CHAPTER-IV

ROLE OF HYALURONIC ACID BINDING PROTEINS IN HOST-PARASITE INTERACTION DURING LEISHMANIA INFECTION
7.1 Introduction

Protozoan parasites have evolved a remarkable ability to survive in their host. Parasites resist host defence by antigenic variation, resistance to complement and immunological attack, and/or escape to "safe" compartments such as host cell cytoplasm. Intracellular parasites have become masters at manipulating the structure and pathways of host cells for their own nefarious purposes, which require a more hospitable environment. One strategy commonly adopted, is a major remodelling of host cell compartments, thereby radically changing the cell's architecture and function. Another is a sophisticated manipulation of host cell signalling pathways, inhibiting some in order to preclude hostile responses or, even more remarkably, activating others in order to exploit them for invasion and/or survival (Beverley, 1996).

*Leishmania donovani* is an obligate intracellular protozoan parasite that colonizes the macrophages of the host and causes potentially fatal disease like kala-azar or visceral leishmaniasis. The parasite exists in two forms during its life-cycle, the extracellular flagellated promastigote form in the insect vector (sandfly) and the intracellular non-motile amastigote form in the mammalian host (Chang et al., 1990). The promastigotes are introduced into the mammalian blood stream when the sandfly takes a blood meal. Promastigotes bind to specific receptors on macrophages and enter the cells by receptor mediated endocytosis (Russel et al., 1989). The promastigotes quickly transform to the intracellular amastigotes that replicate in macrophages and spread the infection to neighbouring cells (Mosser, 1990). Thus *L. donovani* adhesion to macrophages plays a central role in the pathogenesis of infection. There are only a few reports indicating the interactions of *L. donovani* with some of the extracellular matrix components like heparin (Butcher et al., 1992), fibronectin (Wyler et al., 1985), collagen (Ljungh et al., 1995) and laminin (Ghosh et al., 1996).

In the previous chapter, we have shown the important role of HA-binding proteins in HA mediated cellular signalling. This, allowed us to speculate its effect in host-parasite interaction, as HA level is known to regulate in different diseased conditions (Knudson and Knudson, 1993). Hyaluronan (HA), is ubiquitously present in the extracellular matrix and is involved in many cellular functions like cell adhesion (Miyake et al., 1990), migration (Turley et al., 1991), differentiation (Knudson and Toole, 1987) and immune responses (Bourguignon et al., 1993). Several groups have demonstrated the presence of different HA-binding proteins,
comprising a family known as Hyaladherins, which may regulate the cellular functions by interacting with HA (Knudson and Knudson, 1993). From our laboratory also we have reported the purification and characterization of a 34 kDa HA-binding protein from rat kidney (Gupta et al., 1991a) and showed its role in cell adhesion (Gupta and Datta, 1991b) and in HA induced lymphocyte signalling (Rao et al., 1996). Recently, we cloned the 34 kDa HA-binding protein (Deb and Datta, 1996) and the sequence analysis revealed it as a novel HA-binding protein having 100% homology with the receptor of C1q complement protein (gC1q-R) (Ghebrehiwet et al., 1994). There are recent reports that the interaction of complement receptors of macrophages with some species of *Leishmania* is essential for the initial establishment of infection in macrophages (Mosser et al., 1992). In the present study, we have examined whether the HA-binding protein plays a role in host-parasite interaction during *Leishmania* infection.

7.2 Results

7.2.1 Enhanced expression of the 34 kDa HA-binding protein in *Leishmania* infected hamster tissues

An equal amount of protein from different tissues of normal and *L. donovani* infected hamsters were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The HA-binding protein was detected using anti-34 kDa HA-binding protein antibodies. Higher expression of the 34 kDa HA-binding protein was observed in liver, spleen, macrophages and serum of infected hamsters as compared to the tissues from normal hamsters. However, the expression of the 68 kDa protein showed the reversal although overall, an increase in the expression of the 34 kDa and the 68 kDa could be seen (Fig. 7.1). The quantitative estimation of the HA-binding protein in various tissues by ELISA was also performed. HA-binding protein levels were found to be significantly higher in liver, spleen, macrophages and serum of *Leishmania* infected hamsters (Table 7.1).

7.2.2 Level of HA-binding protein in serum of kala-azar patients

Since the level of HA-binding protein was found to be elevated in hamster serum, it was of interest to examine the serum HA-binding protein levels in kala-azar patients. Interestingly, a marked increase in 68 kDa, 34 kDa and 22 kDa HA-binding protein levels was observed in kala-azar patients in comparison to the normal human sera (Fig. 7.2). Estimation
Table 7.1  Elevation in the levels of the 34 kDa HA-binding protein during *Leishmania* infections

μg of HA-binding protein/ mg of total protein

<table>
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<tr>
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<th>Normal</th>
<th>Infected</th>
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<tr>
<td>Liver</td>
<td>1.28 ± 0.075</td>
<td>1.54 ± 0.069</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.14 ± 0.046</td>
<td>1.40 ± 0.071</td>
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<tr>
<td>Macrophages</td>
<td>0.83 ± 0.094</td>
<td>1.30 ± 0.09</td>
</tr>
<tr>
<td>Serum</td>
<td>1.35 ± 0.016</td>
<td>1.62 ± 0.031</td>
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<tr>
<td>Human Serum</td>
<td>1.45 ± 0.083</td>
<td>2.085 ± 0.133</td>
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Levels of HA-binding protein in hamster tissues and human sera were estimated by ELISA using anti-HA-binding protein antibodies as mentioned under material and methods.

Results were expressed as mean ± s.d. of six experiments with P value<0.005 vs normal.
Enhanced expression of the 34 kDa HA-binding protein in Leishmania infections. Equal amounts of protein (20 μg) from various tissues were subjected to 12.5% SDS-PAGE, transferred onto a nitrocellulose membrane and probed with anti HA-binding protein antibodies and immunodetected as described in the material and methods. Lane 1 to 4, liver, spleen, macrophages and serum from normal hamsters, respectively. Lane 5 to 8 liver, spleen, macrophages and serum of infected hamsters respectively.

Elevated levels of HA-binding protein in sera of kala-azar patients. Serum proteins (20 μg) from normal and kala-azar patients were subjected to 12.5% SDS-PAGE and other details as mentioned for the Fig. 7.1. Results shown are representative of 6 different experiments.
of serum HA-binding protein by ELISA also showed a significantly higher HA-binding protein level in kala-azar patients (P<0.005) (Table 7.1).

7.2.3 Cellular phosphorylation of 34 kDa HABP in *L. donovani* infected macrophages and lymphocytes.

In the previous chapter, we showed that the HA-binding protein phosphorylation is regulated by its ligand HA and its phosphorylation is involved in cellular signalling (Rao et al., 1996). In order to elucidate the possible role of the 34 kDa HA-binding protein phosphorylation in *Leishmania* infection, we compared its *in vivo* phosphorylation in macrophages and lymphocytes from normal and *Leishmania* infected hamsters. The phosphorylation of the 44 kDa and the 34 kDa HA-binding protein was found to be inhibited in macrophages and enhanced in lymphocytes from infected hamsters in comparison with that of corresponding cells from normal hamsters. A very faint 68 kDa in normal macrophages was also observed (Fig. 7.3). These results suggest the regulation of cellular phosphorylation of HA-binding protein during host-parasite interactions.

7.2.4 Evidence for the existence of HA-binding protein-binding proteins in promastigote extracts

In order to examine if the 34 kDa HA-binding protein is present in promastigotes of *L. donovani*, the promastigote proteins were resolved by SDS-PAGE and transferred onto the nitrocellulose membrane, and probed with anti-HA-binding protein antibodies. As shown in Fig. 7.4A, *L. donovani* promastigotes are devoid of this protein. Further, in order to detect if the promastigotes possessed proteins capable of binding to HA-binding protein, a transblot assay was carried out. The nitrocellulose membrane carrying the promastigote cell extract was probed with biotinylated HA-binding protein. Fig. 7.4B suggests the presence of two proteins of molecular mass 30 kDa and 55kDa having binding affinity for HA-binding protein. The binding specificity of these two promastigote proteins to HA-binding protein was further checked by competing the biotinylated HA-binding protein with excess of unlabelled HA-binding protein (Fig. 7.4C).

7.3 Discussion

During the course of evolution, protozoan parasites have developed strategies to subvert
Fig. 7.3  Cellular phosphorylation of 34 kDa HA-binding protein in macrophages and lymphocytes in *Leishmania* infected hamsters. Freshly isolated macrophages and lymphocytes from normal and infected hamsters were metabolically labelled with $[^{32}\text{P}]$ orthophosphate for 2 h. The cells were washed and lysed with lysis buffer and immunoprecipitated with anti-34 kDa HA-binding protein antibodies. Immunoprecipitates from equal number of cells were separated on 12.5% SDS-PAGE, dried and autoradiographed. Lane 1 and 2, infected and normal macrophages, respectively. Lane 3 and 4 normal and infected lymphocytes, respectively.

Fig. 7.4  Evidence for the existence of HA-binding protein in promastigotes of *L. donovani*. Protein extracts from $1 \times 10^7$ promastigotes of *L. donovani* were separated on 12.5% SDS PAGE, transferred onto nitrocellulose membrane and probed with anti HA-binding protein antibodies [A], Biotinylated-HA-binding protein [B] and excess of unlabelled HA-binding protein followed by Biotinylated-HA-binding protein [C] Bands were visualized as described in material and methods.
the immune response of their hosts in order to multiply, reproduce and survive. One of these inherited strategies is their capacity to modulate the host cell signal transductional mechanisms in their favour. This chapter documents the role of a 34 kDa HA-binding protein in host-parasite interactions during *Leishmania* infection. Firstly, the levels of the HA-binding protein were elevated in spleen, liver, macrophages and serum of *Leishmania* infected hamsters; secondly, the overexpression of the serum HA-binding protein was also reflected in kala-azar patients; thirdly, the phosphorylation status of HA-binding protein was altered in both macrophages and lymphocytes isolated from *Leishmania* infected hamsters and finally, the evidence for the presence of two proteins in the promastigotes which can bind to HA-binding protein was found.

*L. donovani* replicates primarily in macrophages of the spleen, liver and bone marrow (Glew et al., 1988). Interestingly, there is a reversal in expression of the 68 and the 34 kDa HA-binding proteins in infected and normal tissues. As shown in the Western blot, the expression of the 68 kDa is more, as compared to the 34 kDa HA-binding protein in normal tissues while in the infected tissues, it is the reverse. However, in the serum of kala-azar patients this reversal in the expression of the 68 and the 34 kDa HA-binding proteins is not observed. This observation on the overall elevated HA-binding protein levels in spleen, liver and peritoneal macrophages of *Leishmania* infected hamsters, as shown by ELISA indicates a role of HA-binding protein in the pathogenesis of leishmaniasis. Elevation in tissue HA-binding protein levels is reflected in serum HA-binding protein levels since this is a protein which is secretory in nature. This experimental observation is further supported by the finding that there occurs a rise in the level of HA-binding protein in kala-azar patients sera, warranting a detailed clinical study.

In order to find out the possible role of the 34 kDa HA-binding protein phosphorylation in *Leishmania* infection, the macrophages and lymphocytes isolated from *Leishmania* infected hamsters were metabolically labelled with $^{32}$P orthophosphate and immunoprecipitated with anti-HA-binding protein antibodies. A partial inhibition in cellular phosphorylation of the 34 kDa HA-binding protein was seen in infected macrophages, whereas the phosphorylation level of the HA-binding protein was enhanced in the lymphocytes obtained from infected animals as compared to the normal counterparts. Earlier reports have
shown that *Leishmania* infection leads to deactivation of macrophages by inhibiting the activation of PKC, thereby, impairing the intracellular signalling events (Reiner, 1994; Moore et al., 1993). The observed inhibition of cellular phosphorylation of HA-binding protein in *L. donovani* infected macrophages can be explained convincingly, since its phosphorylation is stimulated by PMA, the protein kinase C (PKC) stimulator (Rao et al., 1997). Moreover, our previous report of cloning and sequence data analysis of HA-binding protein also reveals the presence of substrate phosphorylation sites for PKC (Deb and Datta, 1996). *Leishmania* infection of macrophages results in an enhanced production of prostaglandins and TGFβ (Barral et al. 1993). TGFβ is known to stimulate the synthesis of hyaluronan (Ellis and Schor, 1996), thus making it available for binding to lymphocytes. An enhanced phosphorylation of HA-binding protein in infected lymphocytes was observed, which may be due to increased HA synthesis. This inference is made from our earlier observation of HA induced HA-binding protein phosphorylation and IP₃ formation in lymphocytes (Rao et al., 1996). These observations suggest that the differential phosphorylation of HA-binding protein in lymphocytes and macrophages may play a critical role in the pathogenesis of *L. donovani*.

The other aspect of pathogenesis of *L. donovani* is the effective recognition and ingestion of promastigotes by the macrophages, wherein, the promastigotes differentiate into the amastigote form and establish infection (Chang et al., 1990). In view of the possible role of HA-binding protein in parasite adhesion and disease pathogenesis, two proteins of Mr 30 kDa and 55 kDa were detected from promastigotes, which exhibit binding affinity to the 34 kDa HA-binding protein. It is important to mention here that several groups have already identified putative receptor-ligand interactions that mediate the attachment of promastigotes to their host (Chang et al., 1990; Mosser and Edelson, 1985). It has been suggested that gp63 and lipophosphoglycan (LPG) isolated from promastigotes interact directly with macrophage receptors namely, the macrophage complement type 3 receptor, and the fibronectin receptor (Talamas et al., 1990; Rizvi et al., 1988). A fibronectin like domain in gp63 has been implicated in the interaction of gp63 with macrophages (Soteriadou et al., 1992). Recently, Ghosh et al, (1996) reported the existence of a laminin binding protein on *L. donovani* membrane (Wyler et al., 1985). The 34 kDa HA-binding protein being a specific cell surface and a secretory protein has been shown to act as a specific receptor for HA, a major
component of the extracellular matrix (Gupta and Datta, 1991). Thus, the presence of two proteins on *L. donovani* having affinity to HA-binding protein suggest a role of HA-binding protein in host parasite interactions during the course of infection by *L. donovani*. 