The studies on hyaluronic acid-binding protein (HABP or hyaluronectin) conducted during the course of the present investigation has been divided into four chapters. The results and the conclusions obtained for each chapter are accordingly summarized below:

(I) In order to identify a novel binding site for HA in the extracellular matrices and/or on the cell surfaces, an HA-binding protein was purified and characterized from normal rat kidney and polyclonal antibodies were raised against this protein. Attempts were also made to (i) study the tissue distribution of kidney-derived HABP, (ii) compare this protein with HABP isolated from other tissues, (iii) examine the binding characteristics of kidney HABP towards HA and other matrix biopolymers, (iv) study the renal HABP levels in newborn and aging rats, and (v) localize this protein in human fibroblast culture. Keeping the above points in view, the results and conclusions are given below:

(a) The hyaluronic acid-binding protein was purified from the acidic (0.2 M glycine-HCl, pH 2.2) extract of normal adult rat kidney by ammonium sulfate fractionation followed by hyaluronate-Sepharose affinity chromatography step. The homogeneity of the purified protein was confirmed by the criteria of single band in native PAGE, SDS-PAGE, IEF and two-dimensional electrophoresis.

(b) The purified kidney HABP was characterized for some of its basic properties. The apparent molecular mass of the protein in native state was found to be 68 kDa as determined by gel filtration chromatography and native gradient PAGE. SDS-PAGE analysis of kidney HABP under reducing as well as nonreducing conditions revealed a single protein band of 34 kDa, thus indicating that the molecule is a homodimer of 34 kDa subunits and lacks interchain disulfide bridge. Moreover, the HABP was found to be sialic acid-containing glycoprotein having an isoelectric point of 6.

(c) Polyclonal antibodies were raised against the purified kidney HABP and the monospecificity of the antibodies towards this protein was confirmed by Western blot analysis of tissue extracts. Immunoblot analysis using these antibodies elucidated the occurrence of this kidney-derived glycoprotein in various rat tissues such as brain, liver and heart. Moreover, the kidney HABP was found to be identical to the 68 kDa HABP isolated from these tissues with respect to molecular weight, subunit composition, pl value, glycoprotein
nature and immunological characteristics. However, this glycoprotein was found to be antigenically distinct from other well-characterized ECM-associated glycoproteins, e.g., fibronectin, laminin and collagen type IV. Further, the HABP described in this study does not seem to be related to any of the known HA-binding proteins reported by other investigators since it exhibits distinct structural characteristics.

(d) The binding characteristics of this HABP towards HA and other matrix biopolymers were studied with the help of enzyme-linked immunosorbent assay (ELISA) using anti-kidney HABP antibodies and by iodinated \(^{125}\)HABP. It was observed that the HABP binds specifically to HA amongst all the GAGs, though it can interact with various matrix-associated structural proteins e.g., laminin, fibronectin and collagen type IV. In terms of the biopolymers tested in this study, the binding preference of HABP was: HA > laminin > fibronectin > collagen type IV, suggesting highest affinity towards HA. The apparent dissociation constants (Kd) of HABP for its binding substrates were found to be approximately in the range of \(10^{-9}\) M and the kinetic analysis showed that these binding interactions were complex and of positive co-operative nature. These observations on preferential and saturable, high-affinity binding of 68 kDa HABP to HA in vitro fulfil the normal criteria of binding site - ligand interaction in vivo and therefore strongly suggest that this protein described in the present investigation is a possible endogenous binding site for HA.

Further, the ability of HABP to bind also to various ECM-associated structural proteins, although to a lesser extent than to HA, raises the possibility that in vivo HABP in concert with these matrix macromolecules could play an organizing role in structuring the ECM, in tissue morphogenesis and development, and in positioning and anchoring cells in the ECM.

(e) Quantitative analysis by ELISA using anti-kidney HABP antibodies has revealed that the HABP level in the renal tissue is the highest in neonatal rat and decreases concomitantly with aging. This result further suggests the possible involvement of HABP in the process of tissue morphogenesis and development.

(f) The HABP was also detected in human fetus lung fibroblasts by Indirect Immunofluorescence staining using anti-kidney HABP antibodies. In these cells, the protein was not only distributed uniformly over the cell surface but
was also present inside the cells where it occurred predominantly in the peri nuclear region (possibly Golgi apparatus) indicating its secretion to the outer cell surface and/or in the extracellular spaces. Moreover, HABP was found to be only associated with the cell surface but not intercalated into the plasma membrane, as it could be stripped off from the cell surface by urea pretreatment. Detection of 34 kDa HABP in the serum-free supematant culture medium of these fibroblasts was further evident by immunoblot analysis, thus confirming the secretory nature of the HABP and its occurrence in the ECM. Together, these observations strongly suggest that HABP is a cell surface-associated extracellular matrix protein.

Thus, based upon the results summarized above, it can be concluded that the 68 kDa HABP, a homodimeric glycoprotein of 34 kDa subunits derived from normal rat kidney, is a distinct HA-binding protein which is not only widely-distributed in rat tissues but also present in human cells suggesting that this novel protein is not tissue-and species-specific. The results suggest that this 68 kDa HABP belongs to the same family of ECM-and cell surface-associated glycoproteins which are able to bind preferentially and with high-affinity to HA and are possible endogenous binding sites for this polysaccharide. Moreover, the binding characteristics of 68 kDa HABP towards matrix biopolymers suggest a structural role for this protein in the ECM. Its possible involvement in tissue morphogenesis and development is also indicated from the data.

The successful purification of a novel HABP from rat tissues and the availability of polyclonal antibodies against it prompted us to further characterize its physiological functions.

With a view to probe the biological functions of this novel cell surface-and ECM-associated 68 kDa HABP and to begin to ascertain whether it is involved in the regulation of cell behaviour during normal and disease (e.g., tumorigenesis) processes, a study was undertaken to examine the effect of this protein on cell-substratum attachment process in vitro using both normal and tumor cells and on cell-cell adhesion during in vivo solid tumor formation. Keeping this in view, the cell types chosen for these studies were normal rat peritoneal macrophages, and the histiocytic transplantable macrophage tumor cell line AK-5 which is a mixture of four different cell populations and grows both as
intraperitoneal ascites and subcutaneous solid tumors in Wistar rats. Although all the subpopulations of AK-5 cell line have tumorigenic potential by all criteria, only one subpopulation can produce both solid tumors and ascites; whereas the other subpopulations can grow only as ascites. The use of this tumor cell line as a model system provided us with an advantage; it was possible for us to (i) monitor the level of HABP on different tumorigenic cells having differential adhesive properties, and (ii) determine whether it was associated with cell adhesion of the tumor cell line uniformly or only with the solid tumor producing cells. Keeping the above views in mind the results and conclusions are given below:

(a) The presence of the 68 kDa HABP in normal macrophages and in the macrophages of AK-5 tumor cell line was detected by immunoblot analysis using anti-rat kidney HABP antibodies. The protein was found to be associated with the outer cell surface and secreted into the culture medium of these tumor and normal macrophages as investigated by immunodetection techniques, including immunocytochemical and immunofluorescence staining, immunoblot analysis and ELISA, using the anti-HABP antibodies. Quantitative analysis by ELISA as well as by indirect immunofluorescence staining with subsequent analysis on cytofluorometer, using these antibodies, demonstrated that the levels of HABP present intracellularly and associated with the outer cell surface as well as the amount of HABP secreted in the culture medium are significantly higher in AK-5 tumor cell line as compared to normal macrophages. These results thus suggested that the synthesis and expression of this ECM - and cell surface-associated HABP is regulated with cell transformation and tumorigenesis.

(b) Immunocytochemical staining and cytofluorometric analysis using the anti-HABP antibodies revealed a differential expression of HABP on the cell surface of different subpopulations of AK-5 tumor cell line. The cell population which is highly adhesive and responsible for developing both ascites and subcutaneous solid tumors was found to contain significantly higher amounts of HABP than those subpopulations which are less adherent and capable of producing only ascites. These observations imply that the increased expression of surface-associated HABP on the relatively more adhesive subpopulation of AK-5 may be related to the ability of this cell population to produce solid
tumors in vivo, and therefore suggest the possible involvement of this protein in solid tumor formation.

(c) The HABP coating on the substratum (i.e., the wells of tissue culture plates) was found to promote the attachment of trypsinized normal macrophages and AK-5 tumor cells to the substratum in a dose-response profile, but the cell-substratum adhesion could be specifically inhibited by pretreating the HABP-coated wells with anti-HABP antibodies. However, AK-5 tumor cells were observed to display greater attachment than normal macrophages on the HABP-coated surfaces. These results suggested that the HABP has a cell-substratum attachment-promoting activity on both normal and tumor macrophages, which is independent of its ligand i.e., HA (since prior to the cell adhesion assay, the cells were trypsinized in order to remove the cell surface-bound materials including HA) and which can be specifically blocked by its antibodies.

Further, the iodinated $^{125}$I-HABP was found to bind to the surface of intact normal macrophages and ascitic AK-5 tumor cells in a linear and saturable dose-dependent manner, which could be specifically competed with an excess of unlabeled HABP, thus demonstrating the existence of specific binding sites for this protein on the cell surface membranes of these cells. This finding further suggests that the HABP associates with the cell surface and mediates the cell-substratum attachment of normal and tumor macrophages by directly interacting (non-covalently) with its cell-surface binding sites. Moreover, it was observed that $^{125}$I-HABP bound in greater amounts to ascitic AK-5 cells than to normal macrophages, implying that AK-5 tumor cells exhibit more number of cell-surface binding sites ($18.06 \times 10^4$ binding sites per cell with a Kd value of $1.33 \times 10^{-13}$ M) than normal macrophages ($10.34 \times 10^4$ binding sites per cell with a Kd value of $1.56 \times 10^{-13}$ M) for HABP. This observation provides an explanation for the greater attachment of AK-5 tumor cells than normal macrophages to the HABP-coated substratum. In addition, these data also raise the possibility that the expression of cell-surface binding sites for HABP may also be regulated with tumorigenesis.

(d) It was seen that the pretreatment of ascitic AK-5 cells with anti-kidney HABP antibodies abolished their ability to grow as solid tumors at subcutaneous sites in rats; however the cells retained their capacity to grow as ascitic tumor. These observations together with the data of cytofluorometric analysis indicated that
the HABP was necessarily required for the formation of subcutaneous solid tumors, while it was dispensable for the intraperitoneal ascitic growth of these cells. Further, these results not only confirmed the adhesive property of HABP but also suggested that the role of this protein in solid tumor formation may be to mediate cell-cell adhesion of the tumor cells without affecting the tumorigenic potential of the cells.

Thus, based upon all these observations, it can be concluded that this newly isolated cell surface-associated extracellular HABP is a cell attachment protein, which associates with the cell surface and mediates the cell adhesion probably by directly interacting (non-covalently) with its binding sites located on the cell surface membranes, and whose expression along with that of its binding sites may be regulated with cell transformation and tumorigenesis. Moreover, from these results it also appears that the loss or gain of this protein in tumor cells does not affect the tumorigenicity of the cells directly. Instead, the levels of HABP on the cell surface is probably related only to the adhesive properties of the tumor cells whereby high-HABP-containing cells can tightly adhere to each other as well as to the host tissue cells, possibly being mediated through this cell surface-adhesive protein, and therefore can grow in a more organized form; while the lack or scarcity of HABP on a tumor cell surface disallows such a property without affecting the tumorigenic potential of cells. However, more studies will be required to prove it.

(III) Knowing the extensive documentation of biological significance of HA in various extracellular fluids (e.g., plasma) on one side and considering the paucity of adequate information on the presence, properties and role(s) of HA-binding proteins in these fluids on the other, a study was undertaken to purify and characterize an HABP from normal rat blood plasma in order to further investigate its physiological relevance. Attempts were also made to compare the plasma-derived HABP with the tissue-derived HABP. Further, in order to find a clue as to the role(s) of the HABP in the functions of host inflammatory cells, keeping the fact in view that HA and extracellular adhesive proteins contribute significantly to their functions, our initial step was to detect, localize and quantitate this protein in various circulating and tissue macrophages. Keeping the above in mind, the results and conclusions are given below:
The presence of HA-binding protein in normal rat plasma was detected immunologically using the antibodies directed against purified rat kidney HABP.

This immunologically detected HABP was purified from rat plasma by ammonium sulfate fractionation followed by hyaluronate-Sepharose affinity chromatography and then by immunoaffinity chromatography using anti-rat kidney HABP antibodies as the ligand. The procedure resulted in 182-folds purification with an overall yield of about 47%. The protein obtained was homogeneously pure as confirmed by the criteria of single band in native PAGE, SDS-PAGE and two-dimensional electrophoresis.

The purified plasma HABP (the soluble or circulating form) was characterized for some of its basic properties and compared with the kidney-derived HABP (the cellular form) to explore the relationship between these two immunologically related proteins. In fact, the soluble form of HABP was found to be identical to the cellular form with respect to native molecular weight, subunit size and composition, absence of interchain disulfide bridge, pi value, glycoprotein nature, behaviour on molecular sieve chromatography, HA-binding properties and immunological characteristics. These results thus suggest that the same 68 kDa HABP molecule exists in tissues and plasma.

Furthermore, Immunoblot analysis using anti-rat kidney HABP antibodies revealed the occurrence of the 34 kDa HABP also in various circulating leukocytes (e.g., lymphocytes, granulocytes and platelets) as well as tissue macrophages of normal rat. Interestingly, this protein was observed to be absent in erythrocytes and co-incidentally there is no report on the regulation of erythrocyte functions by HA. Moreover, the HABP was found to be associated with the outer surface of these host inflammatory cells as revealed by indirect Immunofluorescence staining using anti-HABP antibodies. Further, it was seen that this HA-binding protein is synthesized de novo in circulating leukocytes and macrophages, and is also released in their culture medium. This finding may be of relevance in understanding the origin of the plasma 68 kDa HABP. Quantitative analysis by ELISA demonstrated that the cellular level of HABP and its rate of secretion is higher in tissue macrophages than in circulating leukocytes. In view of the fact that this cell surface-associated
extracellular glycoprotein has been identified as a cell attachment protein
(Chapter II), its differential level/secretion in leukocytes and macrophages may
be related to their differential adhesive properties.

(d) In an attempt to elucidate the functional role of HABP in the blood physiology,
as an initial step its interaction with plasma glycoproteins, e.g., fibrin and
fibrinogen, was examined by radiolabelling method. It was observed that this
protein, apart from having binding affinity towards HA, laminin, fibronectin
and collagen type IV (as described in Chapter I), also interacts with fibrinogen),
although to a lesser extent than to HA. Although at present the
physiological significance of HABP-fibrinogen interaction is unclear,
presumably the possible involvement of HABP in blood clotting process may be
suggested from this observation. But extensive investigation is required before
any definite conclusion can be drawn.

The results presented above thus prompt us to conclude that the 68 kDa
HA-binding protein, apart from being present as a cellular form in
extracellular matrices and on cell surfaces (results from Chapters I and
II), also (I) occurs as a structurally and immunologically identical soluble
(circulating) form in plasma, and (II) is synthesized and secreted by
various host inflammatory cells. Although at present the physiological
significance of the presence of 68 kDa HABP in plasma has not been fully
characterized, this information will certainly help us in future to
understand the mechanisms of circulating HA-mediated processes.
Furthermore, in light of the cell adhesive, binding and secretory
characteristics of the cell surface-68 kDa HABP, our present results on the
occurrence of this protein on circulating leukocytes and tissue
macrophages perhaps would shed more light on the mechanisms of those
functions of host inflammatory cells in which extracellular adhesive
proteins and HA play crucial roles, e.g., the manner in which these cells
interact with their target (normal and neoplastic) cells and with ECM
components in the basement membranes or stroma, as well as how they
extravasate and infiltrate various tissues during inflammatory and wound
healing processes, angiogenesis, phagocytosis, lymphocyte activation and
immune recognition, and homing of circulating lymphocytes. Future
studies will unravel the precise role(s) of HABP in these processes.
(IV) In order to further elucidate the functional significance of 68 kDa HABP in the altered phenotype (e.g., reduced cell adhesion to matrix) of virally transformed fibroblasts and to probe its role(s) in the regulation of normal cell growth and in the process of cell transformation the following observations were made:

(A) Reduced level of the HABP in virally transformed fibroblasts and its functional contribution to the aberrant behaviour (e.g., reduced adhesion to substratum) of these cells:

(a) Initially, the presence, localization and levels of the 68 kDa HABP in normal rat heart fibroblasts (NRHF) obtained by primary culture, and in a matched pair of normal (F111) and polyoma virus-transformed (PYf) rat skin fibroblast cell lines were studied by immunodetection techniques using the anti-rat kidney HABP antibodies. In immunoblot analysis, these antibodies revealed the occurrence of 34 kDa HABP in both the normal and transformed fibroblasts. Moreover, the protein was found to be associated with the outer surface and secreted in the culture medium of these cells as elucidated by indirect immunofluorescence staining and immunoblot analysis studies. However, the levels of HABP present intracellularly and associated with the cell layer matrix as well as the amount of HABP shed in the culture medium were observed to be markedly reduced in the transformed fibroblasts as compared to their normal counterparts.

These data thus suggested that the viral transformation of fibroblasts is accompanied by decrease in the level of cell surface-and ECM-associated 68 kDa HABP. However, such a reduction in the expression of HABP is not common to all types of transformed or tumorigenic cells, since earlier in Chapter II the level of this protein was shown to be elevated in macrophage tumor cell line. These divergent findings reflect the existence of heterogeneity in the type of transformed cells with respect to HABP-synthesizing capacity. Therefore any comparison between different transformed or tumor cell types with respect to the levels of HABP expression/synthesis requires considerable caution. Nevertheless, taken together these results strongly suggest that the expression of HABP is transformation-sensitive.
In order to identify the functional significance of the loss of surface/matrix-associated 68 kDa HABP from virally transformed fibroblasts on their reduced cell adhesion, the trypsinized normal (NRHF and F111) and transformed (PYF) fibroblasts were compared for their potential to adhere on HABP-coated surfaces as well as to interact with $[^{125}\text{I}]$HABP. Although HABP was found to promote the adhesion of both the cell types (trypsinized) to solid substratum in a dose-response profile, which could be specifically blocked by its antibodies, the transformed PYF cells displayed significantly reduced attachment as compared to normal fibroblasts on the HABP-coated surfaces. These results thus clearly suggest that the endogenous cell surface-associated 68 kDa HABP mediates the adhesion of fibroblasts to the solid substrates (or matrix in vivo) and the loss of this protein in virally-transformed fibroblasts seems likely to be related to the reduced ability of these cells to adhere to substratum in vitro or in vivo.

Moreover, it was also observed that the extent of the binding of exogenous $[^{125}\text{I}]$HABP to the surface of intact PYF cells was considerably lower than for F111 or NRHF fibroblasts, although in both the cases the $[^{125}\text{I}]$HABP binding was linear and saturable, implying that the transformed PYF fibroblasts exhibit reduced affinity as well as number of cell-surface binding sites (34.26 x 10$^3$ binding sites per cell with a Kd value of 1.25 x 10$^{-13}$ M) in comparison to the normal fibroblasts (77 to 90 x 10$^3$ binding sites per cell with a Kd value of 0.4 x 10$^{-13}$ M) for HABP. These data indicate that the viral transformation of fibroblasts is also accompanied by changes in the expression of the cell-surface binding sites for HABP, which may in turn provide a potential molecular mechanism for the reduced association/adhesion of the transformed PYF cells to HABP matrix.

Taken together, these results thus suggest that the decrease in the levels of cell surface-associated extracellular adhesive 68 kDa HABP and its cell-surface binding sites on the virally transformed fibroblasts may contribute to their aberrant behaviour in culture such as altered adhesive and possibly migratory properties and density-independent growth which are associated with invasion and malignancy in vivo.
Possible role of 68 kDa HABP in cellular growth and transformation

(a) Growth-promoting function of HABP

In the hope of identifying further the biological functions of 68 kDa HABP, after establishing its role in cell adhesion, the effect of this protein on the mitogenesis of cultured cells both in monolayers and in suspensions was examined. It was observed that the increasing amounts of exogenous HABP added to the nearly confluent, quiescent monolayer cultures of normal fibroblasts (NRHF and F111) and to the suspensions of lymphocytes (isolated from normal rat blood) stimulated DNA synthesis in these cells in a dose-dependent manner as measured by $[^{3}H]$thymidine incorporation into cellular DNA. However, the stimulation of DNA synthesis by exogenous HABP required the presence of low concentrations of serum in the culture medium, whereas HABP alone in serum-free conditions had a small effect on the mitogenesis. These data suggest that HABP acts as a potent mitogen in a synergistic manner with undefined growth factors present in the serum.

The above results point to the conclusion that the 68 kDa HABP, apart from having cell-substratum attachment-promoting activity in vitro, also has both the anchorage-dependent and anchorage-independent growth-promoting activity on fibroblastic cells and lymphocytes in culture respectively. The ability of HABP to support these two intrinsically different modes of cellular growth in vitro suggests a role for this protein in the growth of a variety of cells during various physiological processes in vivo. This possibility is further supported by its ubiquitous presence in several normal tissues, serum, most macrophages (including tumor cells), lymphocytes, granulocytes, platelets, and normal as well as transformed fibroblasts. However, the present data on the mitogenic function of HABP are preliminary and a number of questions remain to be answered. Undoubtedly, future studies in our laboratory will unravel the various aspects of growth factor-like function of HABP, including its precise mechanisms.
b. Phosphorylation characteristics of HABP and its possible involvement in some signal transduction pathway:

In an effort to gain some insight into the mechanisms involved in the regulation of biological activities of 68 kDa HABP and to further elucidate the role of this protein in cellular growth and transformation, as an initial step, the phosphorylation characteristics of this protein in normal and virally transformed fibroblasts as well as in the cells exposed to various exogenous agents, including HA and tumor promoters, were examined. The HABP was found to be expressed as a phosphoprotein by both normal (F111) and polyoma virus-transformed (PYF) rat skin fibroblasts in culture, as determined by labeling the cells with inorganic $^{32}$P$_{i}$, followed by extraction of the protein from the $^{32}$P$_{i}$-labeled cells by immunoprecipitation with anti-kidney HABP antibodies, electrophoresis of the immunoprecipitated samples on SDS-PAGE and then autoradiography of the dried gels. Moreover, in both the cell types this protein was found to be phosphorylated only at tyrosine residues as revealed by its phosphomino acid analysis as well as immunoblot analysis using anti-phosphoatyrosine antibody. These results thus establish that the 68 kDa HABP exists in vivo as a phosphotyrosine-containing protein. It is an extremely important observation, since the tyrosine-specific phosphorylation of the HABP was found to be enhanced manifold in the polyoma virus-infected fibroblasts (PYF) as compared to their normal counterparts (F111), as determined by measuring the counts of $^{32}$P-labeled immunoprecipitated 34 kDa HABP bands, from both the cell types, as well as of the tyrosine residues extracted from these radiolabeled bands excised from the SDS-PAGE gels.

Thus, our present observations clearly demonstrate that viral transformation of cells markedly stimulates the tyrosine-specific phosphorylation of 68 kDa HABP in a constitutive fashion. While it remains premature to propose a specific physiological function for the tyrosine-specific phosphorylation of HABP, these results probably would be consistent with the regulatory function of this post-translational covalent modification. Moreover in view of the fact that one of the major roles for tyrosine phosphorylation is in the regulation of normal cell growth and in the process of cell transformation, the tyrosine
phosphorylation of HABP and its stimulation by viral transformation appear to have some functional relevance in these processes. Although a formal proof is lacking, a possible function of the tyrosine phosphorylation of endogenous HABP may be to modulate its biological activities (e.g., mitogenic activity) \textit{in vivo}. Therefore, an increased phosphate content at tyrosine residues in endogenous HABP molecules in response to viral transformation may impair the biological functions of this protein, e.g., may enhance its mitogenic activity which may in turn contribute to unrestrained growth of the transformed cells by providing them with a continuous mitogenic signal.

Further, it was observed that the treatment of resting $^{32}$P-labeled fibroblasts with exogenous 68 kDa HABP or HA alone or both in combination, or with tumor promoter PMA resulted in a rapid increase in the phosphorylation of both the endogenous 68 kDa HABP molecules and total cell proteins as determined by measuring the immunoprecipitable-and TCA-precipitable counts.

The doses of exogenous HABP required to stimulate \textit{in vivo} protein phosphorylation were found to be similar to those required to stimulate DNA synthesis in quiescent fibroblasts in a linear and saturable manner as mentioned earlier. Moreover, these biological effects of exogenous HABP are similar to that of soluble growth factors and therefore the results suggest that the mitogenesis stimulated by exogenous HABP, like other growth factors, may also involve the protein phosphorylation, as an early signaling step in the cascade of molecular events leading to DNA synthesis.

The increased phosphorylation of endogenous HABP in intact fibroblasts in response to HA acquires considerable interest in view of the fact that HA plays a vital role in a number of cellular processes, such as proliferation, differentiation and transformation, which are now known to be regulated primarily by protein phosphorylation. These results, therefore, strongly suggest the biological importance of HABP phosphorylation in the transduction of instructive signals (e.g., mitogenic or transforming) which result from the interaction of HA with the cell surface.
Interestingly, the addition of HA in combination with exogenous HABP was found to stimulate the protein phosphorylation in transformed fibroblasts significantly more than the addition of HA or HABP alone. These results indicate that the supplementation with exogenous HABP may enrich the cultured cells with cell surface-hyaluronate-binding sites which may in turn increase the binding of HA to the cells and thus may result in an enhanced biological response to HA.

The rapid increase in the tyrosine-specific phosphorylation of endogenous HABP in the quiescent $^{32}$P-labeled normal fibroblasts treated with phorbol ester PMA (a potent mitogen and tumor promoter) further supports the possibility that this phosphorylation may be involved in some signal transduction pathway leading to cell growth or transformation.

Hence, based upon these observations it can be concluded that the 68 kDa HABP exists in vivo as a phosphotyrosine-containing protein whose tyrosine-specific phosphorylation in intact cells is stimulated in a variety of ways including viral transformation or exposure of the cells to HA, exogenous HABP or tumor-promoting phorbol esters. Taken together, these results not only point to the possibility that this rare posttranslational covalent modification might be modulating the biological activities of HABP in vivo, but also indicate the possible involvement of tyrosine-specific phosphorylation of endogenous HABP in signal transduction mechanisms regulating various cellular processes, particularly cell growth and transformation. However, these aspects need to be looked into in greater detail.

Thus, it may not be an over-statement to submit that the present investigation has provided substantial information with regard to characteristics, tissue-distribution, occurrence in a wide variety of cell types, localization, and physiological functions of this novel cell surface-and ECM-associated 68 kDa HA-binding protein. Ensemble of evidence summarized here clearly suggest that this protein, like other cell surface/ECM-associated glycoproteins (e.g., fibronectin, laminin and collagen etc), has multifunctional roles in various cellular processes. Although the scope of the present work has left many questions unanswered, it certainly would not be an exercise in the realm of
speculation to suggest that further studies would unravel wider implications of this novel protein as relates to its role(s) in the regulation of cell behaviour. Further, there is every reason to remain optimistic that extensive research of this highly interesting molecule could pin-point its precise function in cellular signal transduction mechanisms, which would undoubtedly enhance the overall understanding of the molecular and biochemical mechanisms underlying various cellular processes under normal and pathological conditions.