Effect of sphingosine and phorbol-12-myristate-13-acetate on the growth and dimethylsulfoxide-induced differentiation in the insect trypanosomatid *Herpetomonas samuelpessoai*

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We investigated the effect of two modulators of protein kinase C, sphingosine and phorbol-12-myristate-13-acetate (PMA), on the growth and dimethylsulfoxide (DMSO)-induced differentiation in Herpetomonas samuelpessoai. *Sphingosine did not stimulate the transformation of undifferentiated-promastigotes in differentiated-paramastigotes. PMA alone or in association with DMSO increased the number of paramastigotes in comparison to control cells. DMSO inhibited the parasite growth (35%) and several unusual morphological features resembling aberrant cell division were observed. Sphingosine did not significantly reduce the growth in contrast to PMA. Collectively, our results demonstrated that the reduction of the proliferation translates in an increase of the differentiation rate in the insect trypanosomatid* H. samuelpessoai.

Key words: *Herpetomonas samuelpessoai* - development - dimethylsulfoxide

Herpetomonas samuelpessoai is a non-pathogenic trypanosomatid isolated from the predatory insect *Zelus leucogrammus* that shares important antigens with *Trypanosoma cruzi*, the etiologic agent of Chagas disease (Souza et al. 1974), and with *Leishmania* spp. (reviewed by Santos et al. 2006). Several Brazilian researchers have used this parasite as a very interesting model to study cellular differentiation, since *H. samuelpessoai* possesses three distinct morphological stages (promastigote, paramastigote, and opisthomastigote) during its life cycle either in culture or in the invertebrate vector (Angluster et al. 1977, Castellanos et al. 1980, Souza et al. 1980, Thomas et al. 1981a,b, Santos et al. 2001, 2002a,b, 2003a).

In protozoa as in higher eukaryotes, cellular differentiation occurs as result of selective gene expression, which is controlled in various ways. Differentiation is often initiated in response to external stimuli such as the binding of ligands to the cell surface (Parsons & Ruben 2000). The *H. samuelpessoai* differentiation mechanism is triggered by chemical and physical changes in the growth conditions. Previous studies have shown that the binding and/or interaction to the plasma membrane of drugs such as 2-deoxy-D-glucose (Angluster et al. 1977), Concanavalin A (Souza et al. 1980), cholinergic drugs (Thomas et al. 1981a), local anesthetic (Thomas et al. 1981b), and dimethylsulfoxide (DMSO) (Castellanos et al. 1980) induce the differentiation process in *H. samuelpessoai*, which is associated with several changes in the parasite biochemical machinery (Santos et al. 2001, 2002a,b, 2003a). Nevertheless, very little is known about the signal transduction pathways involved in the differentiation process of this monoxenous flagellate.

Protein phosphorylation-dephosphorylation in eukaryotes is involved in the regulation of signal transduction, metabolism, differentiation, proliferation, death, and other cellular processes (Nishizuka 2003). Protein kinase inhibitors have been used extensively to study the role of these enzymes in higher eukaryotes, but few similar studies on parasites have been carried out. In this context, we have tested the effects of two distinct modulators of protein kinase C (PKC) activity, sphingosine and phorbol-12-myristate-13-acetate (PMA), throughout the cellular growth and DMSO-induced differentiation process of *H. samuelpessoai*.

H. samuelpessoai (CT-IOC-067) was maintained by weekly transfers in chemically defined conditions as previously reported (Santos et al. 2001). Experiments were made in 18 × 150 mm glass tubes containing 5 ml of medium and the inoculum consisted of 2% of a 48 h culture containing about 1.0×10^6 cells. Drugs were dissolved as follows: sphingosine was dissolved in water, at 500 ng/ml, PMA in ethanol, at 200 ng/ml, and DMSO (Sigma Chemical Co.) in the culture medium, at 4% (final concentration). The parasites were grown at 26°C, for 48 h (exponential growth phase), in the absence or presence of DMSO (Santos et al. 2003a) and, alternatively, the cells were incubated with or without DMSO supplemented with the PKC modulators. Ethanol has also been tested for its possible ability to stimulate cellular growth and differentiation in these flagellates, presenting negative results as previously described (Santos et al. 2001). Growth was estimated by determining the cell number in a Neubauer chamber. To quantify the percent-

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age of differentiation, promastigote, paramastigote, and opisthomastigote forms were counted after Giemsa-staining. At least 500 microorganisms were examined in each preparation. Each experiment was performed in triplicate and repeated at least three times. Statistical analysis of data was performed through Student's *t* test. *P* values ≤ 0.05 were considered statistically significant.

The results presented herein corroborated those previous works in which DMSO at 4% was able to induce differentiation in *H. samuelpessoai* (Fig. 1). Differentiation is postulated to require a series of events that include activation of PKC (Nishizuka 2003). In the present work, we analyzed the effect of sphingosine (a PKC inhibitor) and PMA (a PKC stimulator) on the DMSO-induced differentiation of *H. samuelpessoai*. Sphingosine alone did not stimulate the morphological transformation of *H. samuelpessoai*. Moreover, the differentiation process induced by DMSO was reverted in the presence of sphingosine. In a similar way, staurosporine, which is a microbial alkaloid capable of inhibiting PKC activity, was also able to block the promastigote-amastigote and amastigote-promastigote transformations in *Leishmania donovani* (Blumm 1994). Conversely, PMA alone or in association with DMSO increased the number of paramastigote-differentiated forms in comparison to the control cells (Fig. 1). PMA, which has a diacylglycerol-like structure, is able to replace diacylglycerol at extremely low concentration, promoting a dramatic increase in the affinity of the PKC enzyme for Ca^{+2}, resulting in its full activation without detectable cellular mobilization of Ca^{+2}. For this reason, there is a good correlation between the ability of the phorbol esters, such as PMA, to promote differentiation and to activate PKC (Nishizuka 2003). For instance, PMA and DMSO induced HL-60 human promyelocytic leukemia cells to differentiate into monocyte-macrophage like cells (Jacob et al. 2002).

DMSO induces the differentiation of several cell types, including various tumor cell lines, which is always accompanied by growth inhibition and exit from the cell cycle (reviewed by Santos et al. 2003b). In this sense, *H. samuelpessoai* cells were cultured for 48 h in the absence and presence of DMSO and PKC modulators and then we measured the parasite growth (Fig. 2). DMSO inhibited the parasite growth by approximately 35%. Sphingosine alone did not significantly reduce the growth rate ($P > 0.05$) in contrast to PMA ($P < 0.05$). The association between DMSO and PMA promoted a synergistic effect on the parasite proliferation (Fig. 2). K252a, a PKC inhibitor, inhibited the proliferation and DNA synthesis of HL60 cells, and conversely triggered their differentiation process (Taoka et al. 1990).

In *H. samuelpessoai*, the promastigote form is the only stage capable of dividing and the typical process of binary longitudinal fission was currently observed during the first 48 h of cultivation in vitro (Fig. 3a). Interestingly, we detected in the DMSO-treated parasites a significant amount of aberrant dividing cells (Fig. 3b-r), including several pairs of flagellates diametrically opposed and attached by their posterior ends (Fig 3b-p). In such pairs, each cell could retain its own nucleus (Fig. 3b-l), but in some instances one of them was anucleated (but retaining the kinetoplast and the flagellum) while the other had two nuclei (Fig. 3m-o), sometimes in close proximity (Fig. 3m-n) or apparently fused into a single larger one (Fig. 3o). In some instances, pairs were seen

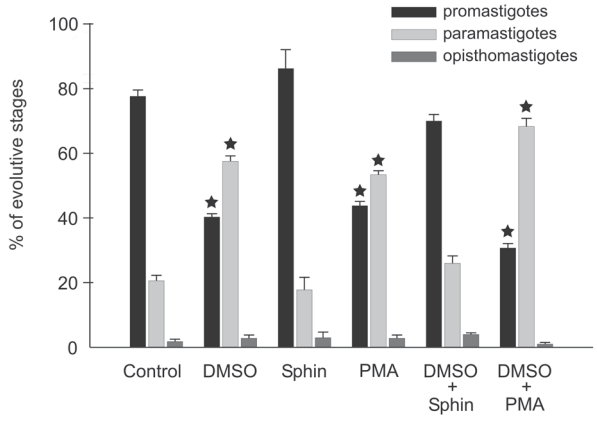

Fig. 1: effect of dimethylsulfoxide (DMSO) and protein kinase C (PKC) modulators on the cellular differentiation of *Herpetomonas samuelpessoai*, cultivated in Roitman's chemically defined medium. The parasites were grown for 48 h in the absence (control) and in the presence of 4% DMSO, 50 ng/ml sphingosine (Sphin), 20 ng/ml phorbol-12-myristate-13-acetate (PMA), DMSO plus Sphin, and DMSO plus PMA. Results were obtained by examination of Giemsa-stained cell preparations. Each bar represents the mean value ± standard error of three independent experiments, which were performed in triplicate. Asterisks denote values statistically different from control ($P < 0.05$, Student's *t* test).

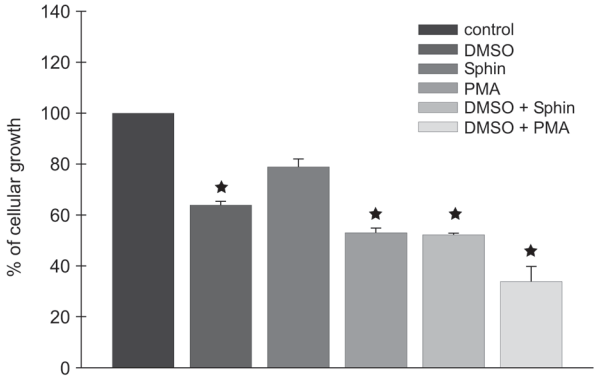

Fig. 2: effect of dimethylsulfoxide (DMSO) and protein kinase C modulators on the cellular growth of *Herpetomonas samuelpessoai*, cultivated in Roitman's chemically defined medium. The parasites were grown for 48 h in the absence (control) and in the presence of 4% DMSO, 50 ng/ml sphingosine (Sphin), 20 ng/ml phorbol-12-myristate-13-acetate (PMA), DMSO plus Sphin, and DMSO plus PMA. Growth was estimated by determining the cell number in a Neubauer chamber. Each bar represents the mean value ± standard error of three independent experiments, which were performed in triplicate. Asterisks denote values statistically different from control ($P < 0.05$, Student's *t* test).

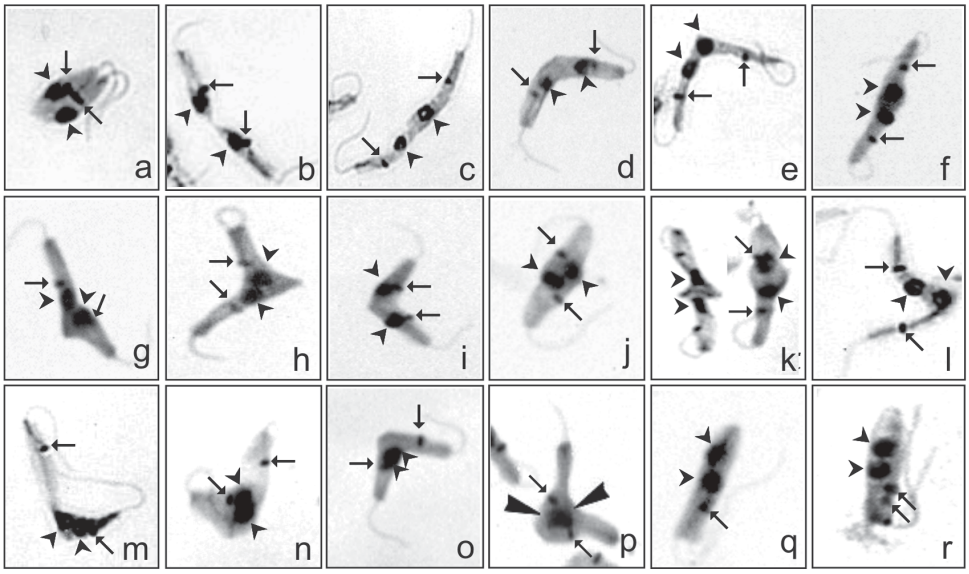

Fig. 3: unusual cytological features observed during the cellular division of *Herpetomonas samuelpessoai*, cultured for 48 h in Roitman's chemically defined medium supplemented with 4% dimethylsulfoxide. a: typically dividing promastigotes; b-l: pairs of opposed promastigotes attached by the posterior ends; m-o: pairs presenting both nuclei in one cell; p: pair with a single large nucleus inside of a bulb-like dilatation; q-r: promastigotes with two nuclei containing only one or two kinetoplasts, respectively. Arrows indicate kinetoplasts and arrowheads show nuclei.

forming an enlargement and/or dilatation, generally bulb-like in shape, at the level of the parasite junction region between the two opposed cells (Fig. 3p). In this situation, the nuclei of the joined cells appeared to be inside of the dilatation region, in such a close proximity that it was difficult to distinguish one from the other (Fig. 3p). Promastigotes with two nuclei containing only one (Fig. 3q) or two (Fig. 3r) kinetoplasts were also frequently observed. Paramastigote forms were also seen as opposed pairs (Fig 3i). In fresh preparations, the pairs were as actively moving as the other forms. The percentage of these aberrant morphologies was statistically different ($P < 0.001$) in DMSO-untreated (1.8 ± 1.1%) and DMSO-treated cells (10.5 ± 1.9%). Sphingosine or PMA alone did not promote an increase in the aberrant cells when compared with control cells. Similarly, both compounds when associated with DMSO did not alter the number of aberrant cells (data not shown). In the same way, DMSO induced structural changes in exponentially multiplying *Tetrahymena pyriformis* including altered nucleolar organization, altered structure of mitochondria and peroxisomes and appearance of lipid droplets (Nilsson 1977). In this latter work, 5% of the dividing *T. pyriformis* cells underwent abnormal nuclear division in the presence of DMSO, resulting in anucleate anterior daughters and in posterior daughter cells containing the entire macronucleus (Nilsson 1977). Similar studies demonstrated that DMSO when used as a cryoprotective agent is able to reduce the lethal effect of freezing and thawing, but marked fine-structural alterations have been observed in various protozoa prior to and after the freezing process (reviewed by Hubálek 2003).

Interactions among various pathways together with the multiplicity of the PKC family can produce enormous variations of the signaling network and cellular responses. Presumably, the function of each member of the PKC family may well be coordinated with dynamic intracellular membrane lipid metabolism (Nishizuka 2003). In this context, DMSO affects directly the lipid metabolism in several biological systems as well as the stability and dynamics of biomembranes (Yu & Quinn 1998, Santos et al. 2003b). Several models are proposed to explain the mechanism whereby DMSO may mediate its effects on the stability and properties of the membrane lipid matrix (Yu & Quinn 1998). As DMSO alters fluidity of phospholipid membranes and PKC is intimately linked to these membrane components, DMSO could alter intracellular signaling processes through the direct modification of the PKC activity. Corroborating these findings, DMSO can positively or negatively change the hydrolytic activity of several classes of enzymes, since if interferes with the water shell around macromolecules that may lead conformational changes and subsequent interference with the proper functioning of the molecules (reviewed by Santos et al. 2003b). DMSO, sphingosine and PMA are able to interfere with several signaling cascades. Interestingly, our results showed that sphingosine reverted the differentiation process induced by DMSO, but no relapsed the growth inhibition. These results suggest that DMSO can be acting in more than one signaling pathway, for example, one of them independent and other dependent of PKC.

Collectively, our results demonstrated that the reduction of the proliferation translates in an increase of the differentiation rate in the insect trypanosomatid *H. samuelpessoai*. The present results showed that PMA and sphingosine, two distinct compounds that interfere with signaling cascades, are able to modulate *H. samuelpessoai* growth and differentiation.

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