Cell-surface changes associated with transformation of human hepatocytes to the malignant phenotype


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ABSTRACT

Hepatocellular carcinoma is one of the leading causes of cancer death in the world. To understand the cellular changes associated with transformation of hepatocytes to the malignant state, we have made several libraries of monoclonal antibodies against the hepatocellular carcinoma cell line FOCUS and have found six antibodies (AF-20, SF-25, SF-31, SF-90, XF-4, and XF-8) that recognize antigens expressed at consistently higher levels on hepatoma cells. We have studied malignant and nontransformed liver tissue from the same individual by using direct 125I-labeled antibody binding and immunoperoxidase staining techniques. For each of these antibodies, we found striking increases in antigen expression on the transformed tissues. These antigens were found to be expressed throughout the tumor and on distant metastases, with little, if any, expression on the nontransformed adjacent liver. These antibodies demonstrate that hepatic transformation may be accompanied by stereotyped and predictable antigenic changes. The uniformity of such antigenic changes suggests an association between these cell-surface alterations and the malignant transformation process.

Epidemiological, serological, and molecular biological evidence links chronic hepatitis B virus infection to acquisition of human hepatocellular carcinoma (HCC). Since there are high chronic carrier rates of hepatitis B virus in many parts of the world, especially in Asia and Africa, HCC is one of most common human malignancies (1). Despite the preponderance of HCC, progress in elucidating the mechanisms of malignant hepatocyte transformation at either the cellular or molecular level has been slow.

One method of studying the transformed phenotype is to search for and characterize alterations in the antigenic composition of the cell surface. Thus, we created a large library of antibodies reactive against a HCC cell line. We then selected antibodies that preferentially bind HCC-derived cells for use in a systematic analysis of antigenic variation in vivo between malignant and normal human liver tissue. To control for antigenic variation between individuals, paired sets of HCC and adjacent nontransformed liver tissue from South African hepatoma patients were employed. By using immunoperoxidase staining and a radioimmunoassay that measures binding of monoclonal antibodies to antigens on cell membranes, we studied the levels of antigen expression on these paired sets of tissue. Some of these antigens were then further characterized by immunoblotting, metabolic labeling, and immunoprecipitation.

MATERIALS AND METHODS

Tissues and Cell Lines. Mycoplasma-free FOCUS, PLC/PRF/5, SK Hep-1, Mahlauv, Hep G2, Hep 3B, and Vero cells were harvested from monolayer cultures by washing with isotonic phosphate-buffered saline (PBS) followed by treatment with EDTA in the absence of trypsin. Leukocytes (WBC) were isolated from fresh human blood by Ficoll/Hypaque centrifugation. Cells used in the binding assays were frozen in 15% (vol/vol) glycerol and stored at –80°C. Hepatoma and adjacent nontransformed liver tissues were obtained from surgically resected or autopsy specimens from South Africa, frozen in liquid nitrogen, and stored at –80°C.

Monoclonal Antibodies Production. Immunizing cells were grown from the second passage of the FOCUS cell line developed in our laboratory and were harvested with EDTA. Six-week-old BALB/c mice were immunized by intraperitoneal injection of 4.0 × 10⁶ FOCUS cells emulsified in 50% (vol/vol) complete Freund’s adjuvant, followed 6–10 weeks later by intravenous injection of 4.0 × 10⁶ cells in 0.2 ml of PBS. Three days later, mouse spleen cells were fused with either SP2/O, NS1, or X63 mouse myeloma cells with 30% (wt/vol) polyethylene glycol. Hybridomas were selected and maintained as described (2). Screening for antibodies was performed by the indirect binding assay described below, and antibody-producing colonies were cloned twice by limiting dilution. Ascites fluid was prepared by injection of hybridoma cells intraperitoneally into 2,6,10,14-tetramethylpentadecane (pristane; Aldrich)-primed BALB/c mice. Purification and iodination of monoclonal antibodies were performed as described (2).

Preparation of Membrane Homogenates. Homogenization was performed at 4°C. Sections (0.5–1.0 g) of frozen hepatoma and remote nontransformed liver tissue were homogenized with a Polytron (Brinkmann) in 10 ml of PBS (pH 7.2) containing 0.1% NaN₃ and centrifuged at 20,000 × g for 30 min. The membrane pellet was homogenized in 10 ml of PBS/NaN₃, centrifuged as above, then resuspended in 10 ml of PBS/NaN₃ with 20% (vol/vol) glycerol, and centrifuged at 1000 × g for 5 min. The fibrous layer was discarded, and the protein concentration of the remaining membrane homogenate was determined by the method of Lowry et al. (3). The membranes were frozen in liquid nitrogen and then stored at –80°C.

Assays. Filter-bottomed 96-well plates (V & P Scientific, San Diego, CA) were preincubated for 15 min with 250 μl of calf serum in each well. With filtration, 50 μg of membrane homogenate protein or 10⁵ cells were trapped in each filter. Indirect assays were performed by incubating each filter in

Abbreviations: HCC, hepatocellular carcinoma; WBC, leukocytes.

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100 µl of monoclonal antibody solution (as a hybridoma supernatant or as a 1:200 dilution of ascites fluid) for 1 hr. The filters were washed with 10% (vol/vol) calf serum in PBS, then incubated in 100 ml of calf serum/PBS, 1:1 (vol/vol), containing 10⁵ cpm of ¹²⁵I-labeled sheep anti-mouse immunoglobulin F(ab)₂. After 1 hr, the filters were washed, and radioactivity was measured as a γ counter. A direct binding assay was performed by preincubating the cells or membranes for 15 min with a 1:100 dilution of either unlabeled relevant monoclonal antibody or unlabeled non-relevant antibody (B2TT, an anti-tetanus toxoid IgG). ¹²⁵I-labeled monoclonal antibody (10⁵ cpm) diluted in calf serum/PBS, 1:1 (vol/vol), was then added. After 1 hr, the filters were washed and counted.

**Statistical Methods.** For indirect assays, the ratio of relevant to nonrelevant monoclonal antibody binding was termed the signal/noise ratio. Direct assays were done in quadruplicate. Here, the specific binding was determined by subtracting radiolabeled antibody binding in the presence of unlabeled antibody (nonspecific binding) from the binding in the presence of a nonrelevant antibody (total binding). The standard deviations reported represent the sum of the standard deviations of the two binding values. P values were calculated using Student’s t test to compare the significance of differences in mean binding values between tissues.

**Immunoperoxidase Staining.** Frozen tissue specimens embedded in OCT compound (Miles Scientific, Naperville, IL) were sectioned, fixed to slides with –20°C acetone, and equilibrated in PBS. The slides were stained with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Briefly, the slides were incubated in dilute horse serum for 20 min dried by blotting, then incubated overnight at 4°C with monoclonal antibody (1:100–1:1000 dilution of ascites fluid in PBS), and washed with PBS. The slides were sequentially incubated in biotinylated anti-mouse IgG (1 hr), 0.3% H₂O₂ in methanol (20 min), and avidin-peroxidase complex (1 hr), with PBS washings between each step. The slides were incubated in 0.1 M Tris phosphate buffer (pH 7.5) containing 0.005% H₂O₂ and 3,3’-diaminobenzidine (0.5 mg/ml) until the positive control slide showed evidence of reaction. After washing, the slides were counterstained with methyl green or hematoxilin.

**Immunoblotting.** Tissue samples were homogenized with a Potter–Elvehjem homogenizer in ice-cold 0.1 M Tris-HCl buffer containing 0.1 M NaCl and 0.1% aprotinin. Nonidet P-40 was added to 0.5% and incubated for 15 min. The lysate was centrifuged for 10 min at 10,000 × g. Washed cells were lysed directly in the Nonidet P-40 solution, then centrifuged for 15 min at 1500 × g. The supernatants were incubated at 95°C for 5 min in 0.025 M Tris-HCl buffer containing 0.1% Na₂D₂SO₄ and 20% (vol/vol) glycerol, then loaded onto 10% NaDdSO₄/polyacrylamide gels and separated as described by Laemmli (4). The protein was electrophoretically transferred overnight onto nitrocellulose sheets at 50 V in 0.025 M Tris containing 0.192 M glycine and 20% (vol/vol) methanol. The nitrocellulose sheets were incubated 1 hr in Tris-HCl-buffered saline (TBS) containing 1% bovine serum albumin, 1% polyvinylpyrrolidone, and 1% nonfat milk, then incubated another 2–3 hr in the same solution with a 1:100 dilution of antibody ascites fluid and 0.05% Tween 20 added. The sheets were washed with TBS containing 0.05% Tween 20 and incubated with ¹²⁵I-labeled sheep anti-mouse immunoglobulin F(ab)₂ at 10⁶ cpm/ml for 2 hr. The sheets were washed, and autoradiographs were made.

**Metabolic Labeling of FOCUS Cells.** Tunicamycin treatment of cell lines. Preliminary experiments suggested that maximal inhibition of N-glycosylation with minimal inhibition of protein synthesis occurred by using tunicamycin at 5 µg/ml. Subconfluent FOCUS cells were starved overnight in methionine-free culture medium containing 10% (vol/vol) dialyzed fetal calf serum. The cells were incubated for 1 hr with tunicamycin at 5 µg/ml (Sigma) and subsequently metabolically pulse-labeled by incubating for 1 hr with [³⁵S]methionine at 20 µCi/ml (Amersham; specific activity, >800 Ci/mmol; 1 Ci = 37 GBq). Cells were then harvested, washed twice with PBS, and stored at –80°C. [³⁵H]Glucosamine labeling. Confluent cells were incubated with 500 µCi of [³⁵H]glucosamine (Amersham; specific activity, 25 Ci/mmol) for 18 hr at 37°C. The cells were then harvested, washed twice with PBS, and stored at –80°C.

**Cell surface ¹²⁵I-labeling.** Cells were incubated for 5–10 min in an EDTA buffer without proteolytic enzymes, and 1–3 × 10⁷ cells were recovered from monolayer culture dishes. The cell suspension was labeled with 1 mCi of Na¹²⁵I in the presence of 40 µg of lactoperoxidase (Sigma) as described (5).

**RESULTS**

**Binding Studies.** We generated 140 hybridomas producing antibodies reactive to FOCUS cells. The FOCUS cell line was chosen because it appears to be a good model for studying hepatitis B virus-associated HCC on the basis of enzymatic and protein synthetic capabilities, solid tumor morphology, and the presence of a single hepatitis B virus integration site (8, 9). For this study we produced monoclonal antibodies against cells grown from the second passage of the FOCUS cell line to represent the antigenicity of the original primary HCC tumor as faithfully as possible.

Seven monoclonal antibodies (AF-20, SF-25, SF-31, SF-58, SF-90, XF-4, and XF-8) were selected on the basis of their binding specificities. The signal/noise ratios for indirect binding of these antibodies to six HCC-derived cell lines, to the Vero cell line, and to normal peripheral WBC are shown in Fig. 1. Four antibodies (AF-20, SF-25, SF-31, and SF-90) bound well to all six HCC cell lines, whereas XF-4 bound to four out of six, and XF-8 bound to four out of five (the sixth HCC cell line was not tested). In contrast to these high rates of binding to HCC cell lines, SF-58 bound to only two of the six HCC cell lines. Antibodies SF-31 and SF-90 bound weakly to Vero and insignificantly to WBCs (signal/noise ratio < 2.5). None of the other antibodies bound to either Vero or WBCs. Thus, with the exception of SF-58, these antibodies bound most or all of the HCC cell lines with minimal or no binding to two nonmalignant cell types.

Competitive inhibition of radiolabeled antibody binding with unlabeled antibody for blocking showed that for all seven antibodies, >90% of the observed binding was specific and could be inhibited. An example of this competitive inhibition for AF-20 is shown in Fig. 2. Other competitive inhibition studies have shown that either SF-31 or SF-90 could completely inhibit binding of the other, suggesting that these two antibodies may recognize the same or adjacent epitopes. The binding of the remaining five antibodies was not blocked by the other antibodies, suggesting that each appears to define a separate membrane-associated epitope (data not shown).

An immunoblot was developed to measure radiolabeled monoclonal antibody binding to membrane preparations derived from hepatoma tissue and adjacent uninvolved liver
Fig. 1. Indirect binding of antibodies to six HCC-derived cell lines, to green monkey kidney cells (Vero), and to normal WBC. Significant binding (----) is at a signal/noise (S/N) ratio >2.5. *, Not tested.

from six patients. Protein concentrations were standardized, and specific binding was determined by subtracting the nonspecific binding (i.e., residual binding in the presence of excess inhibiting unlabeled antibody) from the total binding (i.e., binding in the presence of excess noninhibiting cold antibody). Fig. 3 is a summary of the antibodies binding to all six HCC/nontransformed liver sets. AF-20, SF-31, SF-90, XF-4, and XF-8 had a striking preference for binding to HCC with significant increases in antigen expression on HCC tissue compared to adjacent nontransformed liver tissue. Fig. 4 presents the actual data for binding of AF-20 to the six pairs of tissue, showing that there was a high level of antigen expression on the transformed tissue. In contrast, SF-58 showed an entirely different binding pattern (Fig. 3) with a strong preference for an epitope found on normal liver tissue. With this antigenic determinant, malignant transformation was consistently associated with a decrease in expression. The SF-25 antigen was too labile to adequately demonstrate its HCC selectivity by this method and was studied primarily by immunoperoxidase staining (see below).

Immunohistochemical Staining. Immunoperoxidase studies on as many as 14 paired HCC/nontransformed liver sets confirmed and expanded on the results of the membrane binding assays by showing significant staining of HCC tissue with no staining of adjacent nontransformed liver for all the antibodies except SF-58. Fig. 5 depicts representative staining patterns for a few of these antibodies. Notice the uniform cellular staining suggesting that most transformed cells, including those that are metastatic, express these antigens. Antibody SF-58 was not found to stain HCC. The SF-31, SF-90, XF-4, and XF-8 antigens were stable after formalde-
hydroxide fixation and paraffin embedding. In contrast, the SF-25 and AF-20 antigens were only identifiable in fresh frozen sections of HCC, suggesting that these antigens were labile.

Partial Characterization of FOCUS Antigens. Immunoblots showed that SF-31 and SF-90 recognized the same 40-kDa and 50-kDa protein antigens, thus confirming results of the binding study that suggest these two antibodies recognized the same or closely related epitopes (Fig. 6). As shown in Fig. 7, AF-20 recognized three similar antigens of 116–120 kDa. The middle-sized antigen was highly glycosylated as shown by the \textsuperscript{3}Hglucosamine and tunicamycin experiments. Furthermore, this antigen appeared to exist on the cell surface as demonstrated by \textsuperscript{125}I-labeled protein immunoprecipitation studies. Preliminary immunoprecipitation experiments with SF-25 showed that this antibody recognized a 125-kDa protein on FOCUS cell membranes (10). Glycosylation patterns, competitive inhibition experiments, and specifici-
ties of binding have shown the SF-25 and AF-20 antigens to be unrelated (data not shown). The antigens recognized by the XF-4, XF-8, and SF-58 monoclonal antibodies were not detectable by immunoblotting and have yet to be identified and characterized with respect to their molecular properties.

**DISCUSSION**

Due to the recognized importance of cell surface molecules in regulating cell growth, replication, differentiation, and responsiveness to exogenous information and control (11, 12), several studies of hepatic transformation have centered on generating monoclonal antibodies that recognize antigens present on HCC-derived cell lines. Furthermore, since cell-surface antigenic variations may be manifestations of and thus markers for gene activation or inactivation, it is hoped that such an approach will eventually produce the tools necessary to elucidate some of the genetic changes associated with malignant transformation. This approach has led to the development of several antibodies that recognize HCC-associated antigens (5, 13–16).

The antibodies presented here appear to differ from the reported HCC-selective monoclonal antibodies. Binding specificities, molecular weights, and competitive inhibition experiments (data not shown) have demonstrated that three antibodies described by this laboratory (P215457, PM4E9917, and P232524) do not recognize the same antigens (5, 13). Two other antibodies (K-PLC2 and K-PLC3) bind only to the PLC/PRF/5 cell line and probably also recognize dissimilar antigens (14). Of the remaining three antibodies, 699B-C3 recognizes a 90-kDa membrane protein present on several cell lines (15), and 833-1C4 and K-PLC1, respectively, recognize phospholipid and 115-kDa glycoprotein antigens present on HCC tissue and cell lines but not on normal liver (14, 16). Insufficient specificity data have been reported to permit definitive comparisons between these antibodies and the ones reported here.

The observation that some of these antibodies bound antigens present on HCC tissue and cell lines without binding to normal human liver suggested to us that hepatic transformation is associated with significant antigenic changes. However, these studies were performed on few HCC and normal liver samples and made no attempt to control for variation in antigen expression between individuals or to quantitate the antigenic changes observed. This study was designed to explore both of these aims. In particular, use of malignant and nontransformed liver tissue from individual patients was employed in an attempt to minimize nonantigenic influences on binding activity and thus emphasize those antigenic variations that may be indicative of intrinsic cellular changes. Furthermore, each HCC/nontransformed liver set was assayed against all seven antibodies reported here, thus allowing us to demonstrate that a given pattern of antigen changes found in one HCC/nontransformed liver set may be stereotypically found in other HCC/nontransformed liver tissues.

Indeed, these seven antibodies demonstrate a consistent pattern of antigen alteration. Six antibodies (SF-20, SF-25, SF-31, SF-90, XF-4, and XF-8) recognize antigens that are present on HCC cell lines and on HCC tissues in vivo but are only weakly, if at all, expressed on the adjacent nontransformed liver. Immunoperoxidase staining showed that these antigens were expressed on most, if not all, cells within a given HCC tumor (Fig. 5). The strong association between malignant transformation and the high level of expression of these antigens suggests that they may play an important role on the surface of hepatoma cells. In contrast, SF-58 recognizes an antigen that shows consistent decreases in expression on the transformed tissue with high levels of expression on normal liver tissue.

Studies showing antibody binding to live cells demonstrated that these antibodies recognize cell surface epitopes (Fig. 1). SF-31 and SF-90 recognize the same two 40-kDa and 50-kDa proteins and can competitively inhibit the binding of the other. AF-20 recognized three closely similar antigens of 116–120 kDa, whereas SF-25 recognized a 125-kDa protein (10). The AF-20 and SF-25 antigens are labile and subject to inactivation by mild fixation, denaturation, and detergents. The SF-58, XF-4, and XF-8 antigens have not yet been molecularly characterized.

Thereof, we have identified six HCC-selective antibodies and one normal liver-selective antibody. Of general interest, these seven antibodies demonstrate that human hepatic transformation is accompanied by significant and measurable antigenic changes. Furthermore, alterations in the hepatocyte antigenic composition are predictable and found consistently in all paired comparisons of transformed and adjacent nontransformed tissue studied to date. Such uniformity in antigenic change emphasizes that there is a strong association of these antigens with the cellular transformation process. Further characterization of these antibodies and attempts to identify others will permit a definition of cell surface changes associated with the malignant phenotype.

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