Production of the extracellular domain of Siglec-9 using an *E.coli* expression system and generation of anti-Siglec-9 antibodies

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INTRODUCTION:

- Sialic acid-binding immunoglobulin-like lectins (Siglecs) are a subset of the immunogluobulin superfamily of cell surface receptors.
- Siglecs mediate protein-carbohydrate interactions through their ability to bind sialic acid moieties found on glycolipids and glycoproteins.
- Siglecs are mainly expressed on white blood cells and play a critical role in cell-cell interactions and signaling functions in the hematopoietic, immune, and nervous systems.
- The human Siglec family is composed of 11 genes. In addition to well-characterized Siglecs such as the myeloid receptor (Siglec-3/CD33), seven additional Siglecs (Siglec 5-11) have been identified in recent years.
- All newly identified Siglecs have a high degree of sequence similarity to Siglec-3 and are collectively referred to as Siglec-3-related genes.
- Anti-CD33/Siglec-3 antibodies have recently been used for diagnosis of acute myeloid leukemia (AML).
- The development of Siglec-3 antibody-targeted chemotherapy, as well as emergence of anti-Siglec-5 antibodies as a potential marker and therapeutic reagent for AML has prompted the characterization of other newly identified Siglecs.
- Siglec-9 is one of the newly identified members of the Siglec gene family.
- Similar to other Siglec-3-related genes, Siglec-9 is located on chromosome 19q 13.3-4 in close proximity to the kallikrein gene locus.
- Siglec-9 is composed of an N-terminal V-set Ig domain that mediates sialic acid binding, two C2-set Ig domains, a transmembrane region and a cytoplasmic tail that harbors two immune receptor tyrosine-based inhibitory motifs (ITIMs).
- The Siglec9 protein is expressed in bone marrow, spleen, placenta, and fetal liver. Its high expression in tissues involved in stem-cell differentiation indicates that Siglec-9 may play a role in the activation of several cell types and hence the regulation of tumor growth.
- Siglec-9 protein is composed of 463 aminoacids harboring an extracellular domain, a transmembrane domain, and a cytoplasmic domain.
- The extracellular domain of Siglec-9 is composed of 321 amino acids with a molecular weight of 35 kD.
- Here we report the production of the extracellular domain of Siglec-9 using an E.coli protein expression system.
- This domain was expressed as a fusion protein containing an N-terminal polyhistidine (6xHis) tag.
- Recombinant Siglec-9 was purified to homogeneity using metal affinity chromatography and was used as immunogen for antibody production in rabbit and mice.

METHODOLOGY:

- Commercially available total bone marrow tissue mRNA was reverse-transcribed to cDNA.
- Polymerase chain reaction was conducted using the proof reading enzyme *Pfu* and oligonucleotide primers specific to the extracellular domain of Siglec-9
- The amplified cDNA was purified and cloned into pET/200 TOPO plasmid vector containing an N-terminal polyhistidine (6xHis) tag.
- Plasmid DNA containing the pET-Siglec construct was isolated and sequenced to confirm its identity and then was used to transform the *E.coli* strain BL21(DE3) for protein production.
- Protein expression was induced with isopropyl thiogalactoside (IPTG), and *E.coli* culture was harvested 3 hours post IPTG induction.
- Whole cell extracts of cultured BL21 cells were subjected to SDS-PAGE and Western blotting.
- The most prominent band observed in gels stained with Coomassie blue corresponded to the expected molecular weight for Siglec-9.
- Western blots using anti-histidine antibody resulted in detection of a single band.
- The identity of the Siglec-9 fusion protein was confirmed by mass spectroscopy.
- Siglec-9 was mainly produced in "inclusion bodies". The inclusion bodies were isolated, washed with 2 molar urea, and then dissolved with guanidine hydrochloride.
- The recombinant protein was purified to homogeneity using nickel-nitrilotriacetic (Ni-NTA) metal affinity chromatography.
- Siglec-9 was used as immunogen for production of antibodies in New Zealand White rabbits and female BALB/c mice.
- Specific affinity of anti-Siglec9 antibodies was tested using antibody capture assays and Western blotting.

RESULTS:

Fig.1 Detection of recombinant protein expression as detected by Western blotting using Anti-histidine tag (HisG-AP) antibody

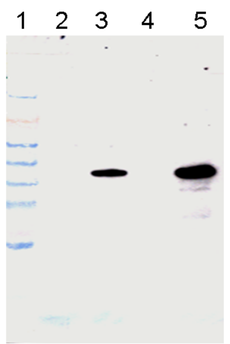
Lane 1. See Blue Marker

Lane 2. BL21 *E. coli* cell lysate (vector only)

Lane 3. BL21 *E. coli* cell lysate (Siglec9 insert + IPTG)

Lane 4. Blank

Lane 5. Purified Siglec-9



RESULTS:

Fig.2 Detection of recombinant protein expression as detected by SDS-PAGE

Lane 1. Mark 12 Marker

Lane 2. *E. coli* lysate (vector only)

Lane 3. *E. coli* lysate (Siglec9 insert)

Lane 4. *E. coli* lysate (Siglec9 Insert + IPTG)

Lane 5. Blank

Lane 6. Purified Siglec9

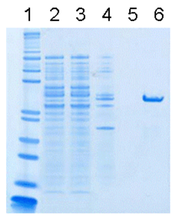
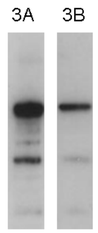


Fig.3 Western blots to detect recombinant Siglec-9

3A) Western blot using anti–Siglec-9 rabbit polyclonal antibody

3B) Western blot using anti-Siglec-9 monoclonal antibody



DISCUSSION:

- In this study the extracellular domain of Siglec-9 was expressed as a fusion protein using an E.coli protein expression system.
- The expected molecular weight for the extracellular domain of Siglec-9 is 35 kD. In our experiments the apparent molecular weight as observed in figure 2 is slightly higher, this is due to the presence of the 3 kD N-terminal tag.
- Purified Siglec-9 was used as an immunogen and polyclonal and monoclonal antibodies were generated.
- Polyclonal and monoclonal antibodies generated were highly specific to Siglec-9 as assessed by Western blot analysis (Figure.3) and antibody capture assays (data not shown).
- polyclonal anti-Siglec-9 antibody was used in flow-cytometric analysis of lymph nodes obtained from patients with hematological malignancies. 7 out of 8 patients with B-cell lymphoma were positive.

CONCLUSION:

- Our results indicate that that Siglec-9 may be a new surface marker of patients with B-cell lymphoma.
- We are currently using the Siglec-9 specific antibodies to develop a sandwich ELISA capable of measuring the levels of this Siglec in various biological fluids.

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