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Cancer vaccine characterization: From bench to clinic

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ABSTRACT

Background: The development of safe, effective, and affordable vaccines has become a global effort due to its vast impact on overall world health conditions. A brief overview of vaccine characterization techniques, especially in the area of high-resolution mass spectrometry, is presented. It is highly conceivable that the proper use of advanced technologies such as high-resolution mass spectrometry, along with the appropriate chemical and physical property evaluations, will yield tremendous in-depth scientific understanding for the characterization of vaccines in various stages of vaccine development. This work presents the physicochemical and biological characterization of cancer vaccine Racotumomab/alumina, a murine anti-idiotypic antibody that mimics *N*-glycolyl-GM3 gangliosides. This antibody has been tested as an anti-idiotypic cancer vaccine, adjuvated in $Al(OH)_3$, in several clinical trials for melanoma, breast, and lung cancer.
Methods: Racotumomab was obtained from ascites fluid, transferred to fermentation in stirred tank at 10 L and followed to a scale up to 41 L. The mass spectrometry was used for the determination of intact molecule, light and heavy chains masses; amino acids sequence analysis, N- and C-terminal, glycosylation and posttranslational modifications. Also we used the DLS for the size distribution and zeta potential analysis. The biological analyses were performed in mice and chickens.
Results: We observed differences in glycosylation pattern, charge heterogeneity and structural stability between in vivo-produced and bioreactor-obtained Racotumomab products. Interestingly, these modifications had no significant impact on the immune responses elicited in two different animal models.
Conclusions: We are demonstrated that this approach could potentially be more efficient and effective for supporting vaccine research and development.

1. Introduction

Racotumomab [1] is a monoclonal antibody (Mab) of murine anti-idiotype (Ab2) generated by immunizing BALB/c mice with the P3 monoclonal antibody [2], a Mab (Ab1) that specifically recognizes *N*-glycolylated gangliosides, sulfated glycolipids, and antigens present in various types of human tumors including those located in lung [3–7]. We hypothesize that Racotumomab Mab contains a structural mimicry of ganglioside *N*-glycolyl GM3 (NeuGc-GM3). The vaccine formulation of Racotumomab/alumina is capable of inducing antitumor effects in murine models [7]. Among the observed antitumor effects in the murine model account a decrease in metastases, increased apoptosis of metastatic cells and a marked decrease in angiogenesis as well as higher amount of CD4 T-lymphocytes and T-tumor infiltrating CD8 [13]. The safety and immunogenicity of vaccination with idiotypic Racotumomab has been shown in clinical phase I trials in patients with breast cancer and melanoma [4,8]. In these vaccinated patients a high titer of antibodies specific to NeuGc-GM3 was detected and an antibody response against NeuGc-GM3 was also found which is not suppressed by the absorption of the serum with Racotumomab Mab suggesting that immunization with Racotumomab idiotype probably induces natural immune response against tumors [4,8]. Phase I clinical trials in NSCLC patients have also been conducted with encouraging clinical results [9]. For phase I and II clinical trials, mAb-Racotumomab was produced in mice ascites, a common practice in the 1990s for small scale antibody production.

We developed a new bioreactor-based process using protein-free media for the production of mAb-Racotumomab. Given the increase in production volume, it was necessary to

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transfer the production process to new facilities with a fermentation scale of 41 L. The mAb-Racotumomab produced from bioreactors (Racotumomab-ST10L and Racotumomab-ST41L) have to be bioequivalent to ascites fluid-produced Racotumomab (Racotumomab-AF) in order to ensure the same effect in the patients. The first bioequivalence study between Racotumomab-AF and Racotumomab-ST10L was reported by Machado and colleagues [10].

In this case, this bioequivalence has to be demonstrated by a set of physicochemical (determination of the molecular weight of the intact molecules and light chains, the sequence of N-terminal peptides, the degree of oxidation by mass spectrometry, and the size and charge of the molecule as determined by dynamic light scattering) and biological methods as required by regulatory authorities for characterization of Mabs [11]. As mAb-Racotumomab is used as an adjuvated vaccine additional characteristics should be taken in to account.

In this study, we present the detailed molecular and immunological characterization of mAb-Racotumomab obtained by two different production methods in two scales in order to determine the impact of the manufacturing process in vaccine performance.

2. Materials and methods

2.1. Materials

The culture, fermentation, purification and conjugation processes by Racotumomab-AF and Racotumomab-ST were described by Machado and colleagues previously [10].

2.2. Analysis of intact molecule by mass spectrometry

For MS analysis of intact Racotumomab, one lot Racotumomab-ST10L and three different lots of Racotumomab-ST41L were taken and desalted by extraction using solid phase Cleanup C18 Pipette Tips as recommended by the manufacturer. The desalted samples were mixed 1:1 with sinapic acid solution at 10 mg/mL and 1.5 μL were applied on a MALDI plate and allowed to dry at room temperature.

The mass spectrometer was operated in the linear mode, at the range of *m*/*z* 1000–200,000, the laser power was 85, for the calibration of the mass range of spectrometer in the experiment, ProteoMass Calibration Standards Kit (Sigma, cat. No-1KT MSCAL2) is acquired for 100 profiles per sample and each profile was the average of 10 laser shots.

2.3. Peptide mass fingerprinting (PMF) of the molecule

To obtain the PMF of Racotumomab ST produced from both scales, were taken, reduced and carboxymethylated with the aim of separating and denaturing the light and heavy chains. This is after digestion was performed with trypsin (Promega, USA) as described by Wang et al. [27]. Tryptic peptides were desalted by using the extraction solid phase Cleanup C18 Pipette Tips recommended by the manufacturer. The desalted peptide was mixed 1:1 with α-cyano-4-hidroxicinnamico acid (CHCA), which is then applied onto a MALDI plate and allowed to dry at room temperature. For PMF analysis spectrometer was operated in the reflectron mode, *m*/*z* range 700–3500, the laser power was 70 and 100 sample profiles were acquired. For each profile there was an average of 20 laser shots. For calibration PeptideMass Calibration Kit (Sigma, Cat. No-1KT MSCAL2) was used. The spectra were processed with the program Axima Biotech Launchpad (Shimadzu, Japan).

2.4. Determination of the oxidation degree

Samples were subjected to enzymatic digestion with trypsin and were subsequently desalted by using extraction solid phase Cleanup C18 Pipette Tips with a gradient of acetonitrile in order to fractionate the sample. The desalted peptide was mixed 1:1 with α-cyano-4-hidroxicinnamico acid (CHCA), then applied onto a MALDI plate and allowed to dry at room temperature. For PMF analysis spectrometer was operated in the reflectron mode, *m*/*z* range 700–3500, the laser power was 70 and 100 sample profiles were acquired, where each profile was the average of 20 laser shots. For calibration PeptideMass Calibration Kit (Sigma, Cat. No-1KT MSCAL2) was used. The spectra were processed with the program Axima Biotech Launchpad (Shimadzu, Japan).

2.5. Analysis of charge heterogeneity

Racotumomab-STs (approximately 100 μg each) were diluted five-fold with buffer A (10 mM sodium acetate pH 5) loaded onto a ProPac10 WCX column (Dionex, Houston, USA) and eluted with buffer B (10 mM sodium acetate, 1 M NaCl pH 5). The gradient was performed in two steps, from 8 to 13% B in 5 min and 13 to 20% B in 20 min. Protein elution was monitored by absorbance at 280 nm.

2.6. N-Glycosylation analysis

N-glycans were released by digestion with peptide-N4-(*N*-acetyl-*b*-D-glucosaminyl) asparagine amidase F (PNGase F,BioLabs, Beverly, MA, USA), using the method described by the manufacturer. Briefly, Mabs-Racotumomab were denatured at 100 °C for 10 min in 0.1% SDS, 5% *b*-mercaptoethanol. Nonidet P-40 (NP-40) was added to a final concentration of 1% before enzyme addition. The digestion was carried out at a ratio of 5 U of PNGase F per milligram of glycoprotein at 37 °C for 2 h. The protein was precipitated by adding three volumes of cold ethanol and the mixture was kept at −20 °C for 30 min. The oligosaccharides were concentrated under vacuum and subjected to 2-aminobenzamide (2AB) labeling. Oligosaccharides were fluorescently labeled with 2AB by reductive amination [30]. Briefly, oligosaccharides were dissolved in 5 μL DMSO-acetic acid (7:3) containing 2AB (0.35 M) and 1 M NaCNBH3 and incubated at 65 °C for 2 h. Excess of fluorophore was removed by 2 h vertical chromatography on Whatman 3 MM paper using acetonitrile. The paper bearing the oligosaccharide signal (application point) was cut. The oligosaccharides were eluted from the paper by adding double-distilled water (2 × 500 μL). The eluate was filtrated in a syringe through a 0.45 μm PTFE filter (Millex-LCR, Millipore) and then concentrated under vacuum.

Normal-phase HPLC (NP-HPLC) of the labeled portion was performed using a TSK-Gel Amide-80 4.6 × 250 mm column (Tosoh BioSep, Japan) on a separation module (Merck–Hitachi, Japan) equipped with a fluorescence detector. Labeled *N*-glycans were separated by a linear gradient of 20–58% of 50 mM ammonium formiate pH 4.4 against acetonitrile over 152 min at a flow rate of 0.4 mL/min. Samples were injected in 80% acetonitrile. The fluorescence detection was carried out using an excitation wavelength of 330 nm and an emission wavelength of 420 nm [31]. The elution positions of the *N*-glycans were determined in glucose units (GU) by comparison with a standard dextran hydrolysate 2AB labeled (dextran ladder) [12]. The glycosylation was also analyzed by mass spectrometry (MALDI-MS) using an Axima Performance mass spectrometer (Shimadzu Biotech, Japan).

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