of immunocytokines to FcγRII-expressing mouse J774 cells was measured as described (1). Effector functions were measured using the hu14.18-IL2 immunocytokines and the GD2-positive human melanoma M21. For ADCC, 51Cr-labeled cells were incubated alone or with dilutions of the immunocytokines and a fixed number of resting peripheral blood mononuclear cells (100:1 E:T ratio) for 4 h at 37°C. For CDC activity, labeled cells were incubated with dilutions of hu14.18-IL2 proteins as well as human plasma (1:8 dilution) as a source of complement. In this case, plates were incubated for 1 h at 37°C. The percentage of specific lysis was calculated by subtracting the background radioactivity from the experimental values, dividing by the total releasable radioactivity obtained with detergent lysis, and multiplying by 100.

**Protease Sensitivity Assays.** KS-IL2 and KS-ala-IL2 (4 μg) were treated with 0.1 or 0.4 milliunits of cathepsin D (Sigma Chemical Co.) for 16 h at 37°C and analyzed by SDS-PAGE. Buffer conditions were according to the manufacturer’s instructions. In a second experiment, 4 μg of KS-IL2 and KS-ala-IL2 were treated with 25 or 50 milliunits of cathepsin L (Enzyme Systems Products, Livermore, CA) for 16 h at 37°C and analyzed by SDS-PAGE.

**Syngeneic CT26-EpCAM Tumor Model.** Mouse colon carcinoma cells expressing human EpCAM (4) were injected s.c. in the backs of BALB/c mice (2 × 10⁶ cells/injection) and observed for rapid tumor growth. Mice with tumors that reached 100 mm³ within 10 days were randomized and treated with PBS, KSγ1-IL2, KSγ1-ala-IL2, or KSγ4-IL2 by i.v. injection for 5 consecutive days. No further treatment was given, and tumor growth or regression was followed until the control mice required sacrifice because of tumor burden. Tumor volumes were measured with calipers and calculated as $V = 4π/3(0.5l × 0.5b × 0.5w)$, where $l$ is the length, $b$ is the height, and $w$ is the width of the tumor.

Mice that had complete tumor regressions were challenged with a lethal dose (1 × 10⁵) of parental CT26 cells on day 60 by i.v. injection. Animals were sacrificed 28 days later, and their lungs were examined for evidence of metastases. Naïve mice served as controls, and all had extensive metastases at this time.

**RESULTS**

We reasoned that two possible mechanisms might account for the decreased half-life of IL-2 immunocytokines once they are taken up by cells: decreased affinity for the protection receptor, FcRν, or increased proteolysis within the intracellular compartment. Although many groups have attempted to mutate the Fc region to increase the affinity of IgG to FcRν, we reasoned that the simplest solution might be to reduce the protease sensitivity of the region between the Fc and IL-2 components. This is based on our earlier studies showing that an immunocytokine containing granulocyte-macrophage colony-stimulating factor was more resistant to proteolytic cleavage at the fusion protein junction than those containing IL-2 or tumor necrosis factor and also had a longer circulating half-life (9). In fact, our first attempt to reduce proteolysis through mutation of the terminal Lys residue to Ala (K-to-A mutant) produced a molecule with a dramatically improved half-life but full retention of affinity for cell-bound FcγRI (Fig. 1). This IL-2 immunocytokine, KS-IL2, targets the human EpCAM molecule expressed on most cancers of epithelial origin (7).

We tested whether the K-to-A mutation reduced effector functions by constructing and testing a second IL-2 immunocytokine, using the V regions of the anti-GD2 antibody 14.18. This antibody mediates both ADCC and CDC activity against GD2-positive tumor cells, whereas the KS-IL2 immunocytokine mediates ADCC, but not CDC. As shown in Fig. 2 there was no significant reduction of ADCC activity and only a minor reduction of CDC activity.

We next tested the effects that other amino acid substitutions in this position have on pharmacokinetic behavior in mice. To do this we determined concentration-time profiles for the different proteins normalized to the initial concentration of KS-IL2. This was necessary because each protein had a slightly different reactivity in the ELISA used to measure the concentration in serum samples. Table 1 shows the systemic exposure and initial half-life of each KS-IL2 variant calculated for each protein. The AUC from the initial injection time until 8 h ($AUC_{0-8}$) varies directly with $t_{1/2a}$, indicating that changes in
the distribution of the proteins (e.g., by differences in cellular uptake) likely account for the differences in systemic exposure. We compared other hydrophobic residues, polar and negatively charged substitutions, and flexible linkers of various lengths (Fig. 3). The results indicate that molecules with hydrophobic residues tended to have better serum half-lives, whereas molecules containing another positively charged residue (Arg), Cys, or flexible linker had shorter half-lives. A negatively charged residue (Asp) had a positive effect on half-life, compared with Lys, but was not as good as the hydrophobic replacements (Leu, Ala, or Ala-Ala-Ala). The Lys-to-Gly mutant was somewhat better than the original molecule but still not as good as the hydrophobic substitutions. These results suggest that pharmacokinetic behavior may be determined by the sensitivity of this region to intracellular proteases; however, another interpretation is that the introduction of hydrophobic residues alters binding to FcRp.

To test whether FcRp plays a role in this phenomenon, we compared the clearance of selected molecules in normal and /H9252 2-microglobulin knockout mice lacking expression of FcRp (3). We included Lys-to-Ala mutant molecules constructed with either the /H9253 1 or /H9253 4 isotype, differing in their binding to Fc /H9253 R. The data showed that FcRp plays a dramatic role in determining the half-life of all of the molecular forms (Fig. 4). The results are expressed as distribution rates and exposure measurements (AUC 0–8) to best reflect the differences in pharmacokinetic behavior (Table 2). Because the relative orders of the individual clearance rates and exposure were not changed, it is unlikely that differences in FcRp binding affinity are responsible for the

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mouse strain</th>
<th>AUC 0–8 (µg·h/ml)</th>
<th>t1/2a (h)</th>
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<td>Balb/c</td>
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<td>KS /H9253 1-ala-IL2</td>
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<td>0.53</td>
</tr>
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<td>KS /H9253 1-IL2</td>
<td>Balb/c</td>
<td>26.6</td>
<td>0.54</td>
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<td>KS /H9253 1-IL2 (K substitutions)</td>
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<td>KS /H9253 1-IL2 11acc</td>
<td>Balb/c</td>
<td>10.0</td>
<td>0.24</td>
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Fig. 1 Pharmacokinetic and FcR binding properties of KS-IL2 with a modified junction sequence. The original KS-IL2 (△) and the KS-ala-IL2 (■) mutant were compared in terms of their clearance rate from the circulation after i.v. injection (A) as well as their ability to bind the FcyRI-expressing mouse macrophage line, J774 (B). Intact immunocytokines in the blood were measured in the blood by capturing them with an EpCAM antigen-coated plate and detecting them with an anti-IL-2 antibody. Conc., concentration.

Fig. 2 Effector function activities of 14.18-IL2 with a modified junction sequence. 51 Cr-labeled, GD2-positive M21 melanoma cells were used as targets together with increasing concentrations of either the original 14.18-IL2 (γ1 isotype; △) or the modified 14.18-ala-IL2 form (○) as indicated. The nonbinding KS-IL2 immunocytokine (□) was used as a negative control. Cells and antibodies (Ab) were mixed with either resting peripheral blood mononuclear cells, for detection of ADCC (A), or with human plasma, for detection of CDC activity (B). Conc., concentration.
The effect of this mutation. Direct comparisons of individual molecules in FcRp-defective mice demonstrate the independent effects of the Lys-to-Ala mutation and the isotype switch from high to low FcR-H9253 binding. For example, the difference in behavior between the δ1-IL2 and δ4-IL2 forms shows a striking difference in the time required to reach two logs of clearance (99% removal). Presumably this reflects the relative differences in the binding and uptake of the molecule into FcγR-bearing cells. The differences between the Lys and Ala forms of both isotypes are even more striking, especially in FcRp knockout mice, clearly showing that the effect of this mutation is independent of intracellular protection via this receptor.

We next tested whether immunocytokines with such altered pharmacokinetic properties had differential susceptibility to intracellular proteases. We treated the purified proteins with increasing concentrations of cathepsin D or L for 16 h and analyzed protein integrity by SDS-PAGE. The data showed a clear decrease in susceptibility to both of these proteases as a consequence of the single Lys-to-Ala mutation (Fig. 5). Interestingly, the reduced sensitivity delayed the degradation of multiple cleavage sites within the molecule, including degradation of the light chain by cathepsin L. These results suggest that an intracellular degradation pathway is responsible for the changes in pharmacokinetic properties and not changes in binding to FcRp.

Finally, we compared the relative antitumor activities of the normal and Lys-to-Ala mutant IL-2 immunocytokines in mice. In these syngeneic models, we first transfected the mouse

Table 2  Distribution and exposure measurement in Balb/c and FcRp-deficient mice

<table>
<thead>
<tr>
<th>Protein</th>
<th>AUC_{0-8} (μg·h/ml)</th>
<th>t_{1/2} (h)</th>
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<tbody>
<tr>
<td></td>
<td>Balb/c mice β2M⁻/⁻</td>
<td>Balb/c mice β2M⁻/⁻</td>
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<tr>
<td>KS Ab</td>
<td>105.3</td>
<td>110.9</td>
</tr>
<tr>
<td>KS δ1-IL2</td>
<td>13.1</td>
<td>2.8</td>
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<tr>
<td>KS δ1-ala-IL2</td>
<td>31.0</td>
<td>7.6</td>
</tr>
<tr>
<td>KS δ4-IL2</td>
<td>26.6</td>
<td>5.2</td>
</tr>
<tr>
<td>KS δ4-ala-IL2</td>
<td>41.8</td>
<td>13.2</td>
</tr>
</tbody>
</table>

a β2M, β2-microglobulin.
tumor cell lines with the human EpCAM antigen, the target of the KS-IL2 immunocytokine (4). Tumor cells were transplanted s.c. and allowed to establish for ~10 days prior to treatment. A single 5-day treatment cycle of daily i.v. injections was used, and mice were observed without further therapy until the control mice required sacrifice. We chose an immunocytokine dose based on our data with the original KS-IL2 and selected one that had only a limited effect on tumor growth. A direct comparison of KS-IL2 and the Lys-to-Ala mutant form (KS<sub>g1</sub>-ala-IL2) using relatively low dosing (10 µg doses) demonstrated a dramatic improvement in efficacy with complete regression in all mice treated with KS<sub>g1</sub>-ala-IL2 (Fig. 6). This effect is likely attributable to the increased half-life of the immunocytokine because, as shown in our previous studies (1), KS<sub>g4</sub>-IL2 demonstrates similar efficacy in this model despite its lack of effector function. Thus, the improved efficacy is attributable to improved targeting of IL-2 to the tumor microenvironment and not changes in effector activity that may have resulted from the mutation.

The mice treated with KS<sub>g1</sub>-ala-IL2 that had complete regression of their primary tumors resisted subsequent challenge with an i.v. dose of the parental CT26 cell line given at day 60 (complete absence of lung metastases; data not shown), strongly suggesting that a T-cell-mediated immune memory response developed as a consequence of the treatment.

The KS<sub>g1</sub>-ala-IL2 form was also more potent in an experimental pulmonary metastasis model using an EpCAM-transfected line of Lewis lung carcinoma (LLC-Ep). Tumor cells were injected i.v. and allowed to establish for 5 days. A single 5-day treatment cycle of daily i.v. injections was used to compare the normal Lys and mutant Ala forms of KS-IL2. Lung metastases were examined on day 24 and compared with those in mice receiving only PBS injections (Table 3). By this time control mice had tumor burdens consisting of nearly three-fourths of the total organ weight. Both immunocytokines had a significant effect on the number and size of lung metastases, reducing the lung weights to within the range of tumor-free mice, but the KS<sub>g1</sub>-ala-IL2 showed a significant difference (P < 0.05) from both the control and the normal KS-IL2 molecule treatment groups (Table 1), when the number of surface metastases were compared.

DISCUSSION

A large body of evidence supports the efficacy of IL-2-based immunocytokines in mouse models of tumor metastases (10–12). In most cases, established micrometastases could be eradicated with a single 5–7-day course of daily injections, and in immunocompetent mice, this leads to the development of T-cell memory, which protects mice from subsequent tumor challenge (13). More recently we have been testing whether larger, established tumors could be treated with this approach. Results indicate that effective treatment of established s.c. tumors requires that the immunocytokine have a longer circulating half-life (1). IL-2 immunocytokines constructed with human γ4 H chains have much improved circulating half-lives because of their reduced affinity for FcR, which in turn reduces the uptake by organs expressing this receptor (primarily the liver). Along with the increased half-life is a marked increase in antitumor activity, despite the loss of antibody effector functions of
ADCC and CDC. We have also found that immunocytokines constructed with the γ2 H chain, which has a lower affinity for FcR, show even longer half-lives and improved efficacy relative to γ4.3

How mouse efficacy data translate into the human clinical situation is always an open question, and one cannot assume that antibody effector function in our IL-2 immunocytokine will be as unnecessary for human therapy as it appears to be in mice. Therefore, we feel more confident developing a molecule that shares both the extended circulating half-life properties of the γ2 and γ4 versions of KS-IL2 as well as the potent effector functions of the original γ1 isotype. Although KSγ1-ala-IL2 and KSγ4-IL2 both have longer circulating half-lives in mice, it is more important that this translates into improvements in the clinic. In this regard we have performed preliminary studies in cynomolgus monkeys4 and found that KSγ1-ala-IL2 has a significantly longer half-life (∼25 h) than either KSγ1-IL2 (∼6 h), or any of the isotypic variants (range, 9–18 h).

The findings in our study are significant from another perspective, beyond improvements in efficacy. They suggest a role for intracellular proteases in determining the catabolic rates of antibodies and their fusion proteins in vivo that is independent of FcRp. We showed that there are at least two processes determining the rates of clearance of the various immunocytokines: one dependent on FcγR binding on the cell surface, and a second intracellular process that is independent of FcγR binding but somehow related to proteolytic cleavage at the fusion protein junction. If this process depended solely on proteolysis, we would expect to see release of free antibody back into the circulation after cleavage of IL-2.

We have observed the presence of recycled antibody in the serum of mice, but not at the levels expected for an efficient process. Rather, a large fraction (∼75%) of the injected protein is removed from the circulation in the first hour after injection, and a small fraction returns in the form of cleaved antibody. This process is altered significantly when the immunocytokine has reduced affinity for FcγR, strongly suggesting that uptake into cells is greatly reduced. It is also altered in a similar fashion when potential junctional protease cleavage sites are removed, despite the fact that FcγR binding is maintained and, presum-

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**Table 3** Tumor burden in mice with LLC-KSA tumors

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<tr>
<th>Group</th>
<th>No. of metastases</th>
<th>Lung weight (g)</th>
</tr>
</thead>
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<tr>
<td>PBS</td>
<td>&gt;250, &gt;250, &gt;250, &gt;250, &gt;250</td>
<td>0.92 ± 0.14</td>
</tr>
<tr>
<td>KS-γ1-IL2</td>
<td>62, 37, 18, 17, 11, 9</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>KS-γ1-ala-IL2</td>
<td>4, 4, 3, 3, 1, 0</td>
<td>0.25 ± 0.02</td>
</tr>
</tbody>
</table>

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REFERENCES


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3 Unpublished data.

4 Jutta Haunschild, MerckKGaA, Grafting, Germany.

5 Unpublished results.


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