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Anthrax Toxin Receptors, Functions and their Possible Use in Therapeutics: A Review

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ABSTRACT

Anthrax is a zoonotic disease and its lethality is due to two secreted exotoxins; lethal toxin and edema toxin. The receptor for anthrax toxin is called Anthrax Toxin Receptor (ATR). Two distinct cellular Anthrax toxin receptors, ANTXR1 (also known as tumour endothelial marker 8, TEM8) and ANTXR2 (also known as capillary morphogenesis protein 2, CMG2) have been identified. TEM8 and CMG2 both are ubiquitous in nature. Apart from their function as anthrax toxin receptor, their ubiquitous presence is suggestive of their physiological role. TEM8 is preferentially expressed in blood vessel of tumours and in vasculature, indicating its probable role during angiogenesis and regulation of neovasculature. CMG2 is present in capillary cells and is associated with capillary morphogenesis. Both the TEM8 and CMG2 are present in different isoforms, share homology in amino acid residues and apart from their role in angiogenesis regulation, are also involved in interaction with extracellular matrix. Mutation in TEM8 result in a condition appeared to alter physical characters in form of growth retardation, alopecia, pseudoanodontia and progressive visual impairment known as GAPO syndrome. Mutation in CMG2 result in autosomal recessive disorder in humans called Hyaline Fibromatosis Syndrome (HFS) and Infantile Systemic Hyalinosis (ISH). Because of the role in physiological functions and participation as toxin receptor, these receptors could be target for several curative therapies both for the anthrax disease as well as for receptor associated physiological disorders. This review presents a detailed insight into isoforms, functions, diseases and therapeutic implications of anthrax toxin receptors.

Key words: Anthrax toxin receptors, TEM8, CMG2, toxin trafficking, physiological functions, angiogenesis, candidate anthrax therapeutics, anti-cancer effects, therapeutic agents

INTRODUCTION

Bacillus anthracis is gram-positive, spore forming, rod-like bacterium causing anthrax disease mainly in ruminants and occasionally, it is transferred to humans due to handling of diseased animals or animal byproducts. Cutaneous, inhalational, gastrointestinal and cerebral forms of anthrax may be present in affected human beings. Death is rare in cases of cutaneous anthrax but common in gastrointestinal, cerebral or inhalational anthrax (Klietmann and Ruoff, 2001). Its spores are extremely tough and resistant to several kinds of disinfectants, UV rays, gamma irradiation etc. (Plotkin and Grabenstein, 2008; Franz, 2009; Bouzianas, 2009). The spores having

such extreme hardy nature, its persistency in nature without decaying for decades and high lethality makes anthrax an ideal bioweapon. It was used as bioweapon in USA in 2001, where anthrax spores in powder form were deliberately released through postal envelopes. In India also, politicians received several powder filled envelopes suspected of anthrax spores which were sent to High Security Animal Disease Laboratory, Indian Veterinary Research Institute (IVRI), Bhopal, Madhya Pradesh for testing. The samples were tested by Polymerse Chain Reaction (PCR) using WHO recommended primers and all were found negative for *B. anthracis* (Khandia *et al*., 2013). Conventional microbiological methods imply parameters like Gram-positivity, non-motility, non β-hemolytic colonies on sheep or horse-blood agar plates, penicillin sensitivity and susceptibility to lysis by gamma phage (Dixon *et al*., 1999). Valuable diagnostics include gamma phage mediated bacterial lysis (Murugkar *et al*., 2008), PCR, Real Time PCR and multiplex PCR detection, (Fasanella *et al*., 2001; Saikaly *et al*., 2007; Antwerpen *et al*., 2008; Batra *et al*., 2013), antibody detection against spores or antigens, ELISA (Leoff *et al*., 2009), fluorescent phage (ScienceDaily, 2014), solid phase oligo probes (Addanki *et al*., 2011), Small Acid Soluble Proteins (SASPs) as protein markers (Chenau *et al*., 2014) and others.

Virulent strains of *B. anthracis* contain two virulence mega plasmids; pX02 and pX01 and both of the plasmid provide excellent target for molecular detection of anthrax bacterium. Out of the two megaplasmids, the 96-kb pX02 plasmid carries genes required for capsule synthesis, protects bacteria from phagocytosis (Hudson *et al*., 2008). The pXO1 codes for three major secretory proteins responsible for the morbidity and mortality are binary exotoxins; anthrax Lethal Toxin (LT) and edema toxin (ET). Protective antigen (PA) is central to these two LF and EF enzymatic moieties. Combination of PA with LF forms LT and PA with EF forms ET (Leppla, 1982; Drum *et al*., 2002). Lethal toxin cleaves Mitogen Activated Protein Kinase (MAPK) (Khandia, 2009; Khandia *et al*., 2010), a known cell cycle regulator signaling molecule and edema toxin elevates intracellular amount of cyclic AMP and causes edema (Bradley *et al*., 2003; Sweeney *et al*., 2011). To intoxicate target cells, PA binds to cell surface receptors, the two well-studied receptors known as anthrax toxin receptor 1 or Tumor Endothelium Marker-8 (TEM8) (Bradley *et al*., 2001) and anthrax toxin receptor 2 or capillary morphogenesis protein-2 (CMG2) (Scobie *et al*., 2003). The crystal structure of both the TEM8 (Scobie *et al*., 2006) and CMG2 (Fu *et al*., 2010) have been completely elucidated. Apart from serving as receptor for toxin binding, several studies have revealed their roles in other physiological and cellular processes like endothelial cell migration (Fu *et al*., 2010), capillary formation (El-Kamah *et al*., 2010) and maintenance of homeostasis in extracellular matrix (Reeves *et al*., 2012). Polymorphism associated with CMG2 has been found to be the basis of variable lethality of anthrax toxin in different ethnic group (Martchenko *et al*., 2012). The current scenario of one health one medicine and emerging drug resistance warrants exploring the health beneficial applications of novel therapeutic regiments and finding out more and more effective treatment modalities/agents to counter various diseases (Dhama *et al*., 2013a, b). In the present review, detailed insight into the isoforms, their functions and therapeutic implications of anthrax toxin receptors is discussed.

MODE OF ANTHRAX TOXIN TRAFFIC INSIDE THE CELL

The action of anthrax toxins starts with the binding of PA83 (83 kDa protein, thus called PA83) to either of TEM8 or CMG2. During the interaction, domain 2 and 4 of PA coordinate with the Mg^{2+} or Ca^{2+} ion of the receptor at metal ion adhesion site (Santelli *et al*., 2004). The proteolytic cleavage of PA83 occurs after the RKKR sequence by furin or furin like cellular proteases. This

cleavage results in removal of a 20 kDa part (PA20) from the N terminus, leaving the 63 kDa part (PA63) bound to the receptor (Liu and Leppla, 2003). The PA63, being free to self-associate, forms a PA63 heptamer (Abrami *et al*., 2003). It is still bound to the receptors, forming PA63 and receptor complex in 7:7 configuration (Klimpel *et al*., 1994). The LF and EF bind to high affinity sites at the interface between two PA molecules of the heptamer. Although seven binding sites are available, steric hindrance prevents binding of 7 molecules of LF/EF at adjacent sites (Klimpel *et al*., 1994). Only three molecules of catalytic moieties, LF and EF can bind to PA63 heptamer prior to lipid raft-dependent and clathrin-mediated endocytosis (Young and Collier, 2007). It is reported that this internalization process requires the presence of low density lipoprotein receptor-related protein 6 (LRP6) which directly associates with CMG2 or TEM8 and promotes internalization by an unknown mechanism (Wei *et al*., 2006). Following internalization, the endocytic vessel is acidified due to fusion with endosome causing a conformational change that converts the pre-pore into 14-stranded β-barrel pore that is responsible for release of active toxin enzymes, LF and EF into cytosol (Abrami *et al*., 2006). The CMG2 bound PA require more acidic pH (approx 5) than its counterpart TEM8 which require less acidic pH (approx 6.0), suggesting that the receptor might be involved in directing the subcellular location of pore formation (Rainey *et al*., 2005; Wolfe *et al*., 2005). Toxin moieties, when released in cytoplasm, modify their respective targets (Van Der Goot and Young, 2009). These enzymatic moieties are believed to unfold partially or completely while passing the narrow pore molecules but probably refolded in the cytosolic compartment to become enzymatically active (Bradley *et al*., 2003).

ANTHRAX TOXIN RECEPTORS (ATR)

The two main receptors for anthrax toxin binding, TEM8 and CMG2 contain a signal peptide, an extracellular von Willebrand factor A (vWA) domain, a single-pass transmembrane alpha helical region of 23 amino acid residue for plasma membrane anchoring and a cytosolic tail of 148 residue. Both the TEM8 and CMG2 proteins bind PA at their vWA site (Bradley *et al*., 2003). The vWA domains are well characterized protein interaction sites found in extracellular matrix components or in cell adhesion proteins, like α integrins (Whittaker and Hynes, 2002). The vWA domains of CMG2 and TEM8 contain a typical Metal Ion-Dependent Adhesion site (MIDAS) motif (DxSxS...T...D, where x can be any amino acid). This site specifically chelates metal ions required for ligand binding. TEM8 and CMG2 proteins share 40% amino acid homology in their overall sequence and 60% homology within their vWA domains (Scobie *et al*., 2006). Variants of both TEM8 and CMG2 proteins, lacking transmembrane anchoring region, are found intracellularly in soluble state and thus cannot serve as receptors for PA. Cytosolic tails of TEM8 and CMG2 although not absolutely required for their function as anthrax toxin receptors but involved in modulation of cytoskeleton and efficiency of the receptor function of these proteins can be positively regulated by post-translational palmitoylation and ubiquitination of their cytosolic tails. A ubiquitously expressed cell surface protein, low density lipoprotein receptor-related protein 6 (LRP6) was found to form a complex with TEM8 and CMG2; and this association is required for internalization of the receptor-toxin complexes into host cells (Wei *et al*., 2006) and is of cell type specific (Ryan and Young, 2008). At the PA binding interface, amino acid residues K51, Y119, H121, E122 and Y158 are absolutely conserved in both ANTXR1 and ANTXR2 and residues S87, R111 and E117 are highly similar with Thr, Lys and Asp, respectively in ANTXR1 (Lacy *et al*., 2004a; Santelli *et al*., 2004). Upon analyzing TEM8 and CMG2 null mice (CMG2-/-, TEM8+/+ and CMG2+/+, TEM8-/-) by deleting their TM domains, TEM8-/-mice succumbed but CMG2-/-mice

survived the challenge completely, confirming that CMG2 is the major anthrax toxin receptor *in vivo* (Liu *et al*., 2012) and TEM8 only plays a minor role in anthrax toxin pathogenesis (Liu *et al*., 2009). This ubiquitous presence of both the TEM8 and CMG2, proposes the role of both the proteins in normal developmental processes and other cell physiology related functions like binding to extracellular matrix components and developmental angiogenesis along with toxin trafficking.

Apart from TEM8 and CMG2, another cell surface vWA domain containing protein, β1 integrin has been reported to mediate killing of the mouse macrophage cell line by lethal toxin challenge, suggesting that integrin β1 could be another anthrax toxin receptor (Martchenko *et al*., 2010). The integrin α4β1 and α5β1 complexes have ability to bind to PA similar to TEM8 (Martchenko *et al*., 2010). Whether integrin β1 complexes or other vWA domain containing proteins functions as anthrax toxin receptors, *in vivo*, it remains to be analyzed and addressed.

Tumour endothelial marker 8 (TEM8/ANTXR1): TEM8 protein is coded by the *tem8* gene, a gene of 190 kb size. The *tem8* gene encodes for 3 different splice variants of 564, 368 and 333 amino acids, respectively. The longest form TEM-564 contains a long proline-rich cytoplasmic tail (Abrami *et al*., 2006). The TEM-368, a medium isoform possesses a short cytoplasmic tail and is dissimilar in last four amino acids at the COOH terminus forming the long isoform. Both the long and medium isoforms are identical in the extracellular domain (Bell *et al*., 2001) and contain the vWA domain that binds to PA and may function as PA receptors. The short isoform comprising of 333-amino acid is identical to the other two isoforms in the extracellular domain but shows divergence just before the transmembrane domain and lacks sequence for membrane anchoring. As a consequence, the short isoform is secreted in the cytosol and does not function as a receptor for the anthrax toxin. Mutation at splice junction in TEM8 transcript cause a frameshift of the complete reading frame of the last exon and resulted in TEM8 with a 118 amino acids long neopeptide present at its C terminus which is the genetic cause of GAPO syndrome, a condition appeared in form of growth retardation, alopecia, pseudoanodontia and progressive visual impairment (Stranecky *et al*., 2013). TEM8 is highly conserved in different species. Mouse and human homologs share 98% sequence identity in the extracellular PA binding domain (Croix *et al*., 2000). TEM8 is selectively upregulated in endothelial cells during blood vessel formation and in endothelial cells of neoplastic tissue (Bonuccelli *et al*., 2005). The TEM8 receptor mutant mice developed mis-aligned incisors and female TEM8 mutant mice bear pregnancy but failed to support normal embryonic development and it was found dispensable for embryonic development (Liu *et al*., 2009). Knockout mice have normal angiogenic development and they develop normally but fail to support strong tumor growth. It has been hypothesized that a key function of TEM8 may be to serve angiogenesis during stress-mediated responses (Liu *et al*., 2008). Expression of TEM8 protein has been tried in *Escherichia coli* and it was found to be present in inclusion body fraction (Bradley *et al*., 2001, 2003). To study the crystal structure of TEM8 in its vWA domain, a C177A mutant was generated which is homologous to residue 175 in CMG2. This cysteine residue is believed to be involved in incorrect folding problems and intermolecular disulfide-bond formation and is believed not to be involved in interaction with PA and have no effect on PA binding ability of ATR (Cai *et al*., 2010).

Capillary morphogenesis gene 2 (CMG2/ANTXR2): CMG2 protein is encoded by *cmg2* gene of 80 kb size, a type I membrane protein that is expressed ubiquitously in human body. The CMG2

was originally identified as a gene expressed at higher levels in endothelial cells of human umbilical vein that were induced to undergo capillary morphogenesis. Also on the other hand, mutations in CMG2 lead to a rare but severe autosomal recessive disorder in humans called Hyaline Fibromatosis Syndrome and Infantile Systemic Hyalinosis (El-Kamah *et al*., 2010). This syndrome is characterized by generalized fibromatosis resulting by deposition of hyaline in the dermis. It contains an N-terminal 180 residue region similar to integrin A domains and therefore belongs to a large family of proteins involved in interaction of cells with their extracellular environment and maintaining homeostasis between intracellular and extracellular environment (Lacy *et al*., 2004b). Several different CMG2 protein isoforms, encoded by alternatively spliced mRNA transcripts, have been identified or predicted. Mainly, 4 isoforms identified so far are of 489, 488, 386 and 322 amino acid residues. CMG2-488 and CMG2-489 are different at their C terminal 12 amino acid tail and both may function as receptor because this difference at C terminal merely affects its function as receptor. CMG2-386 isoform is expressed predominantly within the endoplasmic reticulum of cells and cannot serve as receptor due to its intracellular localization. The CMG-322 is predicted to be a secreted protein without a transmembrane domain (Bell *et al*., 2001). CMG2 is present only in vertebrates and is highly conserved. It showed 84% homology with mice and 62% with zebrafish when compared with human CMG-2 sequence (Deuquet *et al*., 2012). CMG2 expression was detected in heart, lung, liver, skeletal muscle, peripheral blood leukocyte, placenta, small intestine, kidney, colon, spleen, brain and thymus (Scobie *et al*., 2006). Using immortalized cell lines from distal human populations, to understand anthrax toxin entry, the researchers found that anthrax toxin lethality is differed by up to 30,000-fold among cells from people of different ethnic ancestry. Later analysis revealed presence of Single Nucleotide Polymorphism (SNP), where at position 357 amino acid alanine is changed to proline (Martchenko *et al*., 2012) and differential expression of CMG2 is found to be associated with the altered level of lethality.

Physiological functions of ATR: Wide tissue expression pattern of TEM8 and CMG2 receptor exhibits its probable role in anthrax pathogenesis as well as in normal physiological function. Detailed insight into the function of TEM8 and CMG2 came by the experiment carried out in knockout mice. TEM8- and CMG2-null mice (CMG2-/- and TEM8-/- respectively) were produced by deleting their transmembrane domains. CMG2-/- mice are resistant to lethal toxin challenge, whereas TEM8-/- mice are as susceptible to a single dose of lethal toxin like wild type control mice. This experiment revealed that anthrax toxicity is mainly mediated by CMG2 protein as CMG2 is having 11 times higher affinity to PA than TEM8. Due to less affinity towards PA, TEM8 is the minor receptor responsible for direct lethality *in vivo* against anthrax toxin. TEM8 was shown to be highly expressed in tumor vessels and in the vasculature of developing embryos, indicating its potential role in angiogenesis regulation. TEM8 appears to regulate endothelial cell migration and tubule formation whereas a role for CMG2 in endothelial proliferation and morphogenesis during sprouting angiogenesis has been documented (Reeves *et al*., 2012). TEM8 appears to regulate endothelial cell migration and tubule formation whereas a role for CMG2 in endothelial proliferation has been documented (Cryan and Rogers, 2011). *In vitro* studies for physiological functions of TEM8 suggest that TEM8 can bind collagen I, collagen IV and laminin which in turn can promote the migration of endothelial cells (Hotchkiss *et al*., 2005). A recombinantly expressed part of CMG2 was demonstrated to be involved in maintaining homeostasis of collagen type IV and laminin. TEM8 exists in different forms on the cell surface and these forms are influenced by actin and the actin binding protein transgelin (Yang *et al*., 2011). Study carried out by Reeves *et al*.

(2012) proposed that anthrax toxin receptors might be involved in this matrix metalloproteinase activation complex. Matrix metalloproteinase (MMP) family is a group of zinc containing endoproteases that degrade extracellular matrix components including gelatin, collagens, fibronectin, laminin (Reeves *et al*., 2013). Both TEM8 and CMG2 deficient female mice showed normal phenotype with the exception of excess deposition of extracellular matrix reported in ovaries and uteri of these mice including the periodontal ligament of the incisors and the skin (Cullen *et al*., 2009) but were unable to reproduce showing their function involved in embryo development despite the fact that both the receptors are nonessential for life (Liu *et al*., 2009). TEM8 interacts directly with the actin cytoskeleton in a similar manner to the integrins and a cytoskeleton-disrupting drug, cytochalasin D which may decrease spreading of HEK293 cells overexpressing TEM8 (Werner *et al*., 2006). TEM8 and actin co localize at the base of lamellipodia and along with actin filaments extends into the lamellipodia during cell spreading. CMG2 binds to collagen IV, laminin and fibronectin but not to osteopontin, another extracellular matrix protein. It has been suggested that CMG2 may be involved in basement membrane matrix assembly as it also co-localizes with Hsp47, a chaperone protein for collagen I and IV, in the endoplasmic reticulum.

THERAPEUTIC USES OF ANTHRAX TOXIN RECEPTORS

TEM8 was initially identified as a protein upregulated in tumor vessels of different origin in both mice and humans (Carson-Walter *et al*., 2001; Nanda *et al*., 2004; Fernando and Fletcher, 2009) and in tumor vasculature of human colorectal cancer. Some tumor cell itself express TEM8 over their surface (Carson-Walter *et al*., 2001; Jinnin *et al*., 2008; Yang *et al*., 2011). It was initially found to bind with collagens and promote migration of endothelial cells *in vitro* (Werner *et al*., 2006). The fact of ubiquitous presence and over expression of TEM8 receptor on tumor-infiltrating vasculature provides rational strategy for combating cancer as a therapeutic agent. Inhibition in angiogenesis, slow tumor growth and prolonged survival by a soluble TEM8-Fc trap (Duan *et al*., 2007), TEM8 vaccines or sub-lethal doses of anthrax toxin have been demonstrated by different workers (Ruan *et al*., 2009; Felicetti *et al*., 2007; Liu *et al*., 2008; Rouleau *et al*., 2008; Yang *et al*., 2010). Recombinant fab antibody against TEM8 inhibited tumor growth but had no effect on wound healing in immunocompetent mice (Wycoff *et al*., 2011). Anti-TEM8 antibodies were non-toxic and maintained efficacy in combination with various classes of anticancer agents. Therapeutic cellular targets against cancerous tissues often exhibit problem of non specific targeting which kills both the normal as well as malignant cell. Unlike other therapeutic targets, TEM8 was found to be unique as it could not be detected in the normal angiogenic corpus luteum of human ovaries (Nanda *et al*., 2004) and developmental angiogenesis. Deprivation of nutrient or growth factor induce TEM8 expression in cultured endothelial cells suggests that TEM8 might be having some role in survival response pathway. Wound healing is unperturbed in TEM8 Knockout (KO) mice (Cullen *et al*., 2009). So, it could be said that TEM8 is associated with pathological angiogenesis and not with the physiological angiogenesis. Knockout mice have been demonstrated to impair growth of human tumor xenografts of diverse origin including melanoma, breast, colon and lung cancer (Chaudhary *et al*., 2012). The same group of workers demonstrated that antibodies developed against the TEM8 extracellular domain not only blocked anthrax intoxication but also inhibited tumor-induced angiogenesis, while sparing normal healing processes that also require revascularization. This property makes TEM8 an appealing target for the development of novel anti-angiogenic agents. To date, all the anti-angiogenic agents reported are

not able to discriminate between the pathological and physiological angiogenesis (Higa and Abraham, 2009). TEM8 proves its superiority over others in this discrimination process.

Anti-CMG2 antibodies, apart from angiogenic and physiological function related applications, may protect from inhalational anthrax. As PA binds with high affinity to the CMG2 receptor, alternative to anti PA antibodies (which are protective in case of inhalational anthrax), fusion of extracellular domain of human CMG2 and human IgG Fc and expression of them in tobacco plant (transient and stable) offer another vaccine option. Immunization of rabbits with such human IgG Fc fused CMG2 protein provided 100% protection and also such protein has no detrimental effect on growth of rabbits (Wycoff *et al*., 2011). As the intoxication mechanism involves binding of toxin to the receptor, the RNAi molecules against both the receptors should provide protection against lethal challenge. The RNAi molecule induced silencing of receptors significantly protect mouse and human macrophages from anthrax toxins in *in vitro* studies of Arevalo *et al*. (2014). This is proof-of-concept evidence for an effective strategy that can be further developed *in vivo* in animal models.

CONCLUSION

TEM8 and CMG2 are two known receptors for anthrax toxin entry inside the cell. In anthrax intoxication, CMG2 plays the major role and TEM8 is the minor receptor. Antibodies against CMG2 and TEM8 may prove beneficial at time of prophylactic treatment. Apart from their function in toxin trafficking they are also involved in the normal physiological processes like angiogenesis regulation and maintaining extracellular matrix homeostasis. TEM8 is associated with endothelial cell migration and tubule formation and CMG2 is associated with capillary morphogenesis and due to high affinity towards PA, antibodies towards CMG can be alternative to anti-PA antibody. It is supposed that during the process of evolution, toxins utilize normal receptors involved in normal physiological function by using additional receptors like LRP6. The fact that among several TEMs, TEM8 is specifically associated with pathological angiogenesis and thus targeting it, will provide therapeutic means to cure solid tumor where neo-angiogenesis is key to tumor development and by addressing angiogenesis solid tumor growth may be stopped (Chaudhary *et al*., 2012). The soluble fusion protein containing Fc region of IgG and receptor are attractive candidate anthrax therapeutics that is capable to prevent death when it is administered shortly after bacterial infection (Wycoff *et al*., 2011). In future, more advanced modified ligands for these receptors may be constructed synthetically to combat several pathological angiogenesis associated diseases like haemangiomas, psoriasis, kaposi's sarcoma, ocular neovascularization, rheumatoid arthritis, endometriosis, atherosclerosis and tumor growth and metastasis.

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