An in vivo role for *Trypanosoma cruzi* calreticulin in antiangiogenesis

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Abstract

Angiogenesis leads to neovascularization from existing blood vessels. It is associated with tumor growth and metastasis and is regulated by pro- and antiangiogenic molecules, some of them currently under clinical trials for cancer treatment. During the last few years we have cloned, sequenced and expressed a *Trypanosoma cruzi* calreticulin gene (*TcCRT*). Its product, TcCRT, a 45 kDa protein, is more than 50% identical to human CRT (HuCRT). TcCRT, present on the surface of trypomastigotes, binds both C1q and mannan binding lectin and inhibits the classical activation pathway of human complement. Since TcCRT is highly homologous to a functional antiangiogenic fragment from HuCRT (aa 120–180), recombinant (r) and native (n) TcCRT were tested in their antiangiogenic effects, in the chick embryonic chorioallantoid membrane (CAM) assay. Both proteins mediated highly significant antiangiogenic effects in the in vivo CAM assay. This effect was further substantiated in experiments showing that the plasmid construct pSecTag/*TcCRT* also displayed significant antiangiogenic properties, as compared to the empty vector. Most likely, the fact that antiangiogenic substances act preferentially on growing neoplasic tissues, but not on already established tumors, is due to their effects on emerging blood vessels. The results shown here indicate that TcCRT, like its human counterpart, has antiangiogenic properties. These properties may explain, at least partly, the reported antineoplasic effect of experimental *T. cruzi* infection.

Keywords: *Trypanosoma cruzi*; Calreticulin; Antiangiogenesis

1. Introduction

Angiogenesis is a complex multi-step process leading to neovascularization from existing blood vessels. It is associated with inflammation, wound healing, tumor growth, and metastasis. The generation of new blood vessels is regulated by pro- and antiangiogenic molecules, some of them currently under clinical and preclinical trials for cancer treatment [1–4].

Several angiogenic factors have been identified and divided into three groups. The first one includes soluble growth molecules such as acid and basic fibroblast growth factors (aFGF and bFGF, respectively) and vascular endothelial growth factor (VEGF), which affect endothelial cell growth and differentiation [5]. The second group inhibits proliferation and enhances differentiation of endothelial cells and includes transforming growth factor β (TGFβ) [6], angiogenin, as well as several low-molecular weight substances [7]. The third group comprises extracellular matrix-bound cytokines,

Abbreviations: CAM, embryonic chorioallantoid membrane; HuCRT, human calreticulin; IWB, Immuno Western Blots; kDa, kiloDaltons; nTcCRT, immunoaffinity purified native *T. cruzi* calreticulin; pGM-CSF, plasmid expressing the gene coding for the granulocyte and macrophage colony stimulating factor; pSecTag/*TcCRT*, plasmid expressing the gene coding for TcCRT without its leader and KEDL sequences; rTcCRT, recombinant *T. cruzi* calreticulin; TcCRT, *T. cruzi* calreticulin; TcS, TcCRT S functional domain

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which may contribute to angiogenic regulation [7]. The prevailing notion is that angiogenesis is governed by a balance among positive and negative regulators and the microenvironment [1].

During the last few years we have cloned, sequenced and expressed a *Trypanosoma cruzi* calreticulin gene (*TcCRT*) that was localized in several chromosomes. We have named its product TcCRT, a 45 kDa, electrophoretically dimorphic and immunogenic protein [8–12], more than 50% identical to the human counterpart (HuCRT) [10,13,14]. TcCRT is present on the surface of trypomastigotes and binds both C1q and mannan binding lectin (MBL), resulting in the inhibition of the classical activation pathway of the human complement system [15].

Over 40 functions have been described for HuCRT [13,14]. Three fragments derived from HuCRT (aa 120–180, aa 1–180 or vasostatin, and aa 120–400 or D120CRT), as well as the whole molecule, display antiangiogenic effects. They inhibit the proliferation of endothelial cells, the generation of new blood vessels and in vitro and in vivo tumoral growth [16–19]. Specifically, vasostatin inhibits the endothelial cell binding to laminin, thus reducing the basic fibroblast growth factor (bFGF)—dependent proliferation of these cells [16]. In TcCRT we find segments with 46% identity and 60% positivity with a functional fragment from HuCRT (aa 120–180).

In the chorioallantoic membrane (CAM) assay, performed in fertilized chicken eggs, sterile methyl cellulose discs, instilled with solutions of pro- or anti-angiogenic molecules, are placed on the embryonic membrane. Using conventional histological procedures, the number of blood vessels is counted in the areas adjacent to the filters. Many investigators have studied the histological and morphological changes associated with the proliferation of new vessels and tumor neovascularization by direct observation using the CAM assay [20–25]. For example, the angiostatic effect of betamethasone has been reported [26,27] by using this assay. Here, using this assay, we show that both native and recombinant TcCRT, as well as its coding gene, have antiangiogenic effects, possibly explaining, at least in part, the reported antineoplasic effects of experimental *T. cruzi* infection [28–31].

2. Materials and methods

2.1. Recombinant TcCRT

DNA coding for TcCRT was isolated from a genomic library in the λgt11 phage [10]. *TcCRT*, without its leader and KEDL endoplasmic reticulum retention sequence, was amplified by PCR and ligated in the pET-28b(+) vector (Novagen, Darmstadt, Germany) in *Eco*RI restriction sites. *TcCRT* was amplified by using forward (gga att cca cgg tgt act tcc acg ag) and reverse (tgg aat tcg ggt gca gca att tt) primers. Recombinant TcCRT (rTcCRT) was expressed from *E.coli* BL21(DE3)pLysS, transformed with the pET-28b(+)/*TcCRT* plasmid and cultivated in Luria Bertani medium (containing $34\,mg \times ml^{-1}$ chloramphenicol and $50\,mg \times ml^{-1}$ kanamycin) at 37 °C, until reaching an O.D. of 0.5 at 600 nm. rTcCRT expression was induced with 1 mM IPTG, during 3 h, at 37 °C and the protein was purified by affinity chromatography in a nickel column (HisBind Resin, Novagen, Darmstadt, Germany). Purity of rTcCRT was determined both by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and by immunowestern blotting (IWB), developed with an anti-TcCRT monoclonal antibody (E2G7) [10] and also with rabbit polyclonal antibodies anti-rTcCRT and anti its S functional domain (TcS, aa 159–281) [15], all generated in our laboratory. A whole epimastigote extract was used as a positive control in the IWB, as described in [10]. The recombinant protein preparations were tested for the presence of contaminating endotoxin (Limulus Amebocyte Lysate, Pyrogent Plus® kit, BioWhittaker Inc., Cambrex Company, MD, USA).

2.2. Native TcCRT

Native TcCRT (nTcCRT) was purified by immunoaffinity chromatography, from a whole epimastigote (Tulahuén strain) extract, in a cyanogen bromide-activated Sepharose (Pharmacia, Stockholm, Sweden) column, prepared with polyclonal rabbit antibodies anti-rTcCRT. Purity of nTcCRT was determined as described in Section 2.1.

2.3. Plasmid constructs

The PCR-amplified *TcCRT* gene product, without its leader and KEDL sequences, was ligated to the pSecTag2B vector (Invitrogen, La Jolla, CA, USA) in *Eco*RI restriction sites. This construct was designated as pSecTag/*TcCRT*. The empty vector (pSecTag2B) was used as a negative control.

A pGM-CSF plasmid, containing and expressing the gene coding for the granulocyte–macrophage colony stimulating factor was donated by Dr. Oscar R. Burrone (International Center for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy). Endotoxin free plasmid preparations were obtained from *E.coli* DH5α, transformed with the pSecTag/*TcCRT*, pSecTag2B or pGM-CSF plasmids, using conventional technology [32,33].

2.4. Chick embryonic chorioallantoid membrane assay

The chick embryonic chorioallantoid membrane assay was performed as previously described by us [26,27] and by another laboratory [34]. Briefly, 10 fertilized White Leghorn hen eggs (National Public Health Institute, Santiago, Chile) were used in protein and DNA assays. The eggs were incubated for 48 h in a humid 38.5 °C atmosphere. After extracting 2–3 ml of albumin, a small window was opened in the egg, in order to allow separation of the CAM from the shell during the embryo development. The window was temporarily sealed with adherent tape and the eggs were incubated for additional 5 days. Then, sterile methyl cellulose discs

(5 mm diameter, 0.25 μm pore size, 125 μm thickness) (Advantec MFS Inc., CA, USA), were deposited on the CAMs. Immediately, 10 μl of the corresponding samples were directly added onto the filters. The windows were then sealed and the eggs were incubated for additional 72 h, as indicated above. Then, the CAMs were sliced, following the filters contours, and fixed in 10% (v/v) formaldehyde. Tissue sections were prepared, by standard procedures, for conventional light microscopy aimed at detecting mainly acid polysaccharides, nuclei and cytoskeleton. Blood vessels were counted in a light microscope with a 1 cm^2 micrometric grid, divided in 1 mm^2 sections. Ten of these sections, corresponding to a tissue area of 9000 μm^2 were counted. Blood vessels were identified by their histological characteristics, mainly by their endothelial cells, and by the presence of red blood cells in their lumens. Counting, carried out in a double-blind fashion, was performed in 35 microscopic fields of CAM tissue segments, adjacent to the filter edge.

The above procedures were used in three different experimental approaches:

(a) In one experiment, in order to build a dose–response curve, rTcCRT amounts, ranging between 1–1000 pg, were tested. Separate filters contained 10 pg of nTcCRT.
(b) A second experiment was designed in order to test the possible role of contaminating lipopolysaccharide (LPS) in the recombinant protein preparations. Some filters contained 4.9 and 49 ng of rTcCRT, endogenously contaminated with 0.02 and 0.2 endotoxin EUs. Other filters contained only 0.02 and 0.2 EU of lipopolysaccharide (Sigma, MO, USA).
(c) A third experiment was designed in order to further substantiate the antiangiogenic effect of TcCRT. Filters containing alternatively 10 μg of the endotoxin-free DNA pSegTag/*TcCRT* or 10 μg pSecTag2B empty plasmid, used as negative control. DNA plasmids were diluted in endotoxin-free water (Laboratorio Chile®, Santiago, Chile). In order to maximize the stringency of this experiment, 10 μg of a pGM-CSF plasmid, coding for the proangiogenic cytokine granulocyte/macrophage colony stimulating factor [35,36], were added to all DNA-containing filters.

In all experiments, PBS prepared with endotoxin-free distilled water was used as a negative control. Betamethasone (Laboratorio Chile®, Santiago, Chile), a steroidal anti-inflamatory agent, with known anti-angiogenic properties [26,27], was used as a positive control.

2.5. In vivo validation of the productive properties of the pSecTag/TcCRT construct using genetic immunization of mice

The productive properties of the pSecTag/*TcCRT* construct were validated by assessing its capacity to induce a humoral polyclonal anti-TcCRT response in BALB/c mice immunized three times i.m., every 15 days, with 50 μg of the construct. As a negative control, mice were immunized with the empty vector, pSecTag2B, under the same conditions.

The presence of specific anti TcCRT antibodies in these murine sera (a demonstration that the pSecTag2B/*TcCRT* construct was indeed productive in vivo) was detected in two independent experimental approaches: The first one involved flow cytometric indirect immunofluorescence assays, against 4% paraformaldehyde-fixed and live MF strain *T. cruzi* trypomastigotes (donated by Dr. Gittith Sánchez, Cellular and Molecular Biology Program, ICBM, Faculty of Medicine, University of Chile), as previously described in our laboratory [15]. In the second approach, the murine anti TcCRT antibodies were detected in a conventional ELISA [37], against the recombinant molecule.

2.6. Statistical analysis

Conventional one-tailed Student's *t* tests were used to assess the statistical significance of the antiangiogenic effects. Data are expressed as their means and standard errors. Exact *p*-values are provided.

3. Results

3.1. Analysis of rTcCRT and nTcCRT by SDS-PAGE and IWB

rTcCRT and immunoaffinity purified nTcCRT were analyzed in Coomasie Blue stained SDS-PAGE (Fig. 1A, lanes 1 and 2, respectively). rTcCRT showed a main 60 kDa band, while nTcCRT presented a single 46 kDa component.

In Fig. 1B, an IWB developed with the E2G7 monoclonal antibody, the antigens were rTcCRT, visualized as a main 60 kDa component (lane 1), and whole epimastigote extract, where a 46 kDa band was seen (lane 2).

In Fig. 1C, an anti-rTcCRT rabbit polyclonal antiserum was used to develop the IWB, and the antigens were immunoaffinity purified nTcCRT (visualized as a main 46 kDa component) and rTcCRT, where a main 60 kDa band was detected (lanes 1 and 2, respectively).

In Fig. 1D (lane 1), the IWB was developed with an anti-TcS rabbit polyclonal antiserum, the antigen was rTcCRT and a main 60 kDa band was observed. These bands were not detected by the corresponding preimmune serum (lane 2).

A 71 kDa band was also prominent and consistently recognized by both polyclonal antisera in the rTcCRT preparations (Fig. 1C (lane 2) and D (lane 1)) and was visible in a Coomasie Blue stained SDS-PAGE (Fig. 1A (lane 1)). With one exception, both monoclonal and polyclonal antibodies, recognized the same degradation products in rTcCRT (Fig. 1B–D, lanes 1, 2, and 1, respectively). The exception, a 29 kDa band (Fig. 1A (lane 1)), reacted with both polyclonal antibodies (Fig. 1C (lane 2) and D (lane 1)). The lack of recognition of this 29 kDa band by the E2G7 monoclonal antibody was most likely due to enzymatic loss of the cor-

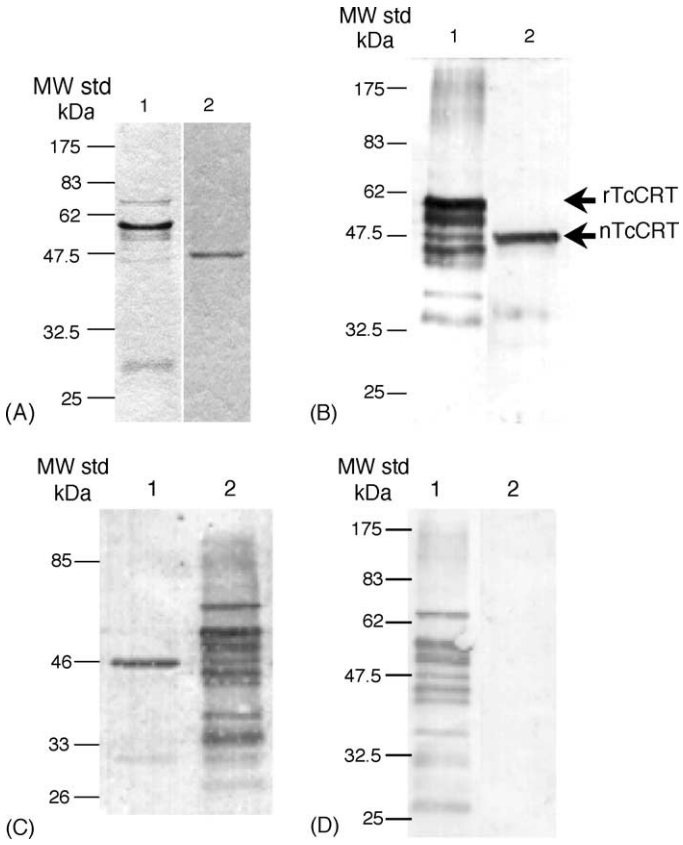

Fig. 1. Analysis of purity of recombinant (r) and native (n) TcCRT by SDS-PAGE and IWB. (A) Coomasie Blue stained SDS-PAGE showing 10 μg rTcCRT (lane 1) and 3 μg nTcCRT; (B) IWB developed with the monoclonal antibody E2G7 for the detection of rTcCRT (lane 1) and native TcCRT, present in an epimastigote extract (lane 2); (C) IWB of immunoaffinity purified nTcCRT (lane 1) and rTcCRT (lane 2), developed with rabbit polyclonal antibodies anti-rTcCRT; (D) IWB of rTcCRT developed with rabbit polyclonal antibodies anti the S domain of TcCRT (lane 1) and with the corresponding preimmune serum (lane 2). Molecular weight standards (MW std) are indicated at the left side of each panel.

responding epitope. None of the bands, recognized only by the rabbit polyclonal antibodies (71 and 29 kDa), decreased their intensity after exhaustive absorption (three times, 2 h each, at 37 °C) with saturated, solid phase-bound, whole *E. coli* extract (results not shown).

3.2. Antiangiogenic effect of TcCRT, as determined in the CAM assay

Fig. 2 summarizes the antiangiogenic properties of rTcCRT and nTcCRT, as determined in the CAM assay. 10 pg of nTcCRT and 1–1000 pg of rTcCRT were assayed. PBS and 10 ng betamethasone were used as negative and positive controls, respectively. A dose/response relationship between several rTcCRT concentrations and angiogenesis inhibition was obtained. At the extreme concentrations (1 pg–1 ng) of the recombinant parasite molecule, angiogenesis inhibitions of 35% ($p = 6 \times 10^{-9}$) and 55% ($p = 10^{-16}$) were respectively observed, while nTcCRT, at 10 pg per filter, induced about 30% inhibition ($p = 6 \times 10^{-7}$). Intermediate concentrations of rTcCRT also induced different inhibition degrees, thus statistically validating a dose-response relationship, as indicated in Fig. 2.

Table 1
Antiangiogenic effect of rTcCRT vs. LPS endotoxin, in a CAM assay

Group	Quantity per filter	% Inhibition
LPS	0.02 EU	39.72
	0.2 EU	48.50
rTcCRT + (LPS)	4.9 ng (0.02 EU)	65.99
	49 ng (0.2 EU)	68.56

$p_{(\text{rTcCRT (4.9 ng) vs. LPS (0.02 EU)})} = 4.5 \times 10^{-7}$;
$p_{(\text{rTcCRT (49 ng) vs. LPS(0.2 EU)})} = 1.4 \times 10^{-3}$.

Since endotoxin (LPS), with known antiangiogenic effects [38] often contaminates *E. coli* recombinant protein preparations, we determined the levels of endogenous LPS contamination present in our samples, as described in Section 2.1. We then carried out the CAM assays including eggs to which only pure LPS was added to the filters, in the same concentrations present in our rTcCRT preparations. As shown in Table 1, 4.9 ng of rTcCRT containing 0.02 EU of endogenous endotoxin, showed a significantly higher ($p = 4.5 \times 10^{-7}$) antiangiogenic effect than 0.02 EU pure LPS. Moreover, 49 ng of rTcCRT containing 0.2 EU of endogenous endotoxin also mediated a significantly higher ($p = 1.4 \times 10^{-3}$) antiangiogenic effect as compared to 0.2 EU pure LPS.

3.3. Antiangiogenic effect of pSecTag/TcCRT

In an effort to complement our studies with rTcCRT and nTcCRT, the antiangiogenic effect of the *TcCRT* gene was assessed in the CAM assay. The average number of blood vessels per microscopic field (3.19 ± 0.13) (35 counted), obtained with equal concentrations of the plasmid mixture pSecTag/*TcCRT* plus pGM-CSF, and with the empty pSecTag2B plus pGM-CSF plasmids (3.80 ± 0.23), were significantly different ($p = 1.2 \times 10^{-2}$). Similar results were obtained in a second experiment.

Since the pSecTag/*TcCRT* plasmid was used as a TcCRT delivery system in the CAM assay, it was necessary to demonstrate that this construct was productive. As shown in Fig. 3, the pSecTag/*TcCRT* construct included the kappa murine immunoglobulin light chain leader sequence and, therefore, the parasite protein should be secreted. Fig. 4 shows FACS results indicating that the anti-TcCRT murine antisera recognized the native protein in the context of fixed and live cultured trypomastigotes. The polyclonal murine anti-TcCRT antibodies, elicited by genetic immunization with the pSecTag/*TcCRT* plasmid, reacted with both intracellular (Fig. 4, left panel) and surface (Fig. 4, right panel) native TcCRT.

Moreover, in a standard ELISA the sera from animals immunized with the pSecTag/*TcCRT* construct, diluted 1/100, recognized rTcCRT bound to the solid phase. Marginal signals were obtained with the sera from animals injected with

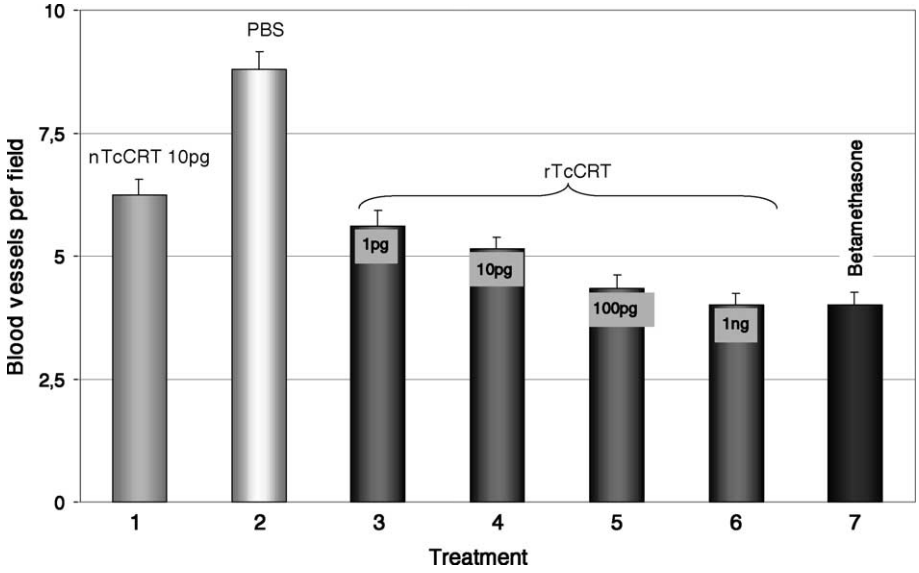

Fig. 2. Antiangiogenic effect of recombinant (r) and native (n) TcCRT determined in a CAM assay. 10 pg of the native protein and 1–1000 pg of rTcCRT were assayed, as described in Section 2. PBS and 10 ng betamethasone were used as negative and positive controls, respectively. Each column represents the mean of 35 blind readings. Each bar over the columns represents the standard error. p values of relevant comparisons: 1 vs. $2 = 6 \times 10^{-7}$; 2 vs. $3 = 6 \times 10^{-9}$; 2 vs. $6 = 1 \times 10^{-16}$; 2 vs. $7 = 4.6 \times 10^{-17}$; 1 vs. $3 = 1.1 \times 10^{-1}$; 1 vs. $4 = 2 \times 10^{-3}$; 3 vs. $4 = 4.8 \times 10^{-2}$; 3 vs. $5 = 1 \times 10^{-3}$; 3 vs. $6 = 1 \times 10^{-3}$; 4 vs. $5 = 4.4 \times 10^{-2}$; 4 vs. $6 = 3 \times 10^{-3}$; 5 vs. $7 = 1.8 \times 10^{-1}$; 6 vs. $7 = 5 \times 10^{-1}$.

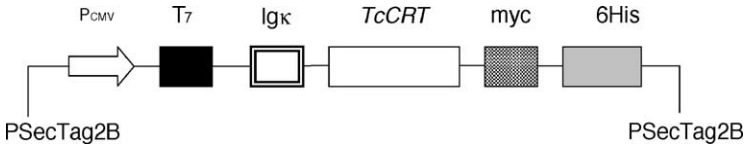

Fig. 3. Schematic diagram of the pSecTag/*TcCRT* vector. This vector contains the cytomegalovirus promoter (P_{CMV}) and incorporates *TcCRT* with six histidines (6His) in the 3′ end. At the 5′, the leader sequence for the murine light kappa chain (Igκ), was introduced, in order to have TcCRT secretion.

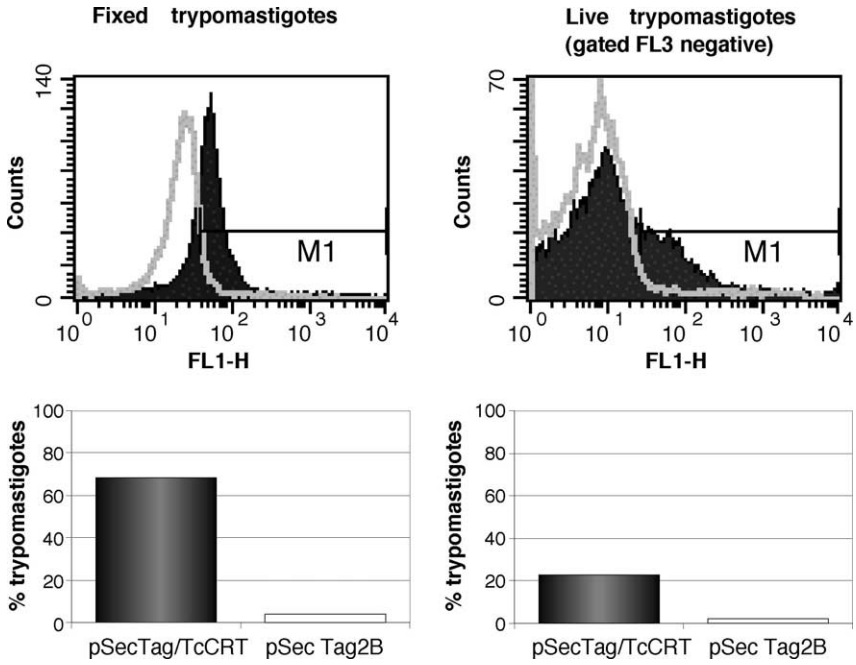

Fig. 4. Polyclonal mouse antibodies, generated by DNA immunization, recognize native TcCRT on live (right top panel) and fixed (left top panel) trypomastigotes (FACs). In this last case, the graph represents values gated based on negative selection with propidium iodide. Closed and open graphs correspond to results obtained with the immune serum from mice immunized with pSecTag/*TcCRT* and pSecTag2B, respectively. The bottom panels correspond to the percentage of selected cells (M1), as indicated in the upper figures.

the empty vector ($p = 1.6 \times 10^{-6}$, for the comparison of both groups).

4. Discussion

Several reports [28–31] indicate that *T. cruzi* infection may have antineoplasic effects. On the other hand, neoplasic growth and metastasis are intimately related with neoangiogenesis [1,3,4], and human calreticulin (HuCRT) has important antiangiogenic properties [16–19]. Since in *T. cruzi* calreticulin (TcCRT) there is a segment with 46% identity and 60% positivity with a functional antiangiogenic fragment from HuCRT (aa 120–180), we asked whether the parasite molecule shares this property.

Fig. 2 and Table 1 show that, in the CAM assay, the difference in the number of blood vessels between the groups treated with rTcCRT and their controls is highly significant, a fact further substantiated by the use of the native purified parasite protein (nTcCRT). The dose-response curve included in Fig. 2 (columns 2–6) shows significant differences not only between the extreme TcCRT amounts, but also among most of the intermediate doses. Most important, nTcCRT has a highly significant antiangiogenic effect ($p = 6 \times 10^{-7}$), although lower than the recombinant counterpart. Since nTcCRT was purified from *T. cruzi* epimastigotes, not known to contain endogenous LPS, a residual endotoxin activity may explain the higher antiangiogenic effect of rTcCRT (expressed in *E. coli*).

When dealing with molecules that modulate angiogenesis, their purity is of utmost relevance. Possible contaminants should be identified and their effect on angiogenesis should be assessed. Immunoaffinity purified nTcCRT, showing homogeneous behavior in SDS-PAGE and in IWB (Fig. 1A (lane 2) and C (lane 1)), had an important and highly significant antiangiogenic effect.

The recombinant protein used in the studies presented is over 90% pure, as shown by SDS-PAGE (Fig. 1A). The 60 kDa protein band, along with bands corresponding to rTcCRT degradation, are recognized by the anti-TcCRT monoclonal antibody (E2G7) in IWB (Fig. 1B(lane 1)). On the other hand, E2G7 recognizes a single band in the total epimastigote extract. Since the recombinant protein preparation does not contain protease inhibitors, due to its intended use in the in vivo CAM assays, degradation products are unavoidable. Likewise, the generation of variable amounts of homo oligomers is a frequent finding in SDS-PAGE and derived IWB technologies. Our results, shown in Fig. 1, detect the presence of two bands (apparent Mws 71 and 29 kDa), both of them not recognized by the MoAb, but clearly detected by the two polyclonal rabbit antibodies used (anti-rTcCRT and anti-TcS). We have also observed this oligomerization tendency with the TcS and TcR (aa 136–281) recombinant domains of TcCRT (results not shown). Thus, aggregation in the 71 kDa band could result in masking of the epitope recognized by the AcMo.

Since all our polyclonal antisera recognize the 29 kDa band, the lack of detection by the AcMo could be due to enzymatic loss of the relevant epitope. This possibility is consistent with the fact that the recombinant protein is more susceptible to degradation than the native counterpart, an issue evident when tracks 1A versus 2A, 1B versus 2B, and 2C versus 1C, are compared in Fig. 1.

Moreover, the possibility that the 71 and 29 kDa molecules, detected in rTcCRT, arise from *E. coli*, is highly unlikely. We prepared a solid-phase immunoabsorbent with excess total bacterial antigens. With this preparation we exhaustively absorbed our polyclonal antiserum, at the working 1/500 dilution (three rounds, 2 h each, at 37 °C). When used in Westerns, both the absorbed and non-absorbed reagents detected the 71 and 29 kDa bands with equal intensity. Likewise, the recognition of rTcCRT was not altered (results not shown).

The observed molecular weight of the recombinant protein is higher than that of the native protein (Fig. 1) since the procedure to obtain the recombinant protein involves, among other, the addition of extra aa from the plasmid vector and a histidine tail, for purification purposes. These extra aa, could explain at least an important part of the observed difference.

Given the known antiangiogenic effects of endotoxin (LPS) [38], a frequent contaminant in *E. coli*-derived recombinant protein preparations, we determined the levels of endogenous contamination present in our samples, as described in Section 2.1. We then carried out the CAM assays including eggs to which only pure LPS was added to the filters, in the same concentrations present in our rTcCRT preparations. As shown in Table 1, the antiangiogenic effect observed with our rTcCRT preparation is mainly due to the recombinant parasite molecule and, to a lesser extent, to contaminating LPS.

In an effort to further support the antiangiogenic results obtained with both the recombinant and native parasite molecules, we asked whether the *TcCRT* gene, in the context of a productive plasmid, mediates a similar effect in the CAM assay. The degree of significance decreases when treatment is performed with the *TcCRT* gene. Most likely, the actual amount of available protein is lower in this case, as compared with the situation when the recombinant or native proteins are directly provided. Alternatively or concomitantly, the presence of a proangiogenic cytokine (GM-CSF) increased the stringency of the assay. The productive property of the pSecTag/*TcCRT* construct, in an eukaryotic murine model, is demonstrated by the detection of specific serum antibodies against the parasite molecule in a conventional ELISA against the recombinant molecule. These antibodies were also detected by FACS analysis, where the native molecule is recognized both at the parasite surface and interior (Fig. 4). The results of this experiment support the possibility that, in the CAM assay used here, this genetic construct mediates TcCRT production by the CAM cells, thus explaining the observed inhibition of angiogenesis. Our results are in accordance with those previously described for HuCRT, in

which antiangiogenic effects were detected under the same experimental conditions [34]. As mentioned above and in previous results from our laboratory [15], TcCRT is expressed on the trypomastigote surface, in spite of having the KEDL endoplasmic reticulum retention signal and lacking a transmembrane region, a fact also observed with mammal CRT [39,40].

Most likely, the fact that antiangiogenic substances act preferentially on growing neoplasic tissues and not on already established tumors, is due to their effects on emerging blood vessels [41]. This may explain why in the CAM assay used, rTcCRT mediated an antiangiogenic effect that, although highly significant, reached a maximum of approximately 50%. In other words, it is possible that most of the remaining vessels were already established and thus resistant to the parasite molecule.

For decades, it has been speculated about possible mechanisms involved in the in vivo experimental growth inhibitory effect that several *T. cruzi* strains have on a variety of transplanted and spontaneous tumors, in animals and humans [28,29,31]. In order to explain the antineoplasic effect of *T. cruzi* infection, induction of a specific anti-tumoral immune response [30] and secretion of a "toxic substance" [29] by the parasite have been proposed, but not experimentally substantiated. Since the *T. cruzi* life cycle involves an important intracellular phase, this putative molecule should have a preferential toxic effect for neoplasic cells. Both explanations are compatible with an evolutionary speculation that this antineoplasic effect would protect the host, with evident benefits for the parasite.

In synthesis, our results indicate that, similar to HuCRT [16–19,42,43], TcCRT has an antiangiogenic effect in the in vivo CAM assay proposed here, even at the low concentrations tested. It could be speculated that these properties, together with the accessibility of this molecule on the trypomastigote surface, may explain, at least partly, the reported [30,31] antineoplasic effect of experimental *T. cruzi* infection, an intriguing and maybe important issue. The results with DNA, although less telling, are presented here as evidence that supports the results obtained with the recombinant and native parasite molecules.

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