CHAPTER-II

Possible role of HA-binding protein on cell adhesion in rat histiocytoma
5.1 INTRODUCTION

Cellular interactions with extracellular matrix components are crucial for a number of biological processes that require cell adhesion or migration, including embryogenesis, development, maintenance of tissue integrity, wound healing, homeostasis, coagulation, immunological recognition, angiogenesis and tumorigenesis [for reviews see Hay, 1981; Yamada and Akliyama, 1984; Hook et al., 1984; Turley, 1984; Buck and Horwitz, 1987; Duband et al., 1987; Hynes, 1987; Akiyama et al., 1990]. The interaction of cells with ECM not only promotes cell anchorage but also provides positional signals that direct cell migration. The stationary cells require attachment to a substratum (i.e., the extracellular milieu) for support, orientation, differentiation and growth, whereas actively migrating cells use adhesive contact for traction and guidance. Among the well-characterized matrix macromolecules, hyaluronic acid (HA), a large non-sulfated GAG and one of the principal components of ECM, in particular, has been implicated in the regulation of cell-to-cell and cell-to-substratum adhesion, and cell motility both in vitro and during the above-mentioned normal and disease processes [Underhill and Dorfman, 1978; Toole, 1981, 1982, 1990; Underhill, 1982a; Hook et al., 1984; Turley, 1984; Turley et al., 1985, 1991; Laurent and Fraser, 1986; Wiegel et al., 1986; Knudson et al., 1989; Evered and Whelan, 1989].

Although the mechanisms by which HA influences cell behaviour are not well-characterized, the demonstration, via kinetic analysis, of specific cell-surface binding sites [Underhill and Toole, 1980, 1981; Toole, 1982; Hook et al., 1984; Toole et al., 1984] suggests that it exerts at least part of its biological effects by interacting with the cell surface. As described in the earlier chapter, in the past decade several hyaluronic acid-binding glycoproteins of different molecular features, including the 68 kDa HABP identified during the course of the present investigation, which are possible candidates for the HA-binding sites on the cell surface or in the ECM, have been isolated and characterized from a variety of sources [for review see earlier Chapter and Toole, 1990]. Even though, all the recent reports suggest a crucial role for HABPs in cell behaviour, only few of these binding proteins have been studied in some detail in this regard. For example, one characterized HA-receptor, because of its presence in focal adhesions of fibroblasts, has been implicated in cell adhesion [Underhill et al., 1985; 1987]. Another antigenically distinct cell surface/ECM-associated HA-binding protein described by Turley and her co-workers [Turley et al., 1987], which occurs predominantly on locomoting fibroblasts where it is localized in the ruffles, blebs and lamellae of cell processes [Turley and Torrance, 1985] and shows a partial and
temporally regulated co-distribution with meshwork actin [Turley et al., 1990], has been implicated in cell locomotion.

In the previous chapter, we reported the homogeneous purification and characterization of a distinct HABP (a 68 kDa homodimeric glycoprotein) from normal rat tissues and demonstrated that this protein is localized on the cell surface (but not intercalated into plasma membranes), in the ECM as well as inside the cells [Chapter I; Gupta et al., 1991]. Thereafter, with a view to probe the biological functions of this novel cell surface-and ECM-associated HABP (hyaluronectin) and to begin to ascertain whether it regulates cell behaviour during normal physiological and pathological (e.g., tumorigenesis) processes, in the present investigation we planned to study the role of this protein on cell-substratum attachment process in vitro and on cell-cell adhesion during solid tumor formation in vivo. In order to accomplish it, we examined specifically the presence, level of expression and adhesive effect of this protein on tumor cell line and compared it with that on normal cells. Keeping this in view, we chose normal rat peritoneal macrophages, and the histiocytic transplantable macrophage tumor cell line AK-5 [Khar, 1986], which consists of a heterogeneous population of cells and grows both as intraperitoneal ascites and subcutaneous solid tumors in Wistar rats.

As reported earlier [Pande and Khar, 1988], AK-5 tumor cells can be further separated into four subpopulations by buoyant density centrifugation on a Percoll gradient; only one subpopulation can produce a solid tumor when injected subcutaneously, whereas the other subpopulations can grow only as intraperitoneal ascites. Because AK-5 tumor cells are a mixture of different cell populations, both capable and incapable of producing a solid tumor, the use of these cells as a model system provided us with an advantage; it was possible for us to monitor the level of HABP on the different tumorigenic cells having differential adhesive properties and determine whether it was associated with cell adhesion of the tumor cell line uniformly or only with the solid tumor producing cells.

5.2 RESULTS

5.2.1 Presence of 68 kDa HA-binding protein (hyaluronectin) in normal macrophages and AK-5 tumor cells

Since we were interested in identifying the role of 68 kDa HABP on cell attachment using macrophage cells (both normal and tumor), we initially attempted to
detect the presence and localization of this protein in normal intraperitoneal and alveolar macrophages and in the AK-5 tumor cell line by immunoblotting and immunocytochemical staining techniques. As shown in Figure 5.1, by immunoblot analysis, using an antibody raised against the purified rat kidney HABP which is a homodimer of 34 kDa subunits, we could detect 34 kDa HABP in the cell extracts of normal peritoneal and alveolar macrophages as well as of ascitic AK-5 cells. It is evident in the Figure that the electrophoretic mobility of this anti-HABP antibody-detectable 34 kDa protein band in the cell extracts of normal macrophages and total AK-5 cells is identical to the one developed for the purified tissue-derived HABP. However, the intensity of the immunoreactive 34 kDa protein band was more prominent in the extract of AK-5 cells as compared to that of normal macrophages indicating that the HABP level is higher in the ascitic AK-5 tumor cells than in the normal cells. Hence, in order to determine the level of this protein in different macrophage cells, its quantitative analysis was carried out by ELISA using the anti-HABP antibodies. As is evident, the HABP content in ascitic AK-5 cells is almost twice normal macrophages (Table 5.1).

In order to acquire more information about the distribution of the HABP on AK-5 cells, immunocytochemical staining of nonpermeabilized ascitic AK-5 cells (total) was performed by the double antibody staining method using anti-rat kidney HABP antibodies as described under Materials and Methods (Section 3.19.1) (Fig. 5.2). The indirect immunofluorescence staining of normal rat peritoneal and alveolar macrophages for this protein, by using anti-HABP antibodies, is shown in the following Chapter III. As shown in Figure 5.2a, a differential staining for HABP on the surfaces of unseparated ascitic AK-5 cells is clearly visualized. However, it is important to mention that such staining is not seen when erythrocytes, i.e., HABP-negative cells, or normal fibroblasts and macrophages, i.e., HABP-positive cells, were stained with the same antibody. This observation suggests a differential level of HABP content in the mixed population of AK-5 cells. Moreover, the association of this protein with the cell surface was further confirmed by treating the cells with 0.2 M urea in PBS, with 0.2 M glycine-HCl, pH 2.2, or with PBS prior to immunocytochemical staining. Urea pretreatment appears to detach most of the HABP from the cell surface of unseparated ascitic AK-5 cells as judged by highly reduced immunostaining for HABP after this treatment (Fig. 5.2b). The detection of 34 kDa HABP in the urea washings of ascitic AK-5 cells by Western blot analysis using anti-HABP antibodies (Fig. 5.3) confirms that the protein is immunoreactive even after urea
Figure 5.1. Detection of the HABP in normal macrophages and AK-5 tumor cells by immunoblot analysis. Proteins from different cell types were extracted by directly solubilizing an equal number of cells ($10^5$) in Laemmli sample buffer [Laemmli, 1970], and then electrophoresed on a 12.5% SDS-polyacrylamide slab gel (Section 3.10.2).— Lane 1: proteins from normal rat peritoneal macrophages; lane 2: proteins from normal alveolar macrophages; lane 3: proteins from ascitic AK-5 cells; lane 4: 5 μg of purified rat kidney HABP (positive control); lane 5: 5 μg of Pharmacia low-molecular-weight marker proteins (14.4-94 kDa). Proteins in the gel were electrophoretically transferred onto a sheet of nitrocellulose. Blotted proteins were incubated with anti-rat kidney HABP antibodies and the resultant immune complexes were visualized by enzyme-linked immunodetection method using alkaline phosphatase-labeled goat anti-rabbit IgG as described under Materials and Methods (Section 3.16). The blotted marker proteins lane was cut and stained with amido black.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>μg of HABP/mg of total protein in the cell extract&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat peritoneal macrophages</td>
<td>17.0 ± 1.5</td>
</tr>
<tr>
<td>Normal alveolar macrophages</td>
<td>16.0 ± 1.1</td>
</tr>
<tr>
<td>Ascitic AK-5 cells</td>
<td>35.0 ± 1.6</td>
</tr>
</tbody>
</table>

HABP contents of the cell extracts were assayed by ELISA using ani-rat kidney HABP antibodies as mentioned under Materials and Methods (Section 3.17). —<sup>a</sup>The average value of 10 different experiments with standard deviation.
Figure 5.2. Immunocytochemical staining of nonpermeabilized ascitic AK-5 cells for the HABP. (a) Monolayer smears of the unseparated population of ascitic AK-5 cells were fixed with 4% paraformaldehyde, and then stained with anti-rat kidney HABP antibodies and with the complex of biotinylated goat anti-rabbit IgG and streptavidin-alkaline phosphatase conjugate as described under Materials and Methods (Section 3.19.1). Differential levels of staining for the HABP can be observed over the surfaces of unseparated AK-5 cells. (b) Ascitic AK-5 cells (total) were washed with 0.2 M urea in PBS prior to fixation and the immunostaining for HABP. The staining of the cells was highly reduced after the urea treatment. (c) Preimmune rabbit IgG stained very little of the cells. X 940.
treatment. Therefore, it can be concluded that the significant decrease in the immunostaining is due only to the removal of HABP from the cell surface by urea treatment. The association of HABP with the cell surface is resistant to PBS washes as the protein cannot be detected in these washings (Fig. 5.3).

5.2.2 Evidence for the HA-binding protein as a secretory protein

The cell surface-associated proteins generally have a secretory nature, and we were able to detect the 68 kDa HABP on AK-5 cell surfaces. Therefore, by immunoblotting and ELISA using anti-kidney HABP antibodies, we analyzed the growth medium of normal macrophages and ascitic AK-5 cells, taking care that no serum be present in the culture medium. As shown in Figure 5.4, it is evident that the HABP is present in the 0.2 M glycine-HCl, pH 2.2, washings of ascitic AK-5 cells (not the cell extract) and also in the serum-free supernatant culture medium of normal macrophages and total AK-5 cells, since in the immunoblot analysis, the protein band was detected in these samples by anti-HABP antibodies at the identical position (i.e., at 34 kDa) as that of the HABP purified from tissues or identified in the cell extracts of normal macrophages and ascitic AK-5 cells. Based upon this observation, it can be concluded that the HABP is secreted by normal macrophages and AK-5 tumor cells in the culture medium.

In order to further confirm the secretory nature of HABP and its synthesis in normal macrophages and ascitic AK-5 cells, the cells were cultured in the serum-free plain medium with or without cycloheximide (25 µg/ml). The medium was collected during different time intervals and, after being concentrated and dialyzed against PBS, the HABP content was measured by ELISA using anti-kidney HABP antibodies (Fig.5.5). As shown, for the first 6 h the rate of secretion of HABP by ascitic AK-5 cells and normal macrophages was the same; however, in AK-5 cells it was increased twofold from 6 h onward. It is to be noted that in normal macrophages, the secretion of HABP reaches a plateau at 24 h, whereas secretion of the protein by AK-5 cells is linear even up to 48 h. The secretion of HABP in the culture medium was totally blocked in the presence of cycloheximide. These data confirm that HABP which is secreted in the culture medium is synthesized de novo in normal and ascitic AK-5 macrophages.
Figure 5.3. Evidence for the removal of the HABP from the cell surface by urea treatment (immunoblot analysis). — Lane 1: 0.2 M urea (in PBS) washings of ascitic AK-5 cells, 50 μg of proteins; lane 2: PBS washings of ascitic AK-5 cells, 50 μg of proteins; lane 3: 5 μg of pure rat kidney HABP were subjected to 12.5% SDS-PAGE (Section 3.10.2). Proteins in the gel were electrophoretically transferred onto a nitrocellulose sheet. Blotted proteins were incubated with anti-rat kidney HABP antibodies and the resultant immune complexes were visualized by using the enzyme-linked immunodetection system as described under Materials and Methods (Section 3.16). The positions of various molecular-weight standards are shown on the right side of the figure.

Figure 5.4. Evidence for the HABP as secretory protein by Western blot analysis. — Lane 1: 0.2 M glycine HCl, pH 2.2, washings of ascitic AK-5 cells, 50 μg of proteins; lane 2: serum-free supernatant culture medium of normal rat peritoneal macrophages collected after 24 h of culturing the cells, 50 μg of proteins; lane 3: serum-free supernatant culture medium of ascitic AK-5 cells collected after 24 h of culturing the cells, 50 μg of proteins; lane 4: 5 μg of purified rat kidney HABP were subjected to 12.5% SDS-PAGE (Section 3.10.2). Proteins in the gel were electrophoretically transferred onto a nitrocellulose sheet. Blotted proteins were incubated with anti-rat kidney HABP antibodies and the resultant immune complexes were visualized by enzyme-linked immunodetection method using alkaline phosphatase-labeled goat anti-rabbit IgG as described under Materials and Methods (Section 3.16). The positions of various molecular-weight standards are shown on the right side of the figure.
Figure 5.5. Secretion of the HABP by normal macrophages and ascitic AK-5 cells. The normal macrophages and the ascitic AK-5 cells (total) were cultured in DMEM without serum; the supernatant culture media were collected at various times and then assayed for the HABP content by ELISA using anti-rat kidney HABP antibodies as described under Materials and Methods (Section 3.17, 3.21). Amount of the HABP secreted (µg/mg of total secreted proteins) is plotted against time in culture. Ascitic AK-5 cells cultured in serum-free DMEM without (Δ-Δ) or with 25 µg/ml cycloheximide (α-α); normal peritoneal or alveolar macrophages cultured in serum-free DMEM without (●-●) or with 25 µg/ml cycloheximide (○-○). Values represent the mean ± SD of six replicates.
5.2.3 HA-binding protein promotes adhesion of normal macrophages and AK-5 tumor cells to substratum

In order to examine the cell-substratum attachment-promoting activity of 68 kDa HABP on macrophages, trypsinized normal rat peritoneal macrophages and AK-5 tumor cells were studied for their potential to adhere to HABP-coated plates. It is known that trypsinization solubilizes cell surface-bound materials including HA [Underhill and Toole, 1979; Angello and Hauschka, 1980]. As shown in Figure 5.6, on an HABP-coated substratum, normal macrophages and AK-5 cells attached with a dose-response profile. Although the promotion of cell-substratum attachment of both the cell types was linear up to 6 μg of HABP added per well and reached a plateau above that concentration, AK-5 cells displayed greater maximal attachment (i.e., 70-76% of the total seeded cells) as compared to normal macrophages which displayed maximal attachment of 55-63% of the total seeded cells on the wells precoated with 6-8 μg of HABP per well. In the presence of 10% fetal calf serum (positive control) in the culture medium, cell adhesion of both the cell types to the substratum was 93-96% of the total seeded cells. However, the number of cells binding to wells coated with BSA alone (non-specific attachment) was 4-8% of the added cells.

As can be seen in Figure 5.6, the preincubation of HABP-coated wells with purified anti-kidney HABP antibodies (1:20 dilution) inhibited the adhesion of both normal macrophages and AK-5 cells to the HABP-coated substratum. However, purified preimmune rabbit IgG had no inhibitory effect on the cell adhesion onto HABP-coated wells. These results suggest that 68 kDa HABP has a cell-substratum attachment-promoting activity on both normal macrophages and AK-5 tumor cells, which is independent of its ligand (i.e., HA) and can be specifically blocked by its antibodies. The results further suggest that AK-5 tumor cells have, as compared to normal macrophages, higher ability to adhere onto HABP-coated surfaces (i.e., HABP-containing matrix in vivo).

5.2.4 Binding of [¹²⁵I]HABP to normal macrophages and ascitic AK-5 cells

After establishing the association of 68 kDa HABP with the cell surface, its secretion in the culture medium and its cell-substratum attachment-promoting activity on macrophages, we tried to elucidate the mode of association of this extracellular
Figure 5.6. Dose-response profiles of the attachment of ascitic AK-5 cells and normal rat peritoneal macrophages to HABP-coated substrates. Microtiter wells were coated with various concentrations of pure rat kidney HABP and then blocked with 1% BSA. Trypsinized ascitic AK-5 cells or normal rat peritoneal macrophages (4 x 10⁴ cells per 200 μL of serum-free DMEM per well) were plated onto the HABP-coated wells and the numbers of cells attached to the substrates were determined after a 60-min incubation at 37°C as described under Materials and Methods (Section 3.22). For inhibition of attachment of the cells to HABP by antibodies, the HABP-coated wells were preincubated with anti-rat kidney HABP IgG or preimmune rabbit IgG prior to the cell attachment assay as described under Materials and Methods (Section 3.22). Values represent the mean ± SD of six replicates from one assay. Assays for each cell type were repeated at least twice with similar results. Less than 5% of the seeded cells attached to the wells coated with BSA alone.
protein with the cell surface as well as the possible mechanism of HABP-mediated cell-substratum adhesion, particularly the increased adhesion of AK-5 tumor cells to HABP-coated substratum. To accomplish this, the binding of HABP to the cell surface of intact normal macrophages and ascitic AK-5 tumor cells in suspensions was examined by the saturation and competition methods using $^{125}$I-labeled HABP. As shown in Figure 5.7, $[^{125}]$HABP, added in increasing amounts (i.e., 2 to 30 ng/ml per well in serum-free medium) to the suspensions of intact urea-pretreated or untreated normal macrophages or ascitic AK-5 cells, bound to such cells in a linear and saturable manner. However, $[^{125}]$HABP bound in greater amounts to urea-pretreated cells than to untreated cells, implying that urea treatment exposed binding sites for HABP on the cell surface. The immunocytochemical staining experiment has already shown that the urea pretreatment appears to strip most HABP from the surfaces of ascitic AK-5 cells, as judged by highly reduced immunostaining for HABP after this treatment (Fig. 5.2b). The normal macrophages and ascitic AK-5 cells, either with or without previous treatment with urea, showed similar linear and saturation curves for $[^{125}]$HABP binding, but differed considerably in the extent of binding in both the conditions. The binding of $[^{125}]$HABP to ascitic AK-5 cells is 1.5- to 2-fold more than to normal macrophages, irrespective of urea-pretreatment. The proportion of the added radiolabeled HABP that bound to urea-pretreated and untreated ascitic AK-5 cells was approximately 60% and 20%, respectively, in the linear portion of the binding isotherm, as compared to 36% and 12% in the case of normal macrophages. The binding of $[^{125}]$HABP to both urea-pretreated as well as untreated normal and AK-5 macrophages was reduced by 70-75% in the presence of a 50-fold excess of unlabeled HABP (Fig. 5.7), which suggests that binding was specific. Moreover, the addition of increasing amounts of unlabeled HABP along with a constant amount of $[^{125}]$HABP progressively reduced the binding of the radiolabeled HABP to the cells (Fig. 5.8), which further confirms the specificity of the binding.

Scatchard plot analysis [Scatchard 1949] of the binding of $[^{125}]$HABP to intact urea-pretreated ascitic AK-5 cells or normal macrophages, which was measured by the saturation experiment (i.e., the binding data of Fig. 5.7), identified only a single class of binding sites for the HABP on the surface of these cells with apparent dissociation constants (Kd) of 1.33x10-13M and 1.56x10-13M, respectively (Fig. 5.9 and Table 5.2). It is evident from these data that, although, the affinities of $[^{125}]$HABP binding to the surface of both the cell types are almost similar (i.e., similar Kd values), ascitic AK-5 tumor cells exhibit more number of cell-surface binding sites (18.06x104 binding sites
Figure 5.7. Saturation of binding of $[^{125}\text{I}]$HABP to intact urea-pretreated or untreated ascitic AK-5 cells and normal rat peritoneal macrophages in suspension. The assays for $[^{125}\text{I}]$HABP binding to the macrophages in suspension were performed in BSA-treated 1.5-ml polypropylene microfuge tubes (Eppendorf) as described under Materials and Methods (Section 3.24). The cells ($10^6$/ml/tube) were pretreated either with 0.2M urea in DMEM to expose cell-surface binding sites for HABP [ascitic AK-5 cells (●), normal rat peritoneal macrophages (■)], or with culture medium only [ascitic AK-5 cells (○), normal rat peritoneal macrophages (□)]. The cells were washed in DMEM containing 0.2 μg/ml of cycloheximide, and then incubated with increasing amounts of $[^{125}\text{I}]$HABP (specific activity 1.09X10$^3$ cpm/μg of protein) as indicated either in the presence (broken lines) or absence (solid lines) of a 50-fold excess of unlabeled HABP in serum-free DMEM for 90 min at 37°C. After binding incubation, the cells were washed, solubilized in 0.1 M NaOH and then counted for the bound radioactivity in a gamma-counter. The cell-bound radioactivity (i.e., cell-bound $[^{125}\text{I}]$HABP) in cpm/10$^6$ cells is plotted as a function of amount of the added radiolabeled HABP. Values represent the mean ± SD of six replicates from one assay. The entire assay was repeated at least twice with similar results.
Figure 5.8. Inhibition of binding of $[^{125}I]HABP$ to intact urea-pretreated or untreated ascitic AK-5 cells and normal rat peritoneal macrophages in suspension by increasing amounts of unlabeled HABP. The competition binding assays were performed in BSA-treated 1.5-ml polypropylene microfuge tubes as described under Materials and Methods (Section 3.2). The intact urea-pretreated or untreated cells in suspension ($10^6$ cells/ml/tube) were incubated with a constant amount (28 ng/ml/tube) of $[^{125}I]HABP$ plus increasing amounts (5-to 50-fold excess) of unlabeled HABP in serum-free DMEM for 90 min at 37°C. The cells were then washed, solubilized in 0.1 M NaOH, and subsequently counted for the bound radioactivity in a gamma-counter. The cell-bound radioactivity (i.e., cell-bound $[^{125}I]HABP$) in cpm/$10^6$ cells is plotted as a function of amount of the added unlabeled HABP. Values represent the mean ± SD of six replicates from one assay. The entire assay was repeated at least twice with similar results. (●), (○) Urea-pretreated and untreated ascitic AK-5 cells, respectively; (■), (□) urea-pretreated and untreated normal peritoneal macrophages, respectively.
Figure 5.9. Scatchard analysis of the binding of $[^{125}\text{I}]$HABP to urea-pretreated ascitic AK-5 cells and normal rat peritoneal macrophages. The binding data for urea-pretreated cells (obtained in the absence of unlabeled HABP) in Figure 5.7 were analyzed by plotting the ratio of the bound concentration of radiolabeled HABP to the free concentration as a function of the bound concentration of radiolabeled HABP. This Scatchard plot analysis was used to determine $K_d$ (slope) and number of binding sites per cell (x-intercept) for the HABP binding to AK-5 cells and normal macrophages.
### TABLE 5.2: \[^{125}\text{I}]\text{HABP BINDING PARAMETERS FOR UREA-PRETREATED NORMAL MACROPHAGES AND ASCITIC AK-5 CELLS\)

The values for Kd and the number of binding sites per cell were calculated by Scatchard plot analysis (Figure 5.9) of the binding data (obtained in the absence of unlabeled HABP) presented in Figure 5.7.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Apparent dissociation constant (Kd) (\times 10^{-13}) M</th>
<th>Number of binding sites/cell (\times 10^4)</th>
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</thead>
<tbody>
<tr>
<td>Normal rat peritoneal macrophages</td>
<td>1.5</td>
<td>10.34</td>
</tr>
<tr>
<td>Ascitic Ak-5 cells</td>
<td>1.33</td>
<td>18.06</td>
</tr>
</tbody>
</table>
per cell) than normal macrophages (10.34x10^4 binding sites per cell) for the HABP (Fig. 5.9 and Table 5.2).

Thus, the existence of specific binding sites for 68 kDa HABP on the cell surface membranes of normal macrophages and AK-5 tumor cells, as demonstrated by the above experiments, suggests that the HABP associates with the surface of these cells and exerts its cell-substratum attachment-promoting activity on them probably by directly interacting (non-covalently) with these binding sites. Moreover, higher number of cell-surface binding sites for HABP on ascitic AK-5 cells than on normal macrophages may account for the greater adhesion of AK-5 tumor cells than that of normal macrophages on HABP-coated substratum. These data further indicate that the expression cell-surface binding sites for HABP may also be regulated with tumorigenesis.

5.2.5 Possible Involvement of HABP in solid tumor formation by AK-5 cells

5.2.5.1 HABP distribution in AK-5 subpopulations

After identifying the cell-substratum attachment-promoting activity of 68 kDa HABP in relation to AK-5 tumor cells and normal macrophages, our aim was to study its role in tumor formation by AK-5 cells. To achieve this goal we took two different approaches. The ascitic AK-5 tumor consists of a heterogeneous population of cells. These cells can be separated into four different subpopulations by buoyant density centrifugation on a Percoll gradient and are called band I, II, III and IV cells [Pande and Khar, 1988]. Unseparated AK-5 cells are tumorigenic in both subcutaneous and intraperitoneal sites in nearly 100% of the animals. The separated cells on the other hand show a different profile: while band I and II cells form subcutaneous tumors in more than 60% of the animals, band IV cells do not form any solid tumors. Intraperitoneally, however, all four cell populations are tumorigenic and kill 100% of the animals. As our preliminary data of the previously described immunocytochemical staining experiment (Fig. 5.2a) showed a differential immunostaining for HABP on the surfaces of unseparated ascitic AK-5 cells, we carried out a quantitative estimation of the HABP content of separated and unseparated AK-5 cells as well as of normal peritoneal macrophages by indirect immunofluorescence staining of the cells in suspension (under nonpermeabilized condition) using anti-rat kidney HABP IgG as first antibody and FITC-labeled goat anti-rabbit IgG as second antibody, and the
subsequent analysis on the FACStar PLUS cytofluorometer as described under Materials and Methods (Section 3.20). HABP-specific fluorescence profiles of these cells were recorded and are shown in Figure 5.10. As shown in Figure 5.10A, 48-49% of unseparated ascitic AK-5 cells are HABP positive with maximum fluorescence intensity of 150, whereas only 9.0% of these cells show a maximum fluorescence intensity of 60 when stained with normal rabbit IgG and FITC-labeled goat anti-rabbit IgG (Fig. 5.10 B). On the other hand, normal rat peritoneal macrophages show lower intensity of HABP-specific fluorescence as compared to that of unseparated AK-5 cells with 46% of the normal macrophages being fluorescence positive (Fig. 5.10 H). Figure 5.10 I shows the staining of normal macrophages with normal rabbit IgG and FITC-labeled goat anti-rabbit IgG; only 4% of the cells show fluorescence of negligible intensity. Since there is no fluorescence seen on total AK-5 cells and normal macrophages after staining with normal rabbit IgG, the fluorescence peaks on these cells in Figures 5.10 A,C-G, and H, respectively, are proved to be HABP-specific. The association of HABP with the cell surface is further confirmed as urea pretreatment of total AK-5 cells leaves only 5% of the cells (Fig. 5.10 G) to be stained for HABP with highly reduced fluorescence intensity. Furthermore, a differential HABP expression on the subpopulations of AK-5 is also evident from our data. A maximum HABP-specific fluorescence intensity of 193 is obtained from 63% of the cell population of band I (Fig. 5.10C), whereas only 36% of band IV cells show HABP-positive staining with a maximum fluorescence intensity of 63 (Fig.5.10F). The other subpopulations, band II cells (Fig.5.10D) and band III cells (Fig.5.10E), show HABP-specific fluorescence between those of band I and IV cells. In summary, these observations clearly suggest that (a) the AK-5 macrophage tumor cell line contains higher amounts of HABP than normal macrophages, and (b) the different populations of AK-5 cells contain different amounts of HABP on the cell surface. i.e., band I cells, the solid tumor producing subpopulation, have the highest amount of HABP, whereas band IV cells, which are unable to produce solid tumors, have negligible amounts of HABP. These observations imply that the increased expression of cell surface-associated HABP on the relatively more adhesive subpopulation of AK-5 may be related to the ability of this cell population to produce subcutaneous solid tumors.

5.2.5.2 Inhibition of tumorigenesis by pretreatment of ascitic AK-5 cells with anti–HABP antibody

Another approach to examine the role of HABP is to study the inhibition of tumorigenesis by anti-kidney HABP antibodies. To investigate the prerequisite of
Figure 5.10. HABP-specific profiles of nonpermeabilized (paraformaldehyde-fixed) AK-5 cells and normal rat peritoneal macrophages as recorded on the FACStar PLUS cytofluorometer. (A) Total AK-5 cells stained with anti-rat kidney HABP IgG and FITC-labeled goat anti-rabbit IgG; (B) total AK-5 cells stained with preimmune rabbit IgG and FITC-labeled goat anti-rabbit IgG; (C), (D), (E), and (F) are band I, II, III and IV cells, respectively, stained with anti-rat kidney HABP IgG and FITC-labeled goat anti-rabbit IgG; (G) total AK-5 cells were washed with 0.2 M urea in PBS prior to fixation and the immunostaining for HABP; (H) normal rat peritoneal macrophages stained with anti-rat kidney HABP IgG and FITC-labeled goat anti-rabbit IgG; (I) normal rat peritoneal macrophages stained with preimmune rabbit IgG and FITC-labeled goat anti-rabbit IgG.
cell surface-associated HABP in tumorigenesis, we preincubated the ascitic AK-5 cells with anti-HABP antibody, before injecting them into the animals for tumor formation. As can be seen from the data presented in Table 5.3, the pretreatment of unseparated AK-5 cells with anti-HABP antibody abolishes their ability to grow as a subcutaneous solid tumor; however, it does not affect the ascites formation. These results and the data of cytofluorometric analysis, as described earlier, indicate that the cell surface-HABP is necessarily required for the formation of subcutaneous solid tumors by AK-5 cells, while it is dispensable for the intraperitoneal ascitic growth of these cells. Thus, these observations together with the ability of 68 kDa HABP to promote cell adhesion in vitro strongly suggest that the presence of this cell surface-associated adhesive protein in AK-5 cells is related to the adhesive properties of the tumor cells and may be mediating the cell-cell or cell-matrix adhesion during in vivo solid tumor formation without being related to the tumorigenic potential of the cells per se.

5.3 DISCUSSION

The interactions of extracellular matrix macromolecules with the cell surface are important facets of the regulation of such diverse cellular properties as cell adhesion, motility, morphology, polarity, growth and differentiation in a variety of physiologically important processes including maintenance of cellular organization, homeostasis, development, and tumorigenesis [reviewed in Hay, 1981; Yamada and Akiyama, 1984; Hook et al. 1984; Toole et al., 1984; Buck and Horwitz 1987; Hynes, 1987]. Consistent with this multiplicity of function, extracellular matrices exhibit diversity in both composition and three-dimensional structure. The overall phenotypic effects induced by an ECM are the sum of complex actions and interrelationships of the molecules comprising the extracellular microenvironment of cells. Many of the most abundant components of extracellular matrices (e.g., collagens, structural adhesive glycoproteins and PGs/GAGs) have been identified, purified and characterized. The majority of these macromolecules have been shown to support cell adhesion in vitro and to have dramatic regulatory effects on the organization of the cytoskeleton [reviewed in Turley, 1984; Yamada and Akiyama, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Buck and Horwitz, 1987; Akiyama et al., 1990; Humphries, 1990]. As described in the introduction, it has become increasingly clear that hyaluronate, one of the well-characterized and major components of ECM, plays vital roles in each of the biological processes that involve cell adhesion or migration. This glycosaminoglycan, like other molecules of the ECM [Buck and Horwitz, 1987; Hynes
TABLE 5.3: EFFECT OF PRETREATMENT OF UNSEPARATED ASCITIC AK-5 CELLS WITH ANTI-HABP ANTIBODY ON ITS TUMORIGENIC ACTIVITY

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intraperitoneal tumors&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Subcutaneous tumors&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>Pretreated with purified preimmune rabbit IgG</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>Pretreated with purified anti-HABP IgG</td>
<td>95</td>
<td>10</td>
</tr>
</tbody>
</table>

Tumor-inhibition studies were carried out as mentioned under Materials and Methods (Section 3.23).<sup>a</sup> The values shown are the cumulative results from three independent experiments.
is thought to mediate some of its biological effects by non-covalently interacting with its specific binding sites (protein in nature) on the cell surface [Toole, 1981, 1982; Toole et al., 1984; Hook et al., 1984; Underhill and Toole, 1980, 1981]; however, not enough work has been reported to elucidate its mechanism. There is an exception of the recent reports in which one mechanism appears to be the regulation of the structure of the cytoskeleton [Lacy and Underhill, 1987]. There is evidence that the hyaluronate-binding site associates with actin-containing filaments [Lacy and Underhill, 1987], and its expression is regulated with these filaments [Turley et al., 1990].

Although one important cellular function of HA is to regulate cell adhesion in a number of normal and pathological processes, there are not many reports that demonstrate the adhesive property of its binding protein(s) except to show the localization of one of them in focal adhesion of fibroblasts [Underhill et al., 1985]. Therefore, we have done this study with the aim of specifying the role of cell surface-associated 68 kDa HABP (hyaluronectin), independent of HA, in the adhesion of normal and tumor cells using normal macrophages and AK-5 tumor cell line as a model system. In this report we have established that this HABP is an extracellular matrix adhesive protein, which associates with the cell surface and mediates the cell adhesion probably by directly interacting (non-covalently) with its specific binding sites present on the cell surface membranes, and whose expression along with that of its binding sites may be regulated with cell transformation and tumorigenesis. Moreover, on the basis of the results presented in this study it can also be concluded that the loss or gain of this protein in tumor cells does not affect the tumorigenic potential of the cells directly. Instead, the level 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 are allowed to 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 the cells.

The experimental evidence to support these conclusions is as follows: First, 68 kDa HABP can be detected in both normal and tumor macrophages where it is associated with the cell surface and can be detached from the surface by urea or glycine-HCl, pH 2.2, treatments but is resistant to PBS washes. In cell culture it is also secreted into the medium as it can be identified in the culture medium. Its synthesis
and expression on the cell surface increase in the AK-5 tumor cell line. Second, coating the wells of tissue culture plates with 68 kDa HABP promotes the attachment of trypsinized cells to the substrata in a dose-response profile, but the cell-substratum adhesion can be specifically inhibited by pretreating the HABP-coated wells with anti-HABP antibody. However, AK-5 tumor cells display greater attachment than normal macrophages on the HABP-coated surfaces. Third, the specific, linear and saturable binding of exogenous $^{[125]}$I-HABP to the surface of intact normal macrophages and ascitic AK-5 tumor cells clearly demonstrates the existence of specific binding sites for this protein on the cell surface membranes of these cells. This observation further suggests that HABP associates with the surfaces of the above-mentioned cells and exerts its cell-substratum attachment promoting activity on them probably by directly interacting (non-covalently) with its cell-surface binding sites. $^{[125]}$I-HABP binds 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 Kd value of $1.33 \times 10^{-13}$M) than normal macrophages $(10.34 \times 10^4$ binding sites per cell with Kd value of $1.56 \times 10^{-13}$M) for HABP. This observation, thus provides an explanation for the greater attachment of AK-5 tumor cells than normal macrophages to the HABP-coated substratum. Fourth, there is a differential distribution of HABP among ascitic AK-5 cells and its expression is higher on the cell surface of the subpopulation which is highly adhesive and specifically responsible for the subcutaneous solid tumor formation. The involvement of cell surface-associated HABP in cell-to-cell adhesion during solid tumor formation in vivo is further supported by the inhibition of subcutaneous solid tumor formation by total AK-5 cells after pretreatment with anti-HABP antibody without having any effect on their ascitic tumor formation.

The present results imply that tumor cells which contain high levels of HABP are capable of lodging themselves in the subcutaneous tissue and, therefore, can grow as an organized solid tumor while those which have low levels of HABP are not able to do so. This point is also supported by the earlier observation made by Pande and Khar [1988] that there is a differential expression of fibronectin in rat histiocytoma and that fibronectin is probably involved in tumor cell adhesion. It is important to mention here that the HABP purified in the current study is distinct from fibronectin as evident from immunological studies [Chapter I and Gupta et al., 1991].
Further, the results presented in this study provide a molecular basis for proposing a model of the events that occur during tumorigenesis. The adhesive interaction of HABP with the cells and matrix may be crucial for anchoring the tumor cells to the endothelium during the initial phase of tumor formation. Undoubtedly the present study on the identification of this novel cell surface-associated extracellular adhesive protein will significantly contribute to our understanding of the regulatory roles of ECM macromolecules in the cell adhesion process under various normal and pathological conditions. A number of questions however remain concerning the cell adhesive activity of HABP and its molecular basis. First, what are the minimal peptide sequences in HABP molecule which contain the cell adhesion activity. Second, whether it has the key tripeptide arginine-glycine-aspartic acid (RGD sequence) or a closely related cell attachment determinant. Third, what are the cell-surface receptors which mediate the adhesion/association of cells to HABP. Further studies will certainly identify both the cell adhesive recognition sequences in HABP molecule as well as the cell-surface receptors involved in cell-HABP adhesive interactions and therefore unravel the exact mechanisms of HABP-mediated cell adhesion at molecular level.