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Comparative *in vivo* antiangiogenic effects of calreticulin from *Trypanosoma cruzi* and *Homo sapiens sapiens*

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ABSTRACT

Angiogenesis is a complex multi-step process of neovascularization arising from preexisting blood vessels whose generation is regulated by pro- and anti-angiogenic factors. Both *Trypanosoma cruzi* calreticulin (TcCRT) and its human counterpart (HuCRT) are antiangiogenic. This is the first report where the TcCRT and HuCRT anti-angiogenic properties are compared *in vivo*. In the chick embryonic chorioallantoid membrane assay (CAM) and at equimolar concentrations, TcCRT displayed significantly higher antiangiogenic activities than its human counterpart. LPS had marginal effects at the concentrations present in the recombinant protein preparations and the TcCRT antiangiogenic effects were largely inhibited by specific polyclonal antibodies, thus, reinforcing the fact that the observed TcCRT effects can be attributed to the parasite-derived molecule and not to the endotoxin. The antiangiogenic TcCRT effects correlate with its anti-tumor *in vivo* effects, as recently shown in our laboratory.

Key terms: Calreticulin, *T. cruzi*, anti-angiogenesis.

Chagas' disease is a chronic debilitating affliction, of difficult treatment, that in Latin America affects 12 million individuals. In Chile, there are about 150,000 seropositive humans and an indeterminate number of other mammals infected, acting like natural reservoirs of the causal agent, the *Trypanosoma cruzi* protozoan. It is, therefore, a zoonosis of great relevance and in Latin America it constitutes one of the most important public health problems, with a severe socioeconomic impact (Bastien, 1998).

We have cloned, sequenced and expressed the *T. cruzi* calreticulin gene (*TcCRT)* (Aguillon et al., 2000). Its product, TcCRT, is an immunogenic 45 kDa protein, with more than 50% identity (80% in some functional domains) with the human counterpart (HuCRT). Although it locates preferentially in the ER, it is translocated to the parasitic surface, mainly to areas adjacent to the flagellum emergence (Ferreira et al., 2004). TcCRT and HuCRT are also functionally homologous, including negative regulation of the human complement system (Ferreira et al., 2004; Valck et al., 2010) As a consequence, they promote parasite infectivity (Ramirez et al., 2010).

HuCRT is a highly conserved 46-kDa calcium-binding pleiotropic protein present in virtually all nucleated cells of higher organisms. It is located preferentially in the ER (Michalak et al., 1992; Ostwald and MacLennan, 1974), and regulates a series of cellular functions (Fraser et al., 2000; Michalak et al., 1999). Peptides derived from HuCRT, located between amino acids 120-180 (vasostatin), as well as the whole molecule, display antiangiogenic properties *in vivo* and *in vitro* (Cheng et al., 2001; Pike et al., 1998). These molecules also have antitumor effects (Pike et al., 1999).

TcCRT is a powerful angiogenesis inhibitor *in vivo*, in the chick embryonic chorioallantoid membrane assay (CAM) of *Gallus gallus* (Aguilar et al., 2005). There has been speculation for decades on the possible mechanisms involved in the *in vivo* effects that infection with several *T. cruzi* strains may have on a variety of transplanted and spontaneous tumors in animals and humans (Galliard et al., 1950; Oliveira et al., 2001; Roskin, 1946). Given the evolutionary distance between *Trypanosoma* and *Homo* species and the differences in primary sequences between their CRTs, it is of interest to define, *in vivo,* the comparative efficiency in equimolar terms of these molecules to mediate antiangiogenic function. The results may contribute to understanding whether the antiangiogenic properties were first consolidated in the parasite chaperone molecule and then HuCRT conserved some of these properties as an evolutionary relict, or alternatively, the parasite "hijacked" this activity from its vertebrate host.

Figure 1 summarizes *in vivo* results obtained in the CAM assay, performed as described (Aguilar et al., 2005), when the antiangiogenic properties of TcCRT and HuCRT were compared in equivalent terms. Briefly, 10 µl containing different concentrations (10pg to 10ng) of rHuCRT and rTcCRT were added on the filters placed on the chicken egg CAM (White Leghorn, Institute of Public Health, Santiago, Chile), Betamethasone (Lemus et al., 2001) and ovoalbumin were used as positive and negative controls, respectively. A significant difference was observed between both controls and between the human and parasite chaperone molecules, the latter displaying stronger effects, in concentrations ranging from 10pg to 10ng ($p<0.05$).

LPS, a known antiangiogenic molecule (Zetter, 1998) and ubiquous contaminant in *E. coli*- derived recombinant proteins, was tested in the CAM assay in concentrations similar to those present in HuCRT and TcCRT. Under these conditions, the endotoxin had marginal effects (results not shown). This result, together with the fact that polyclonal anti-rTcCRT IgG strongly inhibited the anti-angiogenic effects mediated by the parasite molecule (Figure 2), substantiates the specificity of the antiangiogenic TcCRT property.

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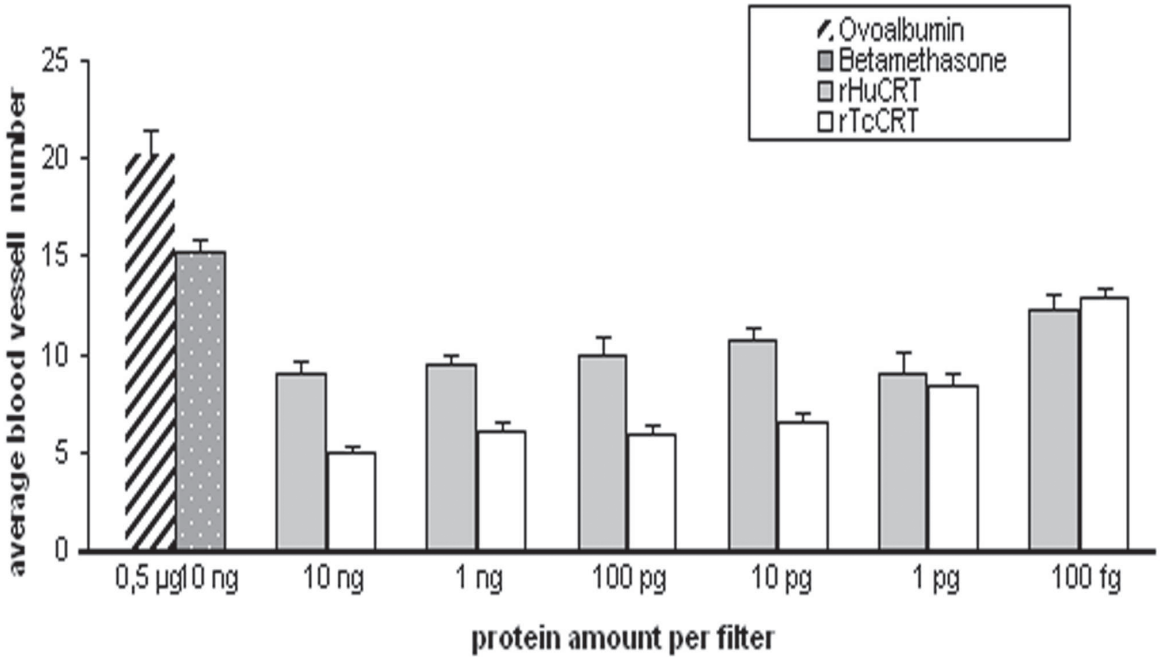

Figure 1: rTCRT mediates a stronger antiangiogenic effect than rHuCRT in the CAM assay. The fertilized eggs were incubated for 10 days at 38.5 ºC. Then, sterile methyl cellulose filters were deposited on the CAMs. On each filter, TcCRT or HuCRT were added at equimolar concentrations. After incubating for an additional 72 h, the number of blood vessels present in 9000 μm^2 was counted, in a double blind procedure, by conventional light microscopy. Bethamethasone and ovoalbumin were used as positive and negative angiogenesis controls, respectively. Data were analyzed by ANOVA and Tukey tests. Results were expressed as means and their standard deviations.

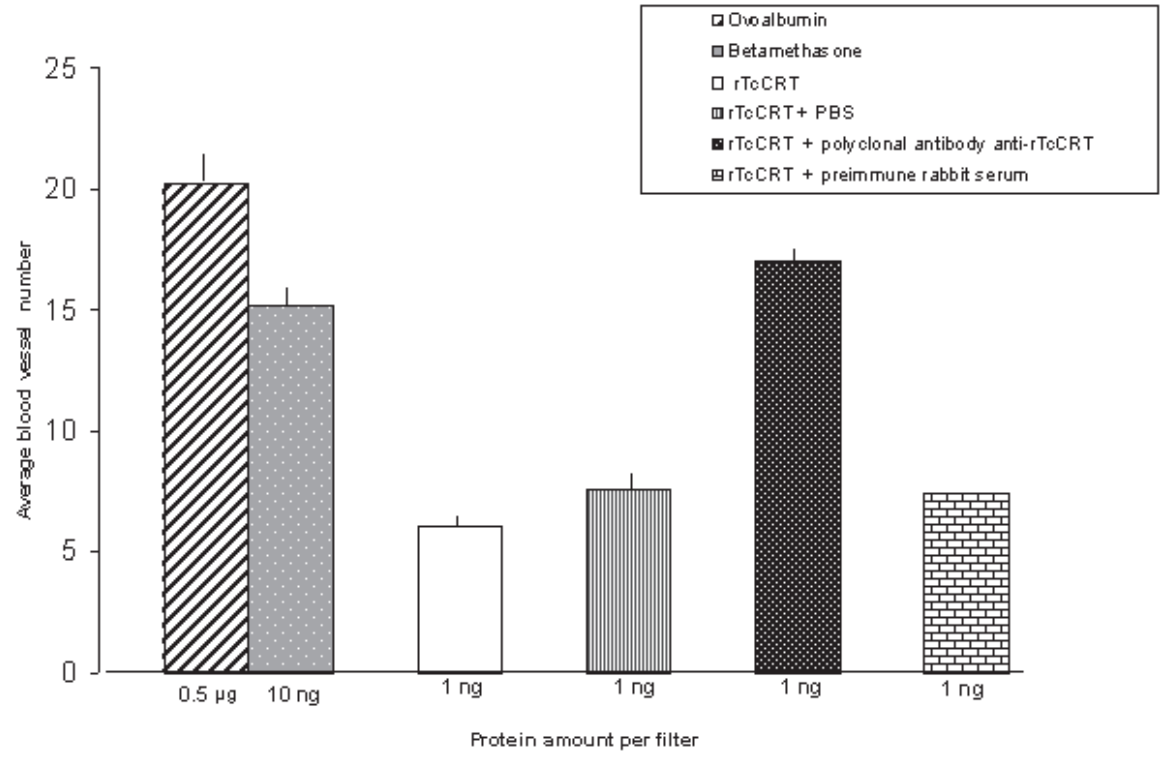

Figure 2: The antiangiogenic rTcCRT effect is inhibited by polyclonal anti-rTcCRT antibodies. Before its addition to the filters, TcCRT was incubated with rabbit polyclonal anti-TcCRT antibodies. Preimmune serum or PBS was used as controls. Data were analyzed by ANOVA and Tukey tests. Results were expressed as means and their standard deviations.

TcCRT, in primary cultures of human endothelial cells, inhibits three fundamental stages of the angiogenic process (migration, proliferation and morphogenesis). In general, in these assays, and also in the *ex vivo* rat aortic ring test, the parasite molecule was more effective than the human counterpart (López et al., 2010). The N-terminal TcCRT domain reproduced these effects (unpublished observations). The *in vivo* results presented here agree with those *in vitro* and *ex vivo* studies. Recently (López et al., 2010), it has been determined *in vitro* that TcCRT enters the endothelial cell and locates perinuclearly. It remains to be defined if this access is a prerequisite for the antiangiogenic TcCRT effect.

The stronger antiangiogenic rTcCRT efficiency, as compared to HuCRT, may have emerged in protozoan forms, as a necessary consequence of their interactions with contemporary vertebrate hosts. The conservation of these functions in HuCRT could represent a non-deleterious evolutionary relict. These TcCRT antiangiogenic and complement inhibitory properties in the parasite may have consolidated to decrease inflammation on the sites of parasite locations, thus impairing the potential immune system capacity to destroy them. Alternatively or concomitantly, since angiogenesis is essential for tumor growth and metastasis, antiangiogenesis would protect the host from these potent neoplastic aggressors, with obvious possible benefits for the parasite. Indeed, there has been speculation for decades about the possible mechanisms involved in the *in vivo* experimental protective effects thatinfection with several *T. cruzi* strains may have on a variety of transplanted and spontaneous tumors, in animals and humans. Secretion by the parasite of "toxic substances" for tumors was proposed (Galliard et al., 1950; Oliveira et al., 2001). In agreement with our results, one such substance may be TcCRT. On the other hand, in the pathogenesis of the chronic inflammatory disease, the immune response triggered by the parasite seems to be an important component (Kierszenbaum, 2005). Moreover, inflammation is an essential part in the protective response against the infection (Pinedo et al., 1998; Teixeira et al., 2002). Thus, the antiangiogenic effect mediated by *T. cruzi* calreticulin may finally result in both, protection for the parasite and, as a collateral effect, indirectly benefiting the parasite, protection for the host.

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