CONCLUSIONS
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Leukocytes are the principal actors in the body’s defense system against invading microorganisms (Weissman and Cooper, 1993; Paul, 1993). This defense system has a specific branch consisting of lymphocytes and a nonspecific branch consisting of granulocytes and macrophages. Granulocytes consist of neutrophils, eosinophils, and basophils which release cytotoxic compounds from their intracellular granules to their local environment when they encounter microorganisms. The random destruction happens rapidly but it may also harm healthy tissues of the body. Macrophages, the other class of defense cells from the nonspecific immune system, can ingest and destroy microorganisms by phagocytosis or, in a similar way to granulocytes, by the secretion of cytotoxic compounds. However, macrophages can also act more specifically by collaborating with lymphocytes and their products.

The lymphoid system comprises of the cellular components responsible for antigen specific immune defense. B lymphocytes produce antibodies that bind to foreign organisms and facilitate their destruction, either by activating the complement system (which in turn perforates the membrane), or by "opsonizing" the microorganisms, i.e., trigger phagocytosis due to receptors for antibodies and the macrophage surface. T lymphocytes act mainly by cell-to-cell contact. One subpopulation of T lymphocytes recognizes and kills cells which bear foreign antigen (e.g., after virus infection); the second subpopulation helps to modulate the activity of other hemopoietic cells in the immune response or helps to multiply effector cells.

All of these leukocytes patrol the body by circulating through the blood and lymphatic system, ensuring a continuous surveillance which is a prerequisite for efficient defense (Anderson et al., 1982). Upon tissue damage and inflammation, leukocytes are recruited from the blood to sites of injury, and this trafficking displays exquisite specificity (Butcher, 1991; Springer, 1994; Dunon et al., 1993).

To efficiently protect the body from infections, the cells of the immune system circulate as nonadherent cells in the blood and lymph, and migrate as adherent cells into tissues, when necessary. Rapid transition between adherent and nonadherent states is the key to the dual functions of immune surveillance and responsiveness. Circulating lymphocytes in the blood have first to adhere to and then, to cross the endothelial lining in order to enter the various lymphoid tissues which are involved in recirculation.

The means by which leukocytes are recruited to the sites of damage and inflammation
CONCLUSIONS

are mediated by the cell adhesion molecules present on the plasma membrane of the leukocytes. Some of these cell surface adhesion molecules have recently been seen to possess carbohydrate binding domains (eg. selectins) or link module (HA-binding domain, eg. CD44). Recently, a consensus structure for the link module has been defined (Kohda et al., 1996) which gives insights into the mode of action of a protein superfamily involved in extracellular matrix assembly and cell migration. The putative HA-binding site which has been identified on the link module indicates that protein carbohydrate interactions form the basis of many important functions of the protein. The structural similarity of the link module and the C-type lectin domain (a carbohydrate binding domain) suggests that they have a common evolutionary origin and this provides a possible explanation for the similar roles of CD44 and the selectins in leukocyte extravasation at the sites of inflammation. The 34 kDa and the other immunologically related high molecular weight HA-binding proteins, for the same reasons, elicit curiosity.

The studies on hyaluronic acid-binding protein conducted during the course of the present investigation have been divided into four chapters. The results and the conclusions obtained for each chapter are accordingly given below:

Presence of high molecular weight HA-binding proteins immunologically related to the 34 kDa HA-binding protein

* Purification of a 34 kDa HA-binding protein from rat spleen by following the established method. The purity of the protein was confirmed by SDS-PAGE analysis. The protein purified by this method was used to develop polyclonal antibodies and the specificity of anti-HA-binding protein antibodies was tested by immunoblot analysis. It was observed that anti-HA-binding protein antibodies recognise a 68 kDa protein in cell lysates and purified samples.

* Co-purification of the 68 kDa HA-binding protein along with the 34 kDa protein by modified method involving protease inhibitors.

* The 2-D analysis of the 68 kDa and the 34 kDa proteins showing the pI of 4.0 and 4.2, respectively.

* Demonstration of the glycoprotein nature of the 34 kDa and the 68 kDa HA-binding proteins by $^{125}$I Con-A blotting.

* Immunoblot analysis with the affinity purified antibodies recognising the 68 kDa, the
55 kDa and the 34 kDa proteins.

* The cell lysates prepared in different combination of protease inhibitors demonstrating the protease sensitivity of high Mr HA-binding proteins.

* The expression of the 34 kDa and the 68 kDa proteins demonstrated in various transformed cells and rat tissues. This shows that these proteins are ubiquitous in nature.

* The Western blot analysis with anti-CD44 and anti-Cdc37 antibodies showing no cross reactivity with the 68 kDa and the 34 kDa proteins, suggesting a distinct identity of these proteins. However, anti-RHAMM antibodies cross reacted with the 68 kDa and the 34 kDa proteins, suggesting a similarity in their epitope structures.

* Cellular localization of the HA-binding protein demonstrated by immunofluorescence and immunochemical staining in MCF-7 cells. The results showed the cell surface and nuclear localization. The subcellular analysis of EL4 cells showed its localization on the plasma membrane, in the nucleus, the cytoplasmic and the Triton X-100 insoluble fractions.

* Studies on the biosynthesis of HA-binding protein in transformed cells with [35S] methionine demonstrating the appearance of the 34 kDa HA-binding protein in 1h. However, the 68 kDa protein could be detected only after 2h.

* [35S] pulse and chase studies showing the processing of the 55 kDa and the 44 kDa proteins, wherein they give rise to the 34 kDa HA-binding protein, confirming the previous observation of Honore et al (1993).

Overall, the results of the first chapter suggest the presence of high molecular HA-binding proteins, which are immunologically related to the 34 kDa HA-binding protein, and these proteins can be purified through HA-affinity column. High Mr HA-binding proteins are acidic, associated with carbohydrates, protease sensitive in nature, are ubiquitously expressed and are distinct from other known HA-binding proteins. The localization and the subcellular analysis also reveals their presence in plasma membrane, cytoplasm and nucleus, thus, suggesting their role in cell-cell adhesion, cell-ECM interactions and also intracellular processes. Finally, from [35S] studies it is also evident that the 55 kDa and the 44 kDa proteins are precursors of the 34 kDa protein, where they undergo post translational processing to produce the mature 34 kDa protein.
Purification and characterisation of the 68 kDa HA-binding protein and its immunological relation to the 34 kDa HA-binding protein from spleen.

* Purification of the 68 kDa HA-binding protein from rat spleen by modified method, using different type of protease inhibitors.

* The PAGE and SDS-PAGE analysis demonstrating Mr as 136 kDa and 68 kDa, respectively. The reducing and non-reducing gel data showed that the 68 kDa protein lacks interchain disulphide bonds.

* HA-Biotin ligand blot assay demonstrating the HA-binding property of the 68 kDa protein. In immunoblotting, the purified protein was cross-reacted with affinity purified anti-34 kDa HA-binding protein antibodies.

* Evidence for the instability of the 68 kDa protein demonstrated by immunoblotting, in the EL4 cell lysates prepared from fresh and stored cells.

* WGA binding studies proving the glycoprotein nature and N-glycosidase treatment showing the presence of N-linked carbohydrates.

* The cell surface localization demonstrated by labelling EL4 cells with [125I] lactoperoxidase and glucose oxidase.

* The treatment of the 68 kDa protein with denaturing agents suggesting that the 68 kDa protein is not a dimer of the 34 kDa HA-binding protein.

* The protein digestion data showing the 68 kDa sensitivity to V8 and CNBr treatments.

* The N-terminal sequence analysis of the 34 kDa and the 68 kDa proteins revealing that the first 15 amino acids are identical.

Interestingly, the presence of identical epitopes and N-terminal amino acid sequence of the 34kDa and the 68 kDa HA-binding proteins, raises the possibility of

- dimerization of the 34 kDa to generate the 68 kDa protein
- the proteolytic cleavage of the 68 kDa protein to generate the 34 kDa protein
- the homotypic crosslinking between the subunits of the the 34 kDa protein to generate the 68 kDa protein

Role of HA-binding proteins in HA-induced cellular signalling

* HA-binding assay and HA-cell binding assay in lymphocytes demonstrating the evidence for the presence of binding sites for high Mr HA on the 34 kDa HA-binding protein. Further, it is demonstrated that the 34 kDa HA-binding protein has the
capacity to bind with HA fragments like HA oligomer and HA hexamer in addition to HA polymer.

* Elevated total cellular protein phosphorylation, tyrosine phosphorylation, cytoskeletal phosphorylation and the IP$_3$ formation in HA induced lymphocytes demonstrating that HA induces early signalling events. These results suggest an activation of cellular signals. Furthermore, the hyperphosphorylation of HA-binding protein in response to HA stimulation implying its involvement in signal transduction pathways.

* Demonstration of the expression of phosphorylated HA-binding protein on cell surface and as well as in the secretory form in EL4 cells.

* The phosphoaminoacid analysis of the 34 kDa protein showing threonine phosphorylation and suggesting the involvement of serine/threonine kinases.

* Regulation of cellular phosphorylation of the 34 kDa and the 68 kDa HA-binding proteins, studied by using different protein kinase modulators.

* Evidence for the existence of HA-binding protein - binding protein in plasma membrane, demonstrated in J774, Raji and EL4 cells.

From the results presented here, it is clear that the 34 kDa HA-binding protein can bind to HA fragments in addition to the HA polymer. The data also suggests that high Mr HA induces early signalling events. The enhanced 34 kDa and 68 kDa HA-binding protein phosphorylation by HA and inhibition of cell aggregation and IP$_3$ formation by anti-HA-binding protein antibodies revealed that the 34 kDa HA-binding protein is a potential mediator in HA induced signal transduction, in addition to the reported involvement of CD44, RHAMM, Cdc 37 and ICAM-1. From these observations, one can speculate that HA-binding protein could have a key role in interacting with the extracellular matrix and mediate important processes like cell proliferation, differentiation and migration. The presence of HA-binding protein ligand on the plasma membrane also suggest that it can act as a counter receptor for the protein and help in mediating cell-cell interactions.

**Role of the HA-binding proteins in host-parasite interaction during Leishmania infection**

* Elevated levels of the HA-binding protein observed in Leishmania infected hamster tissues.

* In kala-azar patients' sera, the levels of the 34 kDa HA-binding protein found to be increased.
* The phosphorylation levels of the 34 kDa HA-binding protein inhibited in macrophages but enhanced in lymphocytes.

* HABP-binding protein presence demonstrated in promastigotes.

The above results suggest that the leishmania infection leads to enhanced expression of HA-binding protein and impairs the host cell signalling machinery thus, resulting in the inhibition of the HA-binding protein phosphorylation. The presence of HABP-binding protein in promastigotes suggests that these molecules might be involved in the recognition of the parasite.

This study conclusively makes an important observation on the presence of 34 kDa HA-binding protein related proteins in various cell types and their participation in HA induced signal transducing events.

Although, the present study indicates the possible role of HA-binding protein in various cell functions, there still exist major gaps in our understanding of the signal transduction mechanisms. Hence, certain issues need to be examined in future in order to bridge the gaps and to have a clearer picture regarding the sequential events that occur in the various cellular processes under normal and pathological conditions.

The present study suggests certain prospective areas of future research:

* Studying the relationship of the 68 kDa, the 55 kDa and the 44 kDa proteins with the 34 kDa protein by micro sequencing the N-terminal amino acids and to examine if the 55 kDa and 44 kDa proteins are pro-proteins as suggested by results of this study.

* Southern blot analysis to check whether the gene encoding the 34 kDa protein is a single copy or a multiple copy gene. Also, the Genomic DNA sequencing and chromosomal localization of this protein has to be done.

* Northern blotting is required to be done in order to show the expression and the transcript size in various transformed cells and to identify the splice variants, if any.

* Studying the role of these proteins during inflammatory processes, phagocytosis, lymphocyte activation, immune recognition and homing in of circulating leukocytes and also in host-parasite interactions in various diseases, could be important areas of investigation.