Archives of Razi Institute, Vol. 76, No. 4 (2021) 847-855

Copyright © 2021 by
Razi Vaccine & Serum Research Institute
DOI: 10.22092/ari.2021.355959.1756

Original Article

Identification of other Cellular Receptors for Edema Factor of *Bacillus anthracis* by Independent Inhibition of Protective Evidenced by Inhibition of Embryo Growth and Antigen Angiogenesis

Khandia, R[1, 2 *], Pattnaik, B[3], Rajukumar, K[1], Pateriya, A. K[1], Puranik, N[2], Bhatia, S[1], Murugkar, H[1]

1. ICAR-National Institute for High Security Animal Diseases, Anand Nagar, Bhopal, MP, India
2. Department of Biochemistry and Genetics, Barkatullah University, Bhopal, MP, India
3. Project Directorate on Foot and Mouth Disease, Indian Council of Agricultural Research, Mukteswar - Kumaon, Nainital, UK

Received 2 September 2021; Accepted 22 September 2021
Corresponding Author: rekha.khandia@bubhopal.ac.in

Abstract

Edema factor (EF) is one of the major secretory proteins of anthrax bacteria along with protective antigen (PA) and lethal factor (LF). Edema factor is a calmodulin-and calcium-dependent adenylate cyclase that increases intracellular levels of cAMP. Intracellular trafficking of EF occurs through PA by binding to ATR/CMG2 receptors, which are also involved in other physiological functions of cells. cAMP is a secondary messenger which activates multiple signaling cascades involved in the cytokinetics of actin molecules and cell junction formation. The present study evaluated the effect of EF on growth and angiogenesis patterns in chicken embryos in the *in ovo* model. Angiogenesis in the chorioallantoic membrane (CAM) of an embryonated chicken egg was decreased and embryo growth was delayed by EF despite the absence of trafficking moiety PA, which is required for transferring the EF molecule inside the cell. Angiogenesis inhibition and embryo growth retardation indicate the use of an alternative receptor by EF to modulate these cellular functions. Additionally, docking was performed between EF as a ligand and hepatocyte growth factor receptor (cMET) and vascular endothelial growth factor (VEGF) receptors, which are mainly involved in growth and angiogenesis. The analysis revealed a very strong binding of EF to cMET receptor (in terms of the number of hydrogen bonds and energy) compared to its ligand hepatocyte growth factor (HGF), which indicates the use of cMET receptor by EF and induction of angiogenesis and embryo growth retardation possibly by competitive inhibition of HGF ligand or receptor-mediated endocytosis.

Keywords: Angiogenesis inhibition, Edema factor, CAM, Embryo growth retardation, cAMP, Signaling molecule

1. Introduction

Bacillus anthracis is a spore-forming, Gram-positive zoonotic bacterium that causes a deadly disease known as anthrax. The vegetative form of bacterium secretes three exotoxins, Protective Antigen (PA), Edema Factor (EF), and Lethal Factor (LF). PA is the major secretory protein along with minor quantities of EF and LF. PA binds to EF or LF to form Edema or Lethal toxin, respectively. PA traffics both catalytic moieties after binding to anthrax toxin receptors of TEM-8 or CMG-2 (1). LF is a metal (zinc) dependent protease enzyme that cleaves and inactivates mitogen-activated

protein kinases (MAPKs) (2) involved in cell cycle signaling pathways. EF is a calmodulin-and calcium-dependent adenylate cyclase, which increases intracellular levels of cyclic AMP (cAMP). cAMP is a secondary messenger that has numerous cellular signaling targets such as protein kinase A (PKA), cAMP-dependent nucleotide-gated ion channels, and cAMP-activated proteins (EPAC) (3), and is involved in cascades of cellular phosphorylation and dephosphorylation inside the cells. These cascades are involved in modulating the actin cytoskeleton, forming cell junction by cadherins, and regulating actin dynamics. Therefore, the effect of EF which disrupts cAMP homeostasis inside the cell probably affects the process of vascularization and growth by altering the cytoskeleton and cell junction formation. Since LF of *B. anthracis* has been reported to independently function in PA (By the author of this manuscript-manuscript communicated), in both *in-vivo* and *in-silico* analysis and affected pattern of growth of chick embryo. Furthermore, possibilities of PA independent functioning of EF can be also assumed in the first 250 residues LF and EF which share significant sequence identity. Alternative cell surface receptors are required for PA independent functioning. cMET (tyrosine-protein kinase Met) also known as HGFR (Hepatocyte growth factor), and VEGF (vascular endothelial growth factor) receptors could be such alternative receptors as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) which are the two major ligands in the development of an embryo. HGF is a potent mitogen for melanocytes, renal tubular cells, and keratinocytes (4, 5) which is also distributed by promoting detachment of epithelial and vascular endothelial cells *in-vitro* (6) and is also designated as an epithelial morphogen (7). This regulates the growth and motility of cells as well as morphogenesis by activating the tyrosine kinase signaling cascade after binding to the cMET receptor. HGF is inactively secreted as a single polypeptide and after cleavage by serine proteases resulted in alpha and beta chains. cMET receptor binds to the alpha chain with a higher affinity compared to the beta chain (8). Additionally, VEGF is a highly specific molecule responsible for endothelial cell proliferation, permeabilization of blood vessels, cell migration, and inhibition of apoptosis besides having a vital role in regulating vasculogenesis (9).

Therefore, HGF and VEGF receptors are also likely to be involved in embryo growth and vascularization. cMET and VEGF receptors were analyzed *in-silico* with EF to detect the receptor-ligand interaction between them to offer PA independent function to find out the possible interaction between these receptors and EF. The protein is expressed in *E. coli* in the experiment to fulfill the requirement of larger quantities of EF protein to use in various experiments.

2. Material and Methods

2.1. Molecular Cloning of *Cya* Gene

B. anthracis Sterne culture was obtained from the Indian Veterinary research institute, Bareilly, India which was grown in nutrient broth containing 2% FBS and used to extract the genomic DNA by alkali lysis method. The PCR amplification of the edema factor gene was conducted using the strain received from ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, Uttar Pradesh, India, by the forward primer *GGATCC*CTCCTCACAGGCATAGAAGTAA and reverse primer *GCGGCCGC*TGAATTTTCAGAAAAACAATTATATATAT contained BamH1 and Not1 restriction site, respectively. The PCR product (2355 bp) was purified and cloned into the pET28a expression vector (Novagen, USA) and transformed into Rosettablue (DE3) Codon plus cells (Stratagene).

2.2. Expression and Purification of Recombinant EF

Bacteria containing plasmids were grown in LB broth including antibiotics chloramphenicol (34 mg/ml), tetracycline (12.5 mg/ml), and kanamycin (30mg/ml) at 37°C under stirring (250 rpm) overnight. The overnight-saturated culture, as inoculum, was then transferred in 1% volume to fresh 2X YT medium

containing respective antibiotics. Expression of recombinant proteins in *E. coli* was induced by adding lactose analog, IPTG (1 mM). Cultures were grown for 5 additional hours post-induction. Cultures were harvested by centrifugation at 4000 rpm for 10 min in a Swinging bucket centrifuge. Bacterial pellets were kept frozen at -70°C until further use. Protein was extracted from bacterial pellet using Bugbuster protein extraction reagent. The supernatant containing the soluble fraction was separated and the insoluble inclusion body fraction was further solubilized in BugBuster Reagent and 20 units of lysozyme were added again and incubated at 25°C for 20 min. Proteins were electrophoresed on 12 % SDS-PAGE and the presence and specificity of expressed protein were determined by tag-specific antibody (anti-His antibody).

The recombinant protein was purified by Ni metal ion chelating chromatography columns (Novagen) in denatured conditions. Protein was further dialyzed against decreasing urea concentration and resulting protein aggregates were solubilized again in solubilization buffer with 0.3% N-lauryl sarcosine detergent (Novagen). The recombinant protein was quantified by the Qubit protein assay kit (Life Technologies) and characterized by SDS-PAGE.

2.3. Effect of EF Protein on Growth and Vascularization of Chicken Embryos

A small 3 mm window was made on the side of the air sac in 7-day-old embryos. Two hundred nanograms of rEF protein were dropped over the Chorioallantoic membrane (CAM) through a window. A total of 12 embryos were included in the treated and control groups. The same solubilization buffer which was used to solubilize recombinant protein served as control. The window was sealed with cellophane tape and incubated in a humidity chamber at 37°C for 72 hours. After incubation, the eggshell was opened by the air sac and gross vasculature was examined. The angiogenesis index was calculated using the following formula.

Angiogenesis index = Mean branch points ± 2SD of new branch points

The number of branch points below the air sac was counted and averaged. Then, the CAM was removed and the embryo was taken out of the egg to be examined for growth.

2.4. In-silico Analysis

For further confirmation, these receptors bind to their natural ligands such as HGF and VEGF, and amplitude of interaction was observed.

Protein-ligand interaction was observed *in-silico* using HEX-8 protein docking software downloaded from http://hex.loria.fr. The PDB for all ligands and receptors was downloaded from the RCSB protein data bank in text format. Docking was set to 2000 solutions per pair and based on a “shape only” correlation. The 3D-FFT (First Fourier transform) model was used with a grid dimension of 0.60 angstroms. The remaining parameters were kept at default. The free-energy change upon binding (the binding affinity) is indicated as E total including other very important descriptors of the I.E hydrogen bond. Root Mean Squares Deviation (RMSD) value -1 was used. The total number of hydrogen bonds (H-bonds) involved in the interactions among the docked proteins and the total free energy for each interaction were compared (Table 3). The occurrence of more H-bonds and less free energy indicates a stronger interaction between the protein and ligand.

3. Results

3.1. Expression and Purification of rEF

Recombinant His tagged EF protein of 89 kDa which was expressed under the control of T7 promoter of pET-28a vector in Rosettablue (DE3) codon plus cells and was found to be localized solely in inclusion body fraction (Figure 1A) with a yield of 8 mg/liter of culture. The purified recombinant protein was found by tag-specific antibody (anti-His antibody) shown in figure 1B.

3.2 Effect of rEF on Growth and Vascularization of Chicken Embryos

The effect of rEF on growth and angiogenesis was evaluated in rEF treated embryonated eggs. The eggshell was opened to visualize the effect after 72 hours of treatment. A significant reduction in vasculature was observed in EF treated group compared to that of the control group (Figure 2A, 2B and 2D). Only major blood vessels and a few sub-vessels were visible by reducing the number of branch points (Table 1). Chorioallantoic membrane became very fragile in the EF treated group, so it could not be harvested and processed for histopathological analysis. Embryos were harvested from the control and treated groups and their size was measured and a size reduction was observed (Table 2). Statistically significant ($p < 0.001$) reduction was observed in both the number of branch points and size of embryos (Figure 2C and 2D).

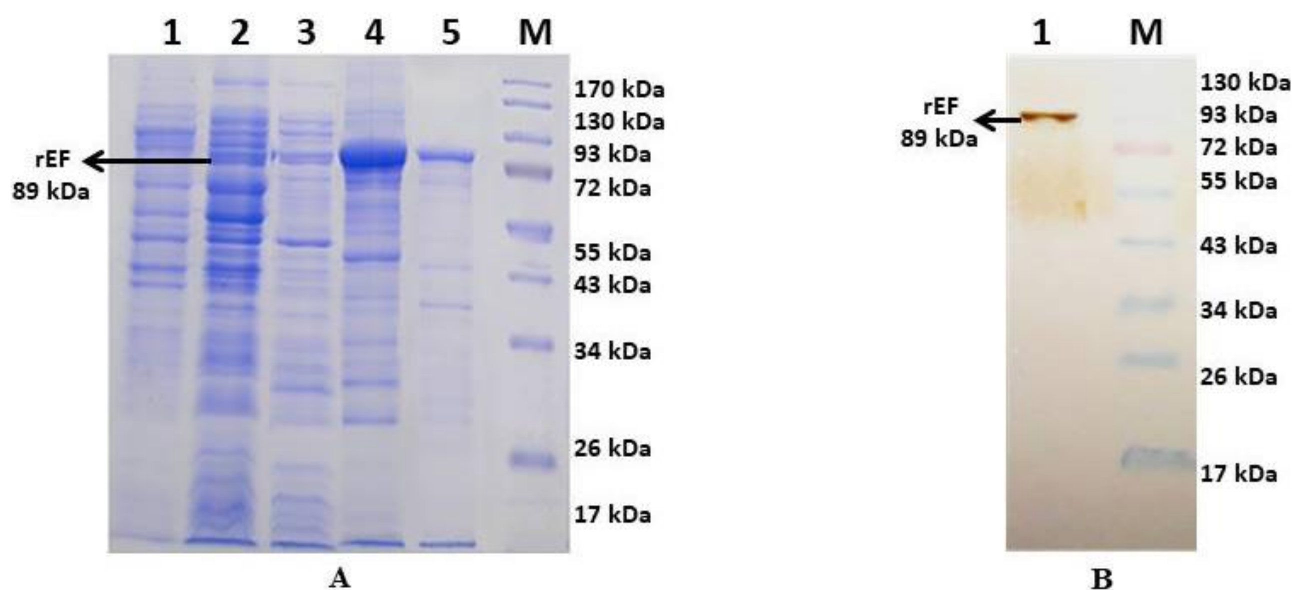

Figure 1. SDS PAGE and Western blot analysis of rEF. A. SDS-PAGE for analysis of EF expression, solubility, and purification. Lane 1: Uninduced culture; lane 2: induced culture; lane 3: soluble fraction; lane 4: insoluble fraction; lane 5: purified rE

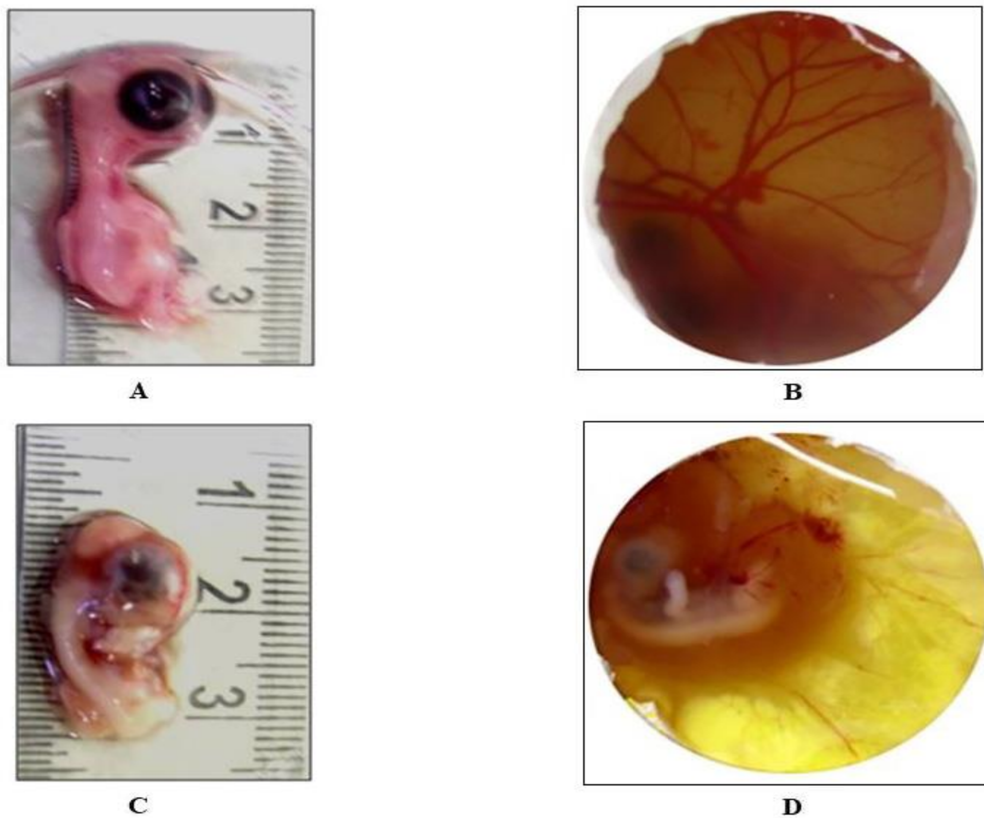

Figure 2. Observation of embryo size and the number of blood vessels. **A:** Size of control embryo. **B:** Number of branch points of blood vessels in control embryo. **C:** Size of EF treated embryo. **D:** Number of branch point of blood vessels in EF treated embryo.

Table 1. The number of branch points in the vasculature of the chorioallantoic membrane of embryonated eggs.

S. No.	Treatment group (Each of 12 embryos)	Mean branch point	Standard deviation	Standard error	Angiogenic index
1	Control	13	0.71	0.202	14.42
2	rEF treated	4	1.40	0.404	6.8

Table 2. Size of the chicken embryo in control and treated groups.

S. No.	Treatment group (Each of 12 embryos)	Average (in cm)	Standard deviation	Standard error
1	Control	11.06	1.40	0.404
2	EF protein treated	3.0875	0.71	0.205

Standard error calculated as mean divided by the square root of N

3.3. In silico Study

The result of comparing the amplitude of interaction of cMET and VEGF receptor with their ligands such as α and β hepatocyte growth factor and VEGF and EF protein is presented in Table 3.

Table 3. Result of receptor-ligand interaction (In silico study).

S. No.	Ligand	Receptor	Hydrogen bonds	E total
1	EF (pdb-1K8T)	cMET (pdb-3DKC)	15	-797.4 (fig. 3.1)
2	β-chain of hepatocyte growth factor (pdb-1SI5)	cMET (pdb-3DKC)	6	574.89 (fig. 3.2)
3	α-chain of hepatocyte growth factor (pdb-1YBW)	cMET (pdb-3DKC)	11	-777.37 (fig. 3.3)
4	EF (pdb-1K8T)	VEGF Receptor 2 (kdr) kinase domain (pdb-1VR2)	9	-610.06 (fig. 3.4)
5	Human Vascular Endothelial Growth Factor (pdf-1VPF)	Vascular Endothelial Growth Factor Receptor 2 (kdr) Kinase Domain (pdb-1VR2.)	16	-712.46 (fig. 3.5)
6	EF (pdb-1K8T)	Fibroblast Growth Factor Receptor_1fgk A	-1	-605.60
7	EF (pdb-1K8T)	Nerve growth factor receptor (NGF) TrkA (1HE7)	-1	-665.3
8	EF (pdb-1K8T)	Human Epidermal Growth Factor (HEGF) HER1 (2ITX)	-14	-736.56
9	EF (pdb-1K8T)	HEGF- HER2 (3PP0)	-19	-442.36

4. Discussion

Anthrax is caused by gram-positive bacteria *B. anthracis*. Its vegetative form secretes 3 major secretary exotoxins LF, PA, and EF. Among all the three exotoxins, EF protein has long been ignored by researchers as LF and PA have received more attention due to high lethality that leads to death and extraction of protective antibody titer, respectively. Nowadays, EF has attracted the attention of researchers and its various functions have been clarified. The amount of secreted EF is limited in the culture medium so that researchers investigate this protein and its function. Therefore, the EF gene was cloned and expressed in *E. coli* for a high amount of EF protein. Eight mg per liter of protein was obtained in inclusion body fraction, which was then solubilized using CAPS buffer, a zwitterionic buffer, which is used in refolding procedures to obtain biologically active protein.

Edema factor is an adenyl cyclase and its catalytic activity requires the binding of calmodulin, a calcium-binding protein. The EF–calmodulin complex catalyzes the synthesis of cAMP in host cells. cAMP is well established secondary messenger for many cellular signaling pathways involved in growth and differentiation, apart from CTP, UTP, and ITP (10) which causes edema in the body due to disturbance in intracellular water homeostasis. Edema factor is supposed to be functionally inactive. EF is internalized inside the cell after complex formation with receptor-bound PA (11).

One of our recent studies demonstrated PA independent functioning of LF, and cMET (a tyrosine kinase which significantly affects various signaling pathways associated with growth, motility, differentiation, movement, and invasion of cells) has

been proposed as an alternative receptor for LF, which is assumed to modulate the LF-mediated signaling cascade if attached to the cMET receptor (unpublished data). It is obvious to assume the similar function exhibited by both based on the fact of N terminal sequencing and similarity of EF and LF genes (12). Therefore the role of EF, independent to PA was observed in embryonated chicken eggs.

In the *in-ovo* experiment, EF protein-treated chorioallantoic membrane was found to be remarkably fragile with weak vasculature. The chicken embryo was also poorly vascularized and reduced in size in the treated group which revealed the effect of EF on angiogenesis and growth of embryo during the growth period. Endothelial cell migration to chemotactic factors is a key feature of angiogenesis. In the experiments of Hong (2007), the effect of edema toxin (PA and EF combination) was determined using HUVECs which indicated that it can significantly alter the morphology and cytoskeletal structure of endothelial cells. Despite morphological and cytoskeletal changes, no growth inhibition was observed with edema toxin (13).

Several other researchers demonstrated inhibition of angiogenesis in rat endothelial cell lines and primary cultures of human umbilical vein endothelial cells by cAMP or cAMP analogs (14). cAMP-mediated endothelial cell apoptosis is also evidenced by DNA fragmentation in TUNEL assay and caspase-3 cleavage in vivo (15).

In the present study, inhibition of angiogenesis through cAMP generation using adenyl cyclase activity of EF failed to play the antiangiogenic role as it must enter the cell for this activity. An alternative mechanism for inhibiting angiogenesis and growth of embryo can be speculated in the absence of EF trafficking molecule (PA). The clue for the PA independent function of EF comes from the evidence that few receptors bind multiple ligands. Physiological functions of TEM-8 (Tumor Endotheliaum Marker-8) receptor is associated with migration of endothelial cells and tubule formation, where the CMG-2 (Capillary Morphogenesis protein- 2) receptor is linked with proliferation and morphogenesis of endothelial cells (16). The TEM-8 and CMG-2 receptors also act as a receptor for anthrax toxin. The GM1 ganglioside receptor involved in neuronal plasticity acts as a receptor for cholera toxin and heat-labile enterotoxin of *E. coli* (Traveller's diarrhea). An alternative receptor for EF was also explored which might be involved in angiogenesis and growth. cMET and VEGF are two potential candidate receptors that dock with their ligands (hepatocyte growth factor VEGF) associated with physiological functions and compared with the docking results with EF. The α and β chains of HGF bind with its cMET receptor, but the interaction was weak in terms of hydrogen bond and E total (αHGF-cMET; H bond=11, E total= 777.37 KiloJoule/Mole and βHGF-cMET; H bond=6, E total=-574.89 KiloJoule/Mole) compared to the interaction of EF and cMET (H bond=15, E total=--797.4)

This suggests that the interaction of EF with the cMET receptor was stronger than that of the natural ligand (HGF α and β chains) which might result in competitive inhibition of HGF by EF and lead to growth inhibition and reduced angiogenesis in embryo.

In contrast, the VEGF receptor strongly interacts with its natural VEGF ligand (H bond=9, E total=-610.06). Although the interaction between EF and VEGF receptors was not strong enough (H bond= 9 and E total -610.06) compared to VEGF and VEGF receptors (H bond= 16 and E total -712.46). The result summarizes the possible existence of a ligand-receptor relationship between EF and cMET receptors, in which EF competes with HGF and blocks the signaling cascade. This competitive inhibition explains how EF can inhibit embryonic growth and angiogenesis without entering inside the cell or exhibiting adenyl cyclase activity.

The findings of this study on EF inhibited angiogenesis and growth are consistent with those of several researchers who have proven that edema toxin inhibits angiogenesis by acting as a chemotaxis

inhibitor of VEGF for endothelial cells (Hong et al., 2007). Hong et al. (2007), demonstrated angiogenesis inhibition in in-vitro cell migration assay (13).

5. Conclusion

The present study demonstrated a complex in-ovo system that consists of several factors such as growth factors and a complex cell to cell signaling system. In this study, the same function of EF was presented without the help of trafficking moiety PA and the use of alternative receptors by EF to perform its activity.

Abbreviations

CAM- Chorio allantioc membrane; cAMP- Cyclic adenosine monophosphate; cMET- Hepatocyte growth factor receptor; CMG-2- Capillary morphogenesis gene-2; EF- Edema factor; FFT- First Fourier transform; HGF- Hepatocyte growth factor; IPTG- Isopropyl β-D-1 thiogalactopyranoside; LF- Lethal factor; MAPK- Mitogen activated protein kinase; PA- Protective antigen; PKA- Protein kinase A; RMSD- Root Mean Squares Deviation; VEGF- Vascular endothelial growth factor; TEM-8- Tumor endothelial marker-8.

Authors' Contribution

Study concept and design: R. K.
Acquisition of data: B. P.
Analysis and interpretation of data: K. R.
Drafting of the manuscript: A. K. P.
Critical revision of the manuscript for important intellectual content: N. P.
Statistical analysis: S. B.
Administrative, technical, and material support: H. M.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

The authors would like to express their sincere gratitude to the director of ICAR-High Security Animal Disease Laboratory, Anand Nagar, Bhopal, for providing the facilities. Dr. Rekha Khandia was the recipient of the Junior Research Fellowship in Science from the Council of Scientific and Industrial Research, India.

References

1. Bradley KA, Mogridge J, Mourez M, Collier RJ, Young JAT. Identification of the cellular receptor for anthrax toxin. Nature. 2001;414(6860):225–9.
2. Young JA and Collier RJ. Anthrax toxin: receptor binding, internalization, pore formation, and translocation. Annu Rev Biochem. 2007;76:243-65.
3. Cheng X, Ji Z, Tsalkova T, Mei F. Epac and PKA: a tale of two intracellular cAMP receptors. Acta biochimica et biophysica Sinica. 2008;(7):651-62.
4. Lokker NA, Mark MR, Luis EA, Bennett GL, Robbins KA, Baker JB, et al. Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding. EMBO J. 1992;11(7):2503-10.
5. Tajima H, Matsumoto K, Nakamura T. Hepatocyte growth factor has potent anti-proliferative activity in various tumor cell lines. FEBS Lett. 1991;291(2):229-32.
6. Naldini L, Weidner KM, Vigna E, Gaudino G, Bardelli A, Ponzetto C, et al. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. EMBO J. 1991;10(10):2867.
7. Montesano RK, Matsumoto K, Nakamura T, Orci L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell. 1991;67(5):901-8.
8. Basilico C, Arnesano A, Galluzzo M, Comoglio PM, Michieli P. A high affinity hepatocyte growth factor-binding site in the immunoglobulin-like region of Met. J Biol Chem. 2008;283(30):21267-77.
9. Goel HL, Mercurio AM. VEGF targets the tumour cell. Nat Rev Cancer. 2013;13(12):871-82.
10. Göttle M, Dove S, Kees F, Schlossmann J, Geduhn J, König B, Shen Y, Tang WJ, Kaever V, Seifert R. Cytidylyl and uridylyl cyclase activity of Bacillus anthracis edema factor and Bordetella pertussis CyaA. Biochemistry. 2010;49(26):5494-503.
11. Tang S, Moayeri M, Chen Z, Harma H, Zhao J, Hu H, Purcell RH, Leppla SH, Hewlett IK. Detection of anthrax toxin by an ultrasensitive immunoassay using

europium nanoparticles. Clin Vaccine Immunol.;16(3):408-13.

12. Quinn CP, Singh Y, Klimpel KR, Leppla SH. Functional mapping of anthrax toxin lethal factor by in-frame insertion mutagenesis. J Biol Chem. 25;266(30):20124-30.
13. Hong J, Doebele RC, Lingen MW, Quilliam LA, Tang WJ, Rosner MR. Anthrax edema toxin inhibits endothelial cell chemotaxis via Epac and Rap1. J Biol Chem. 2007;282(27):19781–7.
14. del Valle-Pérez B, Martínez-Estrada OM, Vilaró S, Ventura F, Viñals F. cAMP inhibits TGFbeta1-induced in vitro angiogenesis. FEBS Lett. 2004;569(1-3):105-11
15. Kim S, Bakre M, Yin H, Varner JA. Inhibition of endothelial cell survival and angiogenesis by protein kinase A. J Clin Investig. 2002;110(7):933-41.
16. Khandia R, Bhatia S, Chanu KV, Sood R, Dhama K. Anthrax toxin receptors, functions and their possible use in therapeutics: a review. Asian J Anim Vet Adv. 2014;9(10):599-609.