Identification of critical cysteine residues in AMOP domain of ISM-C in anti-angiogenesis

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\textbf{ABSTRACT}

Pathological angiogenesis, or abnormal rapid proliferation of blood vessels, is caused by imbalance of signals from growth factors, mural cells, and extracellular matrix proteins. It is implicated in inflammatory diseases such as cancer and rheumatoid arthritis. Isthmin (ISM) is a 60 kDa secreted protein that was identified to be a novel angiogenesis inhibitor. It contains a C-terminal Adhesion-associated domain in MUC4 and Other Proteins (AMOP) domain which has been shown to be responsible for its anti-capillary network formation function, \textit{in vitro}. As sequence alignment showed that AMOP domain contains eight conserved cysteine residues, this study aimed to identify the critical cysteine residues for AMOP domain mediated anti-angiogenesis. Recombinant mouse ISM AMOP domain proteins, with individual cysteine residue mutation, were produced from \textit{Escherichia coli} and their function in \textit{in vitro} capillary network formation was investigated. Of the nine cysteines residues, results showed that C321, C383, C423, C442 and C300 seem to be critical while C294, C319, C365 and C382 do not seem to play an essential role in the anti-angiogenic function. This study hence provided evidence to confirm the role of AMOP domain in the anti-angiogenic function of ISM and also provided useful insights to help in understanding AMOP domain structure.

\textbf{INTRODUCTION}

Angiogenesis is a multifactorial process defined as the formation of new blood vessels from pre-existing vessels. Imbalance of signals from growth factors, mural cells, extracellular matrix (ECM) proteins could result in inappropriate angiogenesis leading or supporting pathologies.

Isthmin has been shown to be a novel angiogenesis inhibitor that plays a role in both physiological and pathological angiogenesis (Xiang, \textit{et al}, 2009). It contains a hydrophobic signal peptide at the N-terminus, three putative O-glycosylation sites, a centrally located thromospondin type 1 repeat (TSR) and a C-terminal Adhesion-associated domain in MUC4 and Other Proteins (AMOP) domain (Rossi, \textit{et al}, 2004). Specifically, studies have demonstrated that AMOP domain is responsible for ISM anti-capillary network formation function (Xiang, \textit{et al}, 2009).

AMOP domain is a novel protein domain and sequence alignment showed that AMOP domain contains eight conserved cysteine residues. We hypothesize that these cysteine residues might play an essential role in AMOP domain function, since cysteine residues are responsible

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for forming disulfide bonds which could affect the folding, stability and structure of a protein. Thus, the objective of this project is to identify the critical cysteine residues in AMOP domain.

**MATERIALS AND METHODS**

Recombinant ISM-C cysteine mutant proteins were expressed in *Escherichia coli* (*E. coli*) strain BL21 with DE3 transformed with recombinant pET-M+ISMC plasmid. Extracted recombinant proteins were purified by Nickel-Nitrilotriacetic acid (Ni-NTA) affinity chromatography followed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC). Purified protein was analysed for its purity, endotoxin content and cytotoxicity to endothelial cells (ECs) before investigating its inhibitory activity in *in vitro* capillary network formation.

**RESULTS**

By comparing the EC capillary tube length of those incubated with ISM-C mutant proteins to the control, effect of ISM-C mutant proteins was categorized into three types: distinct inhibition, mild inhibition and no inhibition.

ISM-C<sup>C294A</sup>, ISM-C<sup>C319A</sup>, ISM-C<sup>C365A</sup> and ISM-C<sup>C382A</sup> showed distinct inhibition on ECs *in vitro* capillary network formation, similar to the effect of ISM-C wild type. Endothelial cells incubated with these mutant proteins for 6 hours appeared either as well separated individual cells or cells that began to migrate and align themselves.

ISM-C<sup>C321A</sup>, ISM-C<sup>C383A</sup> and ISM-C<sup>C442A</sup> showed mild inhibition on ECs *in vitro* capillary network formation where sprouting of new but incomplete capillary tubes were visible but at the same time, some endothelial cells were still well separated.

ISM-C<sup>C300A</sup> and ISM-C<sup>C423A</sup> showed no inhibition on ECs *in vitro* capillary network formation as normal capillary tube formation was observed, similar to those found in the control which was incubated without ISM protein. Endothelial cells incubated with these mutant proteins for 6 hours appeared as closed capillary-like polygon and complex mesh like structures began to develop. In addition, lacunae (areas devoid of cells) developed in these capillary tubes were larger than those observed in mildly inhibited endothelial cells, indicating a higher level of capillary network formation (Vailhe, 1998).

![Graph showing relative mean total capillary tube length for different ISM-C mutants](image)
Figure 1: Quantification of Capillary Tube Length of various ISM-C mutant proteins
ISM-C\textsuperscript{C294A}, ISM-C\textsuperscript{C319A}, ISM-C\textsuperscript{C365A} and ISM-C\textsuperscript{C382A} relative mean total capillary tube length were similar to ISM-C\textsuperscript{WT}. ISM-C\textsuperscript{C321A}, ISM-C\textsuperscript{C383A} and ISM-C\textsuperscript{C442A} relative mean total capillary tube length were 50% longer than ISM-C\textsuperscript{WT}. ISM-C\textsuperscript{C423A} and ISM-C\textsuperscript{C300A} relative mean total capillary tube length were close to the control’s value.

DISCUSSION

Relation of Protein Sequence, Structure and Function

There is a consequential relationship among protein sequence, structure and function; amino acid sequence determines the protein structural features which in turns determine the function of the protein (Sadowski and Jones, 2009). Cysteines are capable of forming covalent disulfide bonds by oxidation of the two thiol side groups. Disulfide bonds could influence the folding, stability and structure formation of a protein.

Identification of Critical Cysteine Residues in AMOP domain function

Cysteines at 294\textsuperscript{th}, 319\textsuperscript{th}, 365\textsuperscript{th} and 382\textsuperscript{nd} residue are concluded to be insignificant for a functional AMOP domain. Cysteines at 321\textsuperscript{st}, 383\textsuperscript{rd} and 442\textsuperscript{nd} residue could be critical cysteine residues as mutation of these cysteines residues resulted in partial function lost in AMOP domain. Lastly, cysteines at 300\textsuperscript{th} and 423\textsuperscript{rd} residues are concluded to be critical cysteine residues in AMOP function. Mutation of cysteines at 300\textsuperscript{th} and 423\textsuperscript{rd} residues could result in significant conformational change in AMOP domain and thus leads to domain loss-of-function.

Implications on AMOP domain structure

Since the anti-angiogenic function of ISM-C\textsuperscript{C294A}, ISM-C\textsuperscript{C319A}, ISM-C\textsuperscript{C365A} and ISM-C\textsuperscript{C382A} was not significantly affected, this indicates that there were insignificant conformational changes in AMOP domain when cysteines at these positions are mutated independently. On the other hand, as the anti-angiogenic function of ISM-C\textsuperscript{C300A}, ISM-C\textsuperscript{C321A}, ISM-C\textsuperscript{C383A}, ISM-C\textsuperscript{C423A} and ISM-C\textsuperscript{C442A} was affected to a substantial extent, this indicates that there were significant conformational changes in AMOP domain. Cysteines at 300\textsuperscript{th} and 423\textsuperscript{rd} residues are very likely to be involved in disulfide bond formation. Absence of this disulfide bond could most probably result in damage to binding site conformation or the collapse of whole domain structure which consequently causes full function abolishment of AMOP domain. However, since mutation of cysteines at 321\textsuperscript{st}, 383\textsuperscript{rd} and 442\textsuperscript{nd} residues respectively did not cause a complete lost in AMOP domain function, it is concluded that conformation of AMOP domain might be altered to a lesser degree by these cysteine mutations, and therefore only affect the binding affinity of AMOP domain to ECs surface receptor(s).

Significance of this project

Results have shown that mutation of cysteine to alanine residue at different amino acid position in AMOP domain could cause the anti-angiogenic function in ISM-C to be abolished. This indicates that these characteristic cysteines residues are important in maintaining the proper structure and function of AMOP domain and hence, results have further confirmed the role of the novel AMOP domain as an angiogenesis inhibitor. In addition, by inferring from the relationship of protein sequence, structure and function, some useful insights to help on the understanding of AMOP domain structure are provided in this study. It is hoped that with better understanding of AMOP domain, the role of ISM as a novel secreted angiogenesis inhibitor would be elucidated.
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