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Analysis of Glycoforms and Amino Acids in Infliximab and a Biosimilar Product using New Method with LC/TOF-MS

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Summary

Biosimilar products of therapeutic antibodies have been launched all over the world. They can relieve some of the economic burden of medicines. Although clinical trials have demonstrated the equivalency of biosimilar products with their reference product, biosimilar products are not commonly used in clinical practice. One reason is that the structural difference between the reference product and a biosimilar one remains unclear.

We analyzed glycoforms and amino acids of an infliximab biosimilar product approved in Japan compared to that of the reference product (Remicade®). By combination of papain digestion and LC/TOF-MS, we established a valuable method to analyze these therapeutic antibodies. Nine glycoforms were detected in infliximab, and a difference in amino acids was observed. In the glycoforms of MMF, MGVF/GnMF, GnGn, GnGnF, AGF/GnAF, and AAF, the relative intensities were significantly different between the reference and biosimilar product. Furthermore, we elucidated that the content rate of the C-terminal lysine was different among glycoforms. In conclusion, our analytical method can analyze not only amino acids but also carbohydrate chains of therapeutic antibodies, and will provide a useful strategy to evaluate bio-medicines including biosimilar antibodies.
Keywords

Infliximab; biosimilar; glycosylation; time-of-flight mass spectrometry
1. Introduction

Biosimilar products have been developed after the expiration of patents for original biological drugs. Novel biological products cause escalating medical costs for patients and nations, and thus the prevalence of biosimilar drugs can relieve some of the economic burden of medicines. However, biosimilar products of therapeutic antibodies, unlike generic drugs, are not commonly used in clinical practice.1,2) One of the reasons is that the structure of biosimilar products is not the same as their original product because of post-translational modifications. Inevitably, the equivalency of structures, such as amino acid sequences, compositions of carbohydrate chains, and higher-order structures, in the manufacturing process should be evaluated. These structural analyses have been performed by mass spectrometry in most cases,3,4) but current analytical methods cannot provide a comprehensive overview of the structures of therapeutic antibodies.

Infliximab, original brand name Remicade®, is an anti-TNF-α antibody and widely used for patients with several inflammatory diseases. Remicade® was first approved for Crohn’s disease in 1998, and a recent expiration of patents is accelerating development of biosimilar products. Remsima®, a biosimilar infliximab, was approved for the first time by the European Medicines Agency in 2013. The similarities between the reference and
biosimilar product were determined in these trials.\textsuperscript{5-8)} For example, the PLANETRA study, which is a phase III randomized study, showed equivalent efficacy of Remsima\textsuperscript{®} with a comparable PK profile, immunogenicity, and a safety profile of the reference product.\textsuperscript{8)} Similarities of pharmacokinetics, pharmacological effects, and safety in clinical trials have also been evaluated in the infliximab biosimilar product manufactured in Japan.\textsuperscript{6, 9)} Thus, it has been demonstrated that the infliximab biosimilar product had similar potential to the reference one in clinical trials.

Some biosimilar products of infliximab have been launched all over the world. However, the structural differences between the reference and biosimilar product remain unclear. In our previous study, we developed a method to analyze monoclonal antibodies after papain digestion by liquid chromatography time-of-flight mass spectrometry (LC/TOF-MS).\textsuperscript{10)} Here, we investigated the structures of an infliximab biosimilar product approved in Japan compared to that of the reference product (Remicade\textsuperscript{®}) in order to understand the clinical implications of the biosimilar one.
2. Materials and Methods

2.1. Materials

Remicade® (Mitsubishi Tanabe Pharma, Osaka, Japan), Infliximab BS (Nippon Kayaku Co., LTD., Tokyo, Japan), and the following reagents were used: Endo S (New England BioLabs, Massachusetts, USA), L-cysteine (Nacalai Tesque, Kyoto, Japan), and papain (Roche Applied Science, Penzberg, Germany).

2.2. Deglycosylation of infliximab

Deglycosylation was performed with Endo S. In brief, 1 µL of Endo S (200 IU/µL) was added to 10 µL of infliximab solution (10 mg/mL). The mixture was incubated at 37°C for 1 h. To stop the reaction, the solution was heated at 55°C for 15 min.

2.3. Papain digestion of infliximab

Papain (10 mg/mL) was diluted to 1 mg/mL with phosphate buffer (pH 7.0) containing 10 mM cysteine. Subsequently, the solution was incubated for 10 min at 37°C, and cysteine was removed by ultrafiltration. Activated papain was added to infliximab solution (200 µg/mL) at a final concentration of 30% (w/w) and incubated at 37°C for 6 h.
In case of deglycosylated infliximab, papain digestion was performed at 37°C for 1 h. At the end of the reaction, 3% (v/v) of 0.5 M iodoacetoamide was added to the mixture.

2.4. Analysis of digested infliximab by LC/TOF-MS

The digested infliximab was analyzed by an Agilent 1200 series LC system coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, California, USA) as previously reported. Data were analyzed using MassHunter Workstation software (Agilent Technologies, California, USA). The mass spectra were deconvoluted by the maximum entropy method to calculate the molecular weight of these fragments. Relative abundance of each glycoform was represented by the ratio of each peak height in the deconvoluted mass spectrum.

2.5. Statistical analysis

Data were expressed as the mean ± standard deviation of three experiments. Statistical analysis was performed using one-way analysis of variance and the Tukey-Kramer test for multiple comparisons, or the Student’s t-test for two-group comparisons.
3. Results

3.1. Analysis of digested infliximab by LC/TOF-MS

Initially, we analyzed the reference product (Remicade®) by papain digestion and LC/TOF-MS analysis. As shown in Figure 1, papain theoretically catabolizes infliximab into 5 fragments; two Fab fragments, two Fc/2 fragments, and one small peptide. Because the molecular weight of the small peptide is smaller than 2000 Da, it was not detected in this method. Two peaks at 6.9 and 7.5 min on the total ion chromatogram were identified as the Fc/2 and Fab fragments of infliximab, respectively (Fig. 2A). Full width at half maximum of these peaks was clearly separated in each extracted ion chromatogram (Fig. 2B). Because the two mass spectra obtained from these peaks did not interfere with each other, deconvolution analysis to obtain their molecular weight was separately conducted with the spectra. In the deconvoluted spectrum of the Fc/2 fragment, multiple peaks were obtained (Fig. 3A). On the basis of the observed molecular weight, 9 types of carbohydrate chains were identified. In each glycoform, the C-terminal lysine was separately deleted. Although only four glycoforms could be visually shown in Fig. 3A, five other glycoforms were also observed by deconvolution analysis. The predictive structure and nomenclature of the carbohydrate chains (I-IX) are summarized in Figure 1 and Table 1. In the
deconvoluted spectrum of the Fab fragment, only one peak was detected at a molecular weight of 47897.99 Da (Fig. 3B). In order to analyze the infliximab structure without carbohydrate chains, deglycosylation was performed with Endo S, which is an endoglycosidase that removes N-linked glycans from the chitobiose core of the heavy chain of IgG. Four peaks corresponding to the Fc/2 fragment were detected in deglycosylated infliximab, and their molecular weights were 24516.42, 24388.38, 24274.99, and 24105.12 Da (Fig. 3C). The peaks observed at 24516.42 and 24388.38 Da were identified as the intact Fc/2 fragment and Fc/2 fragment without the C-terminal lysine, respectively. In addition, the peak at 24274.99 Da was identified as the Fc/2 fragment that had a deletion of the C-terminal lysine and N-terminal leucine in Fc/2 fragment. The peak at 24105.12 Da was the fragment generated by the deletion of the C-terminal lysine and glycine, and two N-terminal leucines from the intact Fc/2 fragment.

3.2. Comparison of Fc/2 and Fab fragments between the biosimilar and reference product

The molecular weight of the Fc/2 and Fab fragments of infliximab were compared between the reference and biosimilar product. A difference was not observed in the molecular weight. Similarly, the mean molecular weights of the Fc/2 fragments in the
The relative intensity of carbohydrate chains in the Fc/2 fragments of infliximab were compared between the reference and biosimilar product from 3 batches each. Abundance compared to all glycoforms shows the sum of both glycoform with and without lysin in C-terminal. Figure 4A shows the difference among batches for each infliximab. The ratio of MMF and AGnF/GnAF was slightly but significantly different among the 3 batches in the reference product. On the other hand, there were no significant differences among the 3 batches in the biosimilar glycoforms (Fig. 4B). Moreover, we compared the reference and biosimilar product in Figure 4C. In the glycoforms of MMF, M GnF/GnMF, GnGn, GnGnF, AGnF/GnAF, and AAF, the relative intensities were significantly different between the reference and biosimilar product. Relative intensity of Fc/2 fragments in the reference and biosimilar product was shown in Table 2.
3.3. Comparison of C-terminal lysine content rate

The deletion of C-terminal lysine usually occurs in the manufacturing process at a certain rate. We compared the C-terminal lysine content rate between the reference and biosimilar product. The mean rate in three batches of the reference product was 46.4%; on the other hand, that of the biosimilar was 35.5% (Table 3). The content rate of C-terminal lysine was significantly different between the two products. Furthermore, the content rate of C-terminal lysine was separately calculated in each glycoform (Table 3). The rates of all glycoforms except for AAF (G2F) were significantly different between the reference and biosimilar product. For each infliximab, the rate was quite different among each glycoform.
4. Discussion

Biosimilar products are required to be equivalent in biological potency and safety to the reference product, and multiple clinical trials have been conducted all over the world to ensure equivalence.\textsuperscript{5, 9, 11-14} In this study, we compared the overall structures between infliximab and its biosimilar approved in Japan. By combination of papain digestion and LC/TOF-MS, we successfully analyzed amino acids and carbohydrate chains, and detected some structural differences between the infliximab biosimilar and the reference product. Both glycoforms and amino acids were different. Our method could be a useful strategy to evaluate new bio-medicines including biosimilar antibodies.

Jung et al. previously reported that the presence of C-terminal lysine was different between Remsima\textsuperscript{®} and the reference product, but it had no effect on the biological potency and safety.\textsuperscript{15} Slight changes in manufacturing conditions can produce differences in post-translational modifications including types of glycoforms and removal of C-terminal lysines.\textsuperscript{16} Unlike previous methods,\textsuperscript{17, 18} our technique reveals differences in not only glycoforms but also amino acid residues and indicated that the content rate of C-terminal lysine in the infliximab biosimilar was significantly different from the reference product. For the first time, we also determined that the content rate of C-terminal lysine was different...
among glycoforms, which suggests that the types of carbohydrate attachment and the modification of amino acids could affect each other.

In addition to the analysis of amino acid residues, a comparison of glycoforms between infliximab and its biosimilar was conducted. We determined that the relative intensity of some types of carbohydrate chains were significantly different by comparing the biosimilar and reference product. The types and relative intensities were similar to that from a previous report. Together with the analytical result of amino acid residues, these results suggest that the analytical method in this study can be used to evaluate not only amino acids but also carbohydrate chains of therapeutic antibodies.

Interestingly, the deletion of amino acids, such as lysine, leucine, and glycine, were observed in the analysis of the deglycosylated Fc/2 fragment in both the biosimilar and reference product as shown in Table 2. In particular, two N-terminal leucines in the Fc/2 fragment were removed by papain digestion when infliximab was deglycosylated by Endo-S. In addition, the second C-terminal glycine was deleted. In the analysis of the Fc/2 fragment without deglycosylation, only deletion of the C-terminal lysine was observed and other amino acids were stable. Deglycosylation can enhance sensitivity to papain. We speculate that the selectivity of the digestion by papain was different between antibodies with and without
glycosylation.

In conclusion, we established a valuable method to analyze a biosimilar and reference product using papain and LC/TOF-MS. For the first time, we discovered that the relative intensity of some glycoforms were significantly different between the two products. Additionally, the content rate of C-terminal lysine was also different between the two products. Our method may be a useful strategy to evaluate bio-medicines including biosimilar antibodies.
Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.
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Figure Legends

Fig. 1. Schematic representation of the cleavage sites in infliximab.

Papain specifically cleaves 2 sites in infliximab and produces 3 types of fragments. The theoretical molecular weight of Fab and Fc/2 fragments are indicated. N_{300} is the only site for N-glycosylation of infliximab. The carbohydrate chains were named according to the proglycan system (www.proglycan.com).

Fig. 2. Chromatograms of the digested reference infliximab product.

(A) Total ion chromatogram of infliximab digested by papain. The peaks at 6.9 and 7.5 min were Fc/2 and Fab fragments, respectively. (B) Overlaid extracted ion chromatograms at 1,416 m/z and 1,452 m/z, which corresponded to the Fc/2 and Fab fragments ions, respectively.

Fig. 3. Deconvoluted mass spectra of the digested reference infliximab.

Typical deconvoluted mass spectra of (A) Fc/2, (B) Fab fragment, and (C) deglycosylated Fc/2 are shown. The observed molecular weight is indicated above each peak.
Fig. 4. Comparison of relative intensities of glycoforms in infliximab.

Values were calculated from the peak height of each glycoform from the deconvoluted mass spectrum. The experiments were performed independently three times. Each sample was measured in triplicate. Batch-to-batch variations were determined from 3 different batches of (A) the reference product and (B) the biosimilar. The relative intensities of MMF and A\text{GnF}/GnAF (G1F) are significantly different among three batches of the reference product. (*: $P < 0.05$, **: $P < 0.01$). (C) Comparison between the reference and biosimilar product was performed. The data for each product show the mean values of three batches. The relative intensities of glycoform of M\text{GnF}/GnMF, GnGnF (G0F), A\text{GnF}/GnAF (G1F), and AAF (G2F) were significantly different between the two products. (*: $P < 0.05$, ***: $P < 0.001$).
Table 1. Molecular weight of the glycoforms from the reference and biosimilar product.

<table>
<thead>
<tr>
<th>Carbohydrate Chain Name</th>
<th>Theoretical Fc/2 M.W.(Da)</th>
<th>Observed Fc/2 Molecular Weight (Da)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lys(-) / Lys(+)</td>
<td>Reference</td>
<td>Biosimilar</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>24915.43</td>
<td>24916.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25043.52</td>
<td>25043.46</td>
</tr>
<tr>
<td>II</td>
<td>MMF</td>
<td>25077.48</td>
<td>25078.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25205.57</td>
<td>25204.8</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>25118.51</td>
<td>25119.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25246.6</td>
<td>25247.07</td>
</tr>
<tr>
<td>IV</td>
<td>MGnF / GnMF</td>
<td>25280.56</td>
<td>25281.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25408.65</td>
<td>25408.99</td>
</tr>
<tr>
<td>V</td>
<td>GnGn (G0)</td>
<td>25337.58</td>
<td>25338.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25465.67</td>
<td>25464.86</td>
</tr>
<tr>
<td>VI</td>
<td>AMF / MAF</td>
<td>25442.61</td>
<td>25443.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25570.7</td>
<td>25569.69</td>
</tr>
</tbody>
</table>
The carbohydrate chains were named according to the proglycan system (www.proglycan.com). Observed molecular weights are the mean value of three independent experiments from 3 batches. Each glycoform molecular weight was separately analyzed depending on the existence of the C-terminal lysine. Observed molecular weights of glycoform of GnGn (G0), AGnF/GnAF (G1F), and AAF (G2F) were significantly different between the two products. (*: \( P < 0.05 \), **: \( P < 0.001 \)).
Table 2. Relative intensity of Fc/2 fragments in the reference and biosimilar product

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Theoretical molecular weight (Da)</th>
<th>Theoretical molecular weight (Da)</th>
<th>Relative intensity (%)</th>
<th>Relative intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reference</td>
<td>Biosimilar</td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>24516.42</td>
<td>36.3 ± 3.2</td>
<td>27.0 ± 0.4**</td>
<td></td>
</tr>
<tr>
<td>K(-)</td>
<td>24388.33</td>
<td>43.5 ± 6.1</td>
<td>49.8 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>K(-)L(-)</td>
<td>24275.25</td>
<td>15.7 ± 1.9</td>
<td>18.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>K(-)G(-)LL(-)</td>
<td>24105.14</td>
<td>4.6 ± 1.0</td>
<td>5.2 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

The percentages are expressed as the mean ± standard deviation of three independent experiments from 3 batches. K(-), the Fc/2 fragment without C-terminal lysine; K(-)L(-), the Fc/2 fragment without C-terminal lysine and N-terminal leucine; K(-)G(-)LL(-), the Fc/2 fragment without C-terminal lysin, C-terminal glycine, and two N-terminal leucines, respectively, as shown in Figure 1. The relative intensity of intact Fc/2 fragment was significantly different between the two products. (**: P < 0.01).
Table 3. Content rate of C-terminal lysine for each infliximab glycoform.

<table>
<thead>
<tr>
<th>Carbohydrate Chain Name</th>
<th>Reference</th>
<th>Biosimilar</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>57.7 ± 1.8</td>
<td>38.4** ± 3.7</td>
</tr>
<tr>
<td>II MMF</td>
<td>57.9 ± 2.1</td>
<td>43.3* ± 5.8</td>
</tr>
<tr>
<td>III -</td>
<td>51.5 ± 1.9</td>
<td>36.5** ± 3.7</td>
</tr>
<tr>
<td>IV MGNF / GNMF</td>
<td>48.7 ± 1.2</td>
<td>36.8** ± 3.3</td>
</tr>
<tr>
<td>V GnGN (G0)</td>
<td>63.0 ± 0.1</td>
<td>59.3* ± 1.5</td>
</tr>
<tr>
<td>VI AMF / MAF</td>
<td>58.6 ± 1.1</td>
<td>51.3* ± 3.1</td>
</tr>
<tr>
<td>VII GnGNF (G0F)</td>
<td>42.9 ± 1.3</td>
<td>33.2** ± 3.1</td>
</tr>
<tr>
<td>VIII AGNF / GNAF (G1F)</td>
<td>39.8 ± 1.2</td>
<td>31.2** ± 2.9</td>
</tr>
<tr>
<td>IX AAF (G2F)</td>
<td>31.2 ± 2.0</td>
<td>27.1 ± 4.2</td>
</tr>
<tr>
<td>Total</td>
<td>46.4 ± 1.6</td>
<td>35.5* ± 4.0</td>
</tr>
</tbody>
</table>

The percentages are expressed as the mean ± standard deviation of three independent experiments from 3 batches. The last line shows the content rate of C-terminal lysine in infliximab (both the reference and biosimilar product). Content rate of C-terminal lysine of
glycoform excluding AAF (G2F) were significantly different between the two products. (*: $P < 0.05$, **: $P < 0.01$).