Factor H Binding to Bone Sialoprotein and Osteopontin Enables Tumor Cell Evasion of Complement-mediated Attack*

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Metastatic cancer cells, like trophoblasts of the developing placenta, are invasive and must escape immune surveillance to survive. Complement has long been thought to play a significant role in the tumor surveillance mechanism. Bone sialoprotein (BSP) and osteopontin (OPN, ETA-1) are expressed by trophoblasts and are strongly up-regulated by many tumors. Indeed, BSP has been shown to be a positive indicator of the invasive potential of some tumors. In this report, we show that BSP and OPN form rapid and tight complexes with complement Factor H. Besides its key role in regulating complement-mediated cell lysis, Factor H also appears to play a role when “hijacked” by invading organisms in enabling cellular evasion of complement. We have investigated whether BSP and OPN may play a similar role in tumor cell complement evasion by testing to see whether these glycoproteins could promote tumor cell survival. Recombinant OPN and BSP can protect murine erythroleukemia cells from attack by human complement as well as human MCF-7 breast cancer cells and U-266 myeloma cells from attack by guinea pig complement. The mechanism of this gain of function by tumor cell expression of BSP or OPN has been defined using specific peptides and antibodies to block BSP and OPN protective activity. The expression of BSP and OPN in tumor cells provides a selective advantage for survival via initial binding to $\alpha_\text{v}\beta_3$ integrin (both) or CD44 (OPN) on the cell surface, followed by sequestration of Factor H to the cell surface and inhibition of complement-mediated cell lysis.

Osteopontin (OPN)$^3$ and bone sialoprotein (BSP) are produced by trophoblasts (1, 2) and are induced in certain neoplasms (3–11). BSP is a phosphoprotein of molecular mass ~70–80 kDa, about half of which is sialic acid rich N- and O-linked carbohydrates, and also contains several glutamic acid-rich domains, tyrosine sulfates, as well as an integrin-binding arginine-glycine-aspartate (RGD) domain (12–14). During normal human development, BSP is produced by cells of the skeleton (osteoblasts, osteoclasts, osteocytes, and hypertrophic chondrocytes) as well as trophoblasts (2). Because of its primary association with cells that produce a mineralized extracellular matrix, BSP has been hypothesized to play a role in mineralization where its high degree of negative charge could function in calcium sequestration or in hydroxypatite crystal nucleation. However, the absence of a clear skeletal phenotype in the BSP knock-out mouse suggests either the existence of molecular redundancy or another as yet undefined functional role for BSP. Because of its apparent restricted expression pattern in trophoblasts and skeletal cells, BSP expression in tumors has been proposed to play a role in either micrometastasis (15–17) or in metastasis homing to bone (3, 9, 18). The level of BSP expression correlates positively with disease severity (9, 18, 19).

The second protein, OPN, is also known as Secreted PhosphoProtein I, 2ar, early T-lymphocyte activation 1, and transformation-associated phosphoprotein. It is a protein of ~60 kDa, and shares with BSP high sialic acid content, highly acidic sequences (but these are aspartic acid-rich), multiple residues with consensus for phosphorylation as well as an integrin-binding RGD motif (6, 12, 14). Because of its RGD tripeptide and adhesive properties, it has been proposed that OPN plays a role in metastasis in certain tumors (20–22). OPN expression is associated with clinical severity in lung cancer (20), lymph node negative breast cancer (23), gastric cancer (24), and perhaps ovarian carcinoma (25). In light of their induction by certain neoplasms, we investigated the possible role of these proteins in one aspect of escaping host humoral surveillance.

EXPERIMENTAL PROCEDURES

Reagents—Rabbit anti-human BSP antibodies LF-83, LF-100, LF-119, LF-120, and LF-125 have been previously described (26). Rabbit anti-BSP peptide-derived antibody LF-142 and a mouse monoclonal antibody LFmAb-11 were raised against the sequence EY*EYE*TVGNEY*DNGY*EITY*ESENGEP (amino acids 258–285) conjugated to horseshoe crab hemocyanin, where the $^*$ denotes tyrosine sulfates. Normal human serum, purified human complement Factor H protein, and mouse monoclonal antibody against Factor H were obtained from Quidel Corp. (San Diego, CA). Polyclonal antibodies against CD44 and a “functional” antibody against $\alpha_\text{v}\beta_3$ were obtained from Chemicon Co. (Temecula, CA). Synthetic purified glycine-arginine-aspartate-serine peptide (GRGDS) was obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Preimmune serum, human serum adsorbed goat anti-rabbit IgG conjugated to horseshad peroxidase (HRP) as well as goat anti-mouse conjugated to HRP were obtained from Kirkegaard & Perry (Gaithersburg, MD). HRP-conjugated streptavidin and sulfosuccinimido-biotin were obtained from Pierce Chemical Co. (Chicago, IL). $\alpha$-Minimal essential medium ($\alpha$-MEM), Dulbecco’s modified essential medium (DMMEM), RPMI 1640, Eagle’s minimal essential medium (EMEM), Earle’s balanced salt solution, Hank’s balanced salt solution, and heat inactivated fetal bovine serum were obtained from BioFluids, Inc. (Rockville, MD).

Western Blotting—Samples diluted in gel sample buffer were resolved by SDS-PAGE 4–20% gradient gels (Novex Corp, San Diego, CA), transferred to nitrocellulose following standard conditions (27).
Nitrocellulose membranes were rinsed with Tris-buffered saline (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 0.05% Tween 20 (TBS-Tween). After a 1-h incubation in blocking solution (TBS-Tween + 5% non-fat powdered milk) at room temperature on rotary shaker, primary antibody was added and incubated overnight at 4 °C. The nitrocellulose sheets were washed with TBS-Tween followed by 5 min of incubation on TBS-Tween and then second antibody in TBS-Tween + 5% milk was added and incubated for 2 h at room temperature. Following removal of the second antibody solution the membrane was washed three times with TBS-Tween and rinsed a final time in enzyme substrate buffer for 5 min. Enhanced chemiluminescence reagents were employed for signal detection. Western blotting with TBS-Tween followed by three times with TBS-Tween and rinsed in 20 mM Tris-gluatamine and EGTA (GVB-MgEGTA, Sigma). Cells were resuspended in GVB-MgEGTA at a density of 5 × 10^5 cells/ml and incubated at 37 °C with different concentrations of normal human serum diluted in GVB-MgEGTA. After 2 h, cells were harvested for trypsin blue exclusion assay by removing a 50-μl aliquot, incubating for 15 min in 0.05% trypsin blue, and counting viable cells under an inverted phase contrast microscope. The thiazolylblue assay was carried out at identical serum dilutions by incubating a 50-μl aliquot of the cell suspension in an equal volume of 1 mg/ml thiazolyl blue (MTT) for 45 min. Cell viability was determined spectrophotometrically by absorbance at 560 nm. Cells in GVB-MgEGTA buffer were preincubated with 10 μg of either rBSP or rOPN in 1 ml for 10 min at 37 °C. Normal human serum collected for good complement activity was then added at a dilution of 1:10 and the cells returned to 37 °C for 2 h and cell viability was determined by trypsin blue exclusion and MTT assays.

For the assay of human cancer cell lines, loosely adherent MCF-7 cells were selected for by sequential growth in EMEM with 2 mM t-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/l glucose, but were unable to detect any BSP. However, when 25-μl aliquots of normal human serum diluted 1:100 were subjected to SDS-PAGE followed by transfer to nitrocellulose and probing with a peptide-derived antibody against BSP, immunoreactive bands were readily apparent (Fig. 1). Immunoprecipitation was then used to further characterize the BSP complex in serum using six different polyclonal antibodies that span the BSP molecule (30). Initially, we adapted an existing competitive ELISA for bone matrix-derived BSP (31) to determine levels of BSP in normal human serum, but were unable to detect any BSP. However, when 25-μl aliquots of normal human serum diluted 1:100 were subjected to SDS-PAGE followed by transfer to nitrocellulose and probing with a peptide-derived antibody against BSP, immunoreactive bands were readily apparent (Fig. 1A). Curiously, in the absence of reducing agent, the BSP immunoreactive band migrated with an estimated molecular mass of 250 kDa, while with reduction a migration position that corresponded authentic BSP (molecular mass ~80 kDa) was evident. BSP contains no cysteine residues hence the shift with reduction suggested that BSP in serum was tightly bound to another serum component.

BSP possesses a high degree of negative charge (pl < 4.0), therefore strong anion exchange HPLC was first employed to isolate the BSP complex. BSP from serum did not, however, bind to QAE resin unless it was previously subject to both heating and reduction (Fig. 1B). Immunoprecipitation was then used to further characterize the BSP complex in serum using six different polyclonal antibodies that span the BSP molecule. When the immunoprecipitates were subjected to SDS-PAGE resolution followed by Western blotting and detection of the BSP with a monoclonal antibody, only certain antibodies were able to immunoprecipitate BSP and even the best could precipitate only a small fraction of the total BSP in the serum. Immunoprecipitates generated by antibodies directed toward the carboxyl-terminal RGD-containing region of the molecule completely failed to immunoprecipitate BSP (Fig. 1, C and D). Together these results indicate that BSP is present in serum as a high molecular weight complex, which masks its negative charge, and that the RGD domain is not surface accessible.

**Identification of the Serum-binding Protein**—To identify the complex constituents, unredosed normal human serum was fractionated by size exclusion chromatography (SEC). The ma-
The requirement of heating and reduction to disrupt the binding complex suggests that the BSP-binding serum component(s) possess multiple disulfide bonds and a stable structure. Subtracting the mass of BSP from the complex yields a mass estimate of 250 ± 30 kDa. Analysis of the same elution profile using premixed serum in place of anti-BSP polyclonal antibodies in the direct ELISA yielded a single peak eluting in the excluded volume of the column, suggesting that immunoreactive material in the void of the anti-BSP profiles represents nonspecific immunoreactivity. Purified rBSP was found to elute as a single peak with a calculated molecular mass of 80 kDa. SEC resolution of a separate aliquot of the same normal human serum that had been incubated with reducing agent and heat to dissociate the binding complex yielded an immunoreactive peak upon direct ELISA analysis was identical to that of free BSP. SEC resolution of a separate aliquot of the same normal human serum that had been incubated with biotinylated-rBSP yielded avidin-HRP immunoreactive and anti-BSP immunoreactive peaks whose elution position corresponded to that of serum-BSP complex (Fig. 3). No free rBSP was measurable by either antibody detection system. Incubation of biotinylated-rBSP with purified human complement Factor H also yielded a SEC profile where a single peak corresponding to that of serum-BSP complex was detected by avidin-HRP. Treatment of reconstituted rBSP-Factor H complex with reducing agent and heat lead to a shift in the BSP immunoreactive peak to that of free BSP.

Thus, complement Factor H has been identified as a BSP-binding protein by immunoprecipitation, Western blotting, and immunoassay. Factor H, a molecular mass 150-kDa protein, is a key regulatory braking mechanism in normal and alternate complement-mediated cell lysis. It dissociates and thereby inactivates the assembled C3 convertase, serves as an essential accelerator of Factor I-mediated cleavage of C3b to iC3b, and sterically inhibits C5 binding to C3b (a prerequisite step for terminal pathway activation). The salient structural features of Factor H include 20 short consensus repeats that contain four cysteine residues forming two disulfide bonds per repeat. In addition, each short consensus repeat contains one conserved tryptophan residue per repeat and Factor H is known to interact with several sialic acid-containing proteins. BSP lacks any tryptophan, OPN has one, while Factor H contains a total of 25 tryptophan residues. Thus, the binding interaction be-
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Fig. 3. Identification of complement Factor H as the BSP serum-binding protein. A, fractions from SEC analysis of unreduced normal human serum were resolved by 4–20% acrylamide gradient gel electrophoresis, transferred to nitrocellulose, and probed with a monoclonal antibody against human complement Factor H. B, immunoreactive bands were visualized by chemiluminescent detection.

Fig. 4. Reconstitution of the BSP-Factor H complex. rBSP was isolated, biotinylated, and, after the indicated incubations, was subjected to SEC analysis. The incubations included: A, biotinylated rBSP alone; B, normal human serum (NHS) alone; C and D, NHS + biotinylated rBSP; E, biotinylated rBSP + purified complement Factor H; and F, biotinylated rBSP + purified complement Factor H treated with heating and reduction (DTT, dithiothreitol). Immunoreactive material was detected by either avidin-horseradish peroxidase (A, C, and E) or by direct ELISA for BSP (B, D, and F).

tween BSP or OPN and Factor H can be readily studied by intrinsic steady state fluorescence.

Titrination of purified human complement Factor H with rBSP or rOPN was followed by excitation at 295 nm and monitoring emission between 300 and 450 nm. The emission profile of Factor H alone yields a peak at 347 nm (Fig. 5). The addition of rBSP or rOPN in nanomolar increments causes a relative fluorescent intensity quenching. Conversion of the fluorescent intensity titration into a binding curve by determining the fraction of binding sites occupied as the fractional change in fluorescence quenching at 347 nm yields a saturable binding curve (Fig. 5C). By steady state fluorescence, the binding of BSP and OPN by Factor H are saturable and possess a 1:1 stoichiometry, have binding constants in the nanomolar range, given the serum concentration of Factor H (~0.5 mg/ml), virtually all BSP and OPN in serum will be complexed with Factor H.

BSP and OPN Protect Tumor Cells from Alternate Complement-mediated Cell Lysis—Besides its key role in regulating complements and alternate complement activity, Factor H also appears to play a role when “hijacked” by invading organisms in enabling cellular evasion of complement. Pathogens such as Streptococcus pyogenes (32, 33), Neisseria gonorrhoeae (34–36), and Echinococcus granulosus (37, 38) bind Factor H to their cell surface and are resistant to complement-mediated cell lysis. In addition, molecular mimicry of Factor H where a pathogen makes a protein that is similar in sequence to Factor H to defend against attack by the host complement system has been described in vaccinia virus (39, 40), herpes simplex virus (41), and Trypanosoma cruzi (42–44). Within this context, it is interesting that Staphylococcus aureus isolated from patients with osteomyelitis has surface bound BSP (45). We have investigated whether BSP and OPN may play a similar role in tumor cell complement evasion by testing to see whether these small integrin-binding glycoproteins could promote tumor cell survival. Having identified the serum-binding component for BSP and OPN and bearing in mind the role of hijacked Factor H in pathogenic resistance to humoral surveillance, the ability of BSP and OPN to protect cells from complement activity was investigated. The first model system employed was a MEL cell line which when incubated with normal human serum can be readily
assayed for ACP-mediated cell lysis (46). Cell survival was measured by both trypan blue dye exclusion and thiazolyl blue (MTT) reduction by living mitochondria. Titration with dilutions of normal human serum and time courses were carried out to define optimal incubation conditions. At 1:10 dilution, human serum totally lysed the MEL cells as measured by both assay systems. The addition of purified recombinant BSP to MEL cells followed by normal human serum completely protected the cells from complement-mediated lysis (Fig. 6). Treatment of MEL cells with recombinant OPN also conferred protection from complement-mediated lysis. For BSP, this is consistent with im-

The mechanism of protection from complement-mediated lysis was investigated. Preincubation of MEL cells with rBSP whose RGD sequence had been mutated to KAE (10 μg/ml), and either GRGDS peptide (400 μM) followed by rBSP or an α3β1 antibody (1:4000) followed by rBSP for 10 min prior to the addition of normal human serum. The cells were then incubated for 10 min after which cell viability determined using the MTT assay. B, a cohort of MEL cells were pretreated with rOPN (10 μg/ml) alone, GRGDS peptide followed by rOPN, the α3β1 antibody followed by rOPN, or an anti-CD44 antibody (Chemicon, Co.) followed by rOPN, or hyaluronan followed by rOPN. Cells were then treated with normal human serum and viability asayed as described in the legend to Fig. 6. Treatment of MEL cells with a pre-formed complex of either BSP-Factor H (BSP+F) or OPN-Factor H (OPN+F) abolished the protection from complement-mediated lysis. Percent cell viability was determined using the absorbance values of various conditions and a control where no serum had been added (100% viable). The cross-hatched region represents that range of values observed when normal human serum (1:10) alone was added (maximal cell death). The data represents the mean and S.E. for three separate experiments. Statistical significance was determined by analysis of variance. Error bars represent the S.E. of the mean. n = number of experiments combined; **, p ≤ 0.01; ***, p ≤ 0.001.

attachment, homing, and aggregation of lymphocytes as well as neoplastic cells. Pretreatment of MEL cells with hyaluronan, a natural ligand for CD44 (48), as well as with an anti-CD44 antibody also reduced the protective effect of added rOPN (Fig. 7). Treatment of MEL cells with a pre-formed complex of either rBSP-Factor H or rOPN-Factor H abolish the protection from complement-mediated lysis. For BSP, this is consistent with immunoprecipitation data that indicates that the RGD moiety is inaccessible in the solution phase Factor H complex. These data suggest that both of these proteins may be entirely masked by Factor H shortly after being secreted by a cell.

To verify that this protective effect of BSP and OPN might be operative in human cancer cells, MCF-7 breast cancer cells selected for nonadherent growth and U-266 myeloma cells were used in the alternate complement pathway-mediated cell lysis assay with guinea pig serum as the source of complement activity. Both cell types exhibited enhanced survival and protection from complement-mediated cell lysis when rBSP or rOPN were present (Fig. 8). Increasing concentration of guinea pig serum lead to decreasing cell viability, while the pretreatment with either 10 μg/ml rBSP or rOPN resulted in increased cell viability.

**DISCUSSION**

The survival of trophoblasts and neoplasms requires resistance to immunologic recognition and subsequent attack by host. Host surveillance pathways include B and T cell lymphocytes and macrophages in immune response as well as complement-mediated attack and lysis. Immune transparency is aided
and abetted in trophoblasts by the lack of expression of major histocompatibility complex antigens which present peptides to CD8+ cytotoxic T cells (49); the expression of nonclassical, nonpolymorphic HLA-G which inhibits natural killer cells (50, 51); the dominance of the type 2 T-helper cells over type 1 T-helper cells (52); the immunosuppressive effect of induced prostaglandin E2 (53); and induction of apoptosis in Fas-bearing activated lymphocytes by placental expression of Fas ligand (54, 55). Similarly, for neoplasms, continued subversion of host surveillance may involve a deficiency or lack of expression of major histocompatibility complex antigens (56, 57); dysregulation between HLA class I antigen expression and natural killer cell activity (58); an expansion in type 2 T-helper cells and a malfunction in type 1 T-helper cells (59, 60); enhanced production of prostaglandin E2 (61, 62); and neoplastic expression of Fas-ligand (63, 64). However, host surveillance mechanisms also include the complement system (65).

The complement system found in the blood of mammals is composed of about 26 proteins that combine with antibodies or cell surfaces as part of host humoral surveillance. Complement plays a role in inflammation, immune adherence, opsonization, viral neutralization, cell lysis, and localization of antigen (66). It is also possible that the expression of these cancer-associated proteins, any other functions that these proteins may serve will likely also be extremely limited in their functional ranges.

The induction of specific genes not usually expressed by a given differentiated cell type is one of the hallmarks of neoplastic transformation. The switching on of these genes can be part of a generalized alteration in phenotypic expression where the de novo production of the gene product is not a necessary part of the neoplastic process (there is no gain of function by its expression). It is also possible that the expression of these cancer-associated genes confers a gain of function or selective survival advantage to the neoplasm. Using MEL cells as well as human breast cancer and myeloma cells and recombinant BSP and OPN, we have found that, following interaction with a specific receptors on the cell surface, BSP and OPN sequester Factor H to the cell surface and that this interaction quenches alternate complement-mediated cell lysis by normal human serum. These results suggest a shared mechanism between trophoblasts and neoplasms for evading host surveillance through “molecular cloaking” via Factor H sequestration and dampening of the complement-mediated cell lysis and opsonization.
