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A neutralizing recombinant single chain antibody, scFv, against BaP1, A P-I hemorrhagic metalloproteinase from *Bothrops asper* snake venom

J.M.A. Castro [a], T.S. Oliveira [a], C.R.F. Silveira [a], M.C. Caporrino [a], D. Rodriguez [b], A.M. Moura-da-Silva [a], O.H.P. Ramos [c], A. Rucavado [d], J.M. Gutiérrez [d], G.S. Magalhães [a], E.L. Faquim-Mauro [a], I. Fernandes [a, *]

[a] *Laboratório de Imunopatologia, Instituto Butantan, Av. Vital Brazil, 1500, Butantã, CEP 05503-900 São Paulo, SP, Brazil*
[b] *Laboratório de Biotecnologia IV, Instituto Butantan, São Paulo, Brazil*
[c] *CEA, iBiTecS, Service d'Ingénierie Moléculaire des Protéines, Gif sur Yvette, France*
[d] *Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica*

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ABSTRACT

BaP1 is a P-I class snake venom metalloproteinase (SVMP) relevant in the local tissue damage associated with envenomings by *Bothrops asper*, a medically important snake species in Central America and parts of South and North America. The main treatment for these accidents is the passive immunotherapy using antibodies raised in horses. In order to obtain more specific and batch-to-batch consistent antivenons, recombinant antibodies are considered a good option compared to animal immunization. We constructed a recombinant single chain variable fragment (scFv) from a monoclonal antibody against BaP1 (MABaP1) formerly secreted by a hybridoma clone. This recombinant antibody was cloned into pMST3 vector in fusion with SUMO protein and contains VH and VL domains linked by a flexible $(G_4S)_3$ polypeptide (scFvBaP1). The aim of this work was to produce scFvBaP1 and to evaluate its potential concerning the neutralization of biologically important activities of BaP1. The cytoplasmic expression of this construct was successfully achieved in C43 (DE3) bacteria. Our results showed that scFvBaP1-SUMO fusion protein presented an electrophoretic band of around 43 kDa from which SUMO alone corresponded to 13.6 kDa, and only the scFv was able to recognize BaP1 as well as the whole venom by ELISA. In contrast, neither an irrelevant scFv anti-LDL nor its MoAb partner recognized it. BaP1-induced fibrinolysis was significantly neutralized by scFvBaP1, but not by SUMO, in a concentration-dependent manner. In addition, scFvBaP1, as well as MaBaP1, completely neutralized *in vivo* hemorrhage, muscle necrosis, and inflammation induced by the toxin. Docking analyses revealed possible modes of interaction of the recombinant antibody with BaP1. Our data showed that scFv recognized BaP1 and whole *B. asper* venom, and neutralized biological effects of this SVMP. This scFv antibody can be used for understanding the molecular mechanisms of neutralization of SVMPs, and for exploring the potential of recombinant antibody fragments for improving the neutralization of local tissue damage in snakebite envenoming.

* Corresponding author. Tel.: +55 11 2627 9783.
E-mail addresses: juciane.castro@gmail.com (J.M.A. Castro), tamiresso@gmail.com (T.S. Oliveira), caio.silveira@butantan.gov.br (C.R.F. Silveira), criscaporrino@gmail.com (M.C. Caporrino), dunia.soto@butantan.gov.br (D. Rodriguez), ana.moura@butantan.gov.br (A.M. Moura-da-Silva), oscarhpr@gmail.com (O.H.P. Ramos), alexandra.rucavado@ucr.ac.cr (A. Rucavado), jose.gutierrez@ucr.ac.cr (J.M. Gutiérrez), geraldo.magalhaes@butantan.gov.br (G.S. Magalhães), eliana.faquim@butantan.gov.br (E.L. Faquim-Mauro), irene.fernandes@butantan.gov.br (I. Fernandes).

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1. Introduction

Envenoming by snakebites constitutes a significant public health problem on a worldwide basis, particularly in tropical regions of Africa, Asia and Latin America (Gutiérrez et al., 2006; WHO, 2013). According to WHO, 2013, the global total of snakebite envenoming cases is estimated to 2, 4 million with deaths ranging from 94,000 to 125,000 per year.

At present, passive immunotherapy is the only specific treatment for snakebite envenoming used worldwide, which is based on the administration of antibodies produced by the hyperimmunization of animals, generally horses, with snake venoms. These antivenoms are preparations of immunoglobulins (Igs), or Ig fragments such as $F(ab')_2$ or Fab, obtained by fractionating hyperimmune plasma either by treatment with caprylic acid to obtain whole IgG preparations (Rojas et al., 1994; Gutiérrez et al., 2005) or by enzymatic digestion followed by ammonium sulfate precipitation and chromatographic steps to obtain IgG fragments (Cardoso, 2000; WHO, 2010).

Antivenoms are generally very efficient for neutralizing the most relevant systemic effects of snakebite envenoming (Warrell, 1992; Lalloo and Theakston, 2003; Gutiérrez and León, 2009); however, they poorly neutralize toxins involved in local pathological effects (edema, dermonecrosis, local hemorrhage and myonecrosis). This is due to the early onset of these effects upon envenoming and, to some degree, to the poor distribution of the Igs and their fragments to the local tissues where venom is injected (Gutiérrez et al., 1998). Furthermore, administration of antivenom may be associated, in a variable percentage of cases, with early and late adverse reactions to the heterologous proteins (Warrell, 1995; León et al., 2013).

A new perspective for the treatment of snakebite envenoming has emerged with the use of recombinant antibodies, particularly the Fv fragment, named the single-chain variable fragment (scFv). The scFv antibody format presents several distinctive features as compared to the whole antibody, such as higher diffusion to the affected tissues, low immunogenicity and faster elimination (Azzazy and Highsmith, 2002; Zhang et al., 2014; Yu et al., 2014).

ScFv antibody is composed exclusively of Ig VL and VH regions joined by a flexible peptide linker, usually composed of 15 amino acid residues with the sequence $(Gly_4Ser)_3$, and is expressed as a single polypeptide chain. The linker allows the association of the VH and VL to form the antigen-binding site (Azzazy and Highsmith, 2002). Recombinant scFv antibodies may be obtained by phage display technology or from hybridoma cells secreting monoclonal antibodies.

Our group has obtained and characterized six monoclonal antibodies (MoAb) against BaP1 (MABaP1) from the venom of the pit viper *Bothrops asper*. BaP1 is an abundant P-I snake venom metalloproteinase (SVMP) in the venom of this species, and plays a relevant role in the local tissue damage associated with envenomings by *B. asper*, a medically important species in Central America and parts of South and North America. We previously showed that three monoclonal antibodies were able to neutralize BaP1-induced hemorrhagic and proteolytic activities (Fernandes et al., 2010).

Herein we describe the generation of a recombinant single chain antibody fragment (scFv) produced from the mRNA isolated from MABaP1-8, expressed in *Escherichia coli* cytoplasm, and possessing neutralizing activities similar to those of the original monoclonal antibody. In addition to its value as a molecular tool to assess the structure–function relationships of this SVMP, this scFv will allow the evaluation of small recombinant antibody fragments in the neutralization of venom-induced local tissue damage.

2. Materials and methods

2.1. Animals, venoms and enzymes

BALB/c female mice (18–20 g) were used throughout. All procedures were approved by the Ethical Committee for Animal Research of Instituto Butantan (661/09) while CGEN (Board of Genetic Heritage Management) provided the license for genetic patrimony access (02001005148/2008–11). The Herpetology Laboratory of Instituto Butantan provided *Bothrops neuwiedi* venom, while Instituto Clodomiro Picado, Costa Rica, provided *B. asper* venom. The venoms corresponded to pools obtained from many specimens and were lyophilized and stored at −20 °C. The SVMPs BaP1 and BnP1 were purified as previously described by Gutiérrez et al. (1995) and by Baldo et al. (2008), respectively.

2.2. Cloning of single-chain variable fragment (scFv) derived from the monoclonal anti-BaP1 antibody

Protocols for DNA manipulation were used as described in Sambrook and Russell (2001). Briefly, MABaP1-8 hybridoma cells secreting anti-BaP1 monoclonal antibody (Fernandes et al., 2010) were cultivated in RPMI medium (Invitrogen, Brazil) plus 10% fetal bovine serum at 37 °C in 5% CO_2. Hybridoma mRNA was extracted (IlustraQuickprep mRNA Purification Kit – GE Healthcare, UK) from the cells and reversely transcribed into cDNA (First Strand cDNA Synthesis Kit – GE Healthcare, UK). Commercially available primers [Light primer mix, Heavy primer 1, Heavy primer 2 (GE Healthcare, UK)] were employed to amplify the variable domain of heavy (VH) and light (VL) chains of the antibody, following the manufacturer's instructions. The cDNA inserts corresponding to VL and VH were cloned into the pGEM-T Easy vector and submitted to sequencing. The synthetic gene ScFvBaP1 was built by GeneArt containing VH sequence joined to VL by a flexible linker sequence that encodes $(Gly_4Ser)_3$ and codon-optimized for *E. coli* with *Bam*HI and *Hind*III sequence flanking the construction.

GeneArt vector holding scFvBaP1 sequence was digested with *Bam*HI and *Hind*III endonucleases to release the construct and cloned into pMST3 vector, a modified pET28b vector with the small ubiquitin-related modifier (SUMO) sequence cloned 3′ to the His6 tag (Yunus and Lima, 2009), resulting in the expression of SUMO-scFvBaP1.

All microorganisms manipulation was approved by CTNBio (National Technical Commission on Biosecurity) (CQB-0039/98 de 31/07/1998).