Cryptic Epitopes on α-Fetoprotein Induce Spontaneous Immune Responses in Hepatocellular Carcinoma, Liver Cirrhosis, and Chronic Hepatitis Patients

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Abstract

To determine α-fetoprotein (AFP) immunogenicity in vitro, the presence of antibodies in sera of 60 hepatocellular carcinoma, 15 liver cirrhosis, and 15 chronic hepatitis patients was evaluated by Western blotting and immunoprecipitation analyses using purified human AFP. High titers of anti-AFP immunoglobulins were detected in 14 hepatocellular carcinomas (P = 0.0006), 3 liver cirrhosis (P = 0.0173), and 1 chronic hepatitis patient, but they were not detected in 40 healthy individuals. Therefore, spontaneous immune responses to AFP are significantly associated to liver diseases (P = 0.0015). Patient immunoglobulins recognized proteic linear epitopes that were cryptic in the native protein, as demonstrated by their restricted reactivity with denatured deglycosylated AFP. Thus, in pathological liver conditions, tolerance to this self-molecule is circumvented. The identification of AFP immunogenic epitopes may contribute to defining novel immunotherapeutic strategies targeting this antigen.

Introduction

AFP, a well-characterized oncofetal antigen structurally related to Hu-SA, is normally expressed during embryogenesis and present only in trace amounts in normal adults (1). AFP appears to function as an osmotic and carrier protein in the fetus the same way that albumin does in adults and to regulate the immune system by playing immunosuppressive functions, such as preventing immunological attacks to the embryo by the maternal immune system (1). AFP has been detected at high levels in sera and tumor tissues of cancer patients (1). In vitro and in vivo treatments with anti-AFP-labeled antibodies have shown a reduction in the growth rate of tumor cells expressing AFP (2, 3). Moreover, radiolabeled antibodies to AFP were used in patients for the detection and localization of HCCs and germ cell carcinomas (4). These observations suggest that AFP may be a useful target in tumor diagnosis and immunotherapy. Accumulating evidence suggests that tolerance to self-proteins and tumor-associated antigens can be spontaneously circumvented in cancer patients (5–9). This evidence prompted us to determine anti-AFP antibodies in HCC, LC, and CH patient sera as well as in normal individuals. The characterization of naturally occurring immune responses to AFP in liver disease patients is an essential step toward the identification of AFP epitopes becoming immunogenic under disease conditions and may bear relevance for immunotherapeutic strategies targeting this antigen.

Materials and Methods

Patient Sera. Sera samples were collected with informed consent from 60 HCC, 15 LC, and 15 CH patients and from 40 apparently healthy donors. Sera were obtained prior to therapy and kept at −20°C until use. HCCs were diagnosed by ultrasound-assisted fine needle biopsies. Sixteen patients with ultrasound evidence of HCC and high AFP did not receive needle biopsy because of C-Child Pugh stage. HCCs were classified into three tumor morphologies: uninodeular (33), multinodeular (7) and massive (20). LC and CH were diagnosed on the basis of histological, clinical, and laboratory findings. The characteristics of patients, including age, etiology, Child-Pugh stage, Clip scores (10), and mean serum levels of AFP are reported in Table 1. Statistical association was evaluated by Fisher’s exact test. P < 0.05 were considered significant.

Purified Antigen and Antibodies. AFP and Hu-SA were purchased from ICN Pharm. Incorporated (Aurora, OH), and purified carcinoembryonic antigen was purchased from Vitro Diagnostic (Littleton, CO). The purity of commercial antigens were analyzed by SDS-PAGE. Mouse antihuman AFP mAb C3, goat antiserum antihuman AFP, and rabbit antiserum to Hu-SA were purchased from ICN Pharmaceuticals Incorporated. Ascitic fluid of mAb D612 (11) and normal rabbit serum and goat antiserum to CD69 (Santa Cruz Biotechnology Incorporated, Santa Cruz, CA) served as negative controls. Enzymatic deglycosylation of AFP was carried out using PNGase F (New England Biolabs, Beverly, MA) as previously described (12).

Serum AFP was determined by a chemiluminescence immunoassay (Immulite Medical System) according to the suggested protocol.

Western Blotting and Immunoprecipitation. Purified proteins (0.5–2.5 μg/lane) were separated by 8% SDS-PAGE or non-denaturing discontinuous Tris-glycine PAGE. Protein for non-denaturing conditions were loaded in a sample buffer devoid of SDS and 2-mercaptoethanol. Following electrophoresis, the proteins were transferred to a nitrocellulose membrane that was blocked overnight with 5% BSA and subsequently incubated overnight at 4°C with either human sera (diluted 1:25–1:250 –1:2500 in wash solution containing 2% BSA), with mAbs C3 and D612 (ascitic fluids at 1:2000), or with antihu-Hu polyclonal antibody or normal rabbit serum at 1:2000 dilutions. After washing, the membranes were incubated with alkaline-phosphatase-conjugated goat antihuman IgG and IgM, goat antimouse or antirabbit antiserum (Life Technologies, Inc., Gaithersburg, MD). Bound antibody was visualized as previously described (7). For immunoprecipitation, goat anti-AFP antiserum (1.5 μl), patient sera (3 μl), and 20 μl of protein G-Sepharose were reacted with 0.5 μg of n-AFP or d-AFP for 2 h at 4°C. d-AFP was diluted in PBS to avoid the effect of 2-mercaptoethanol on immunoglobulins. Protein G-Sepharose complexes containing immunoprecipitated n-AFP or d-AFP were then run on SDS-PAGE (7). mAb C3 was used for immunoblotting.

Because of the lack of standard solutions containing anti-AFP human antibodies, only relative antibody titers could be obtained by Western blotting using patient serum at 1:25, 1:250, and 1:2500 dilutions. The intensity of the specific band was expressed as densitometric unit(s) (D.U) and was obtained using the NIH Pro-Image 1.5 software after blots scanning by a UMAX VISTA SUPER SPEEDY scanner.

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3 AFP, α-fetoprotein; HCC, hepatocellular carcinoma; LC, liver cirrhosis; CH, chronic hepatitis; Hu-SA, human serum albumin; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; n-AFP, native AFP, d-AFP, denatured AFP, D.U, densitometric unit(s); DG-AFP, deglycosylated AFP, Clip, Cancer of the Liver Italian Program.
Results

Detection and Serological Characterization of Human Anti-AFP Immunoglobulins in Serum of Patients with Liver Diseases.

Sera from 60 HCC, 15 LC, and 15 CH patients and sera from 40 apparently healthy donors were analyzed for immunoreactivity with purified AFP. Patient clinicopathological characteristics are reported in Table 1. Initially, all sera were tested by enzyme-linked immunosorbent assay and Western blotting using different serum dilutions and amounts of AFP. Western blotting analysis was chosen to visualize specific detection and to minimize false-positive reactivity detected by enzyme-linked immunosorbent assay due to nonspecific sera absorption of low serum dilutions. To determine patient immunoglobulin reactivity with linear and/or conformational AFP epitopes, Western blotting was performed in antigen denaturing and non-denaturing conditions. Fig. 1A illustrates the reactivity of patient antibodies with n-AFP and d-AFP. Positive reactivity for patient sera comprised the appearance of an immunoreactive band comigrating with that visualized by the anti-AFP mAb C3. Noticeably, none of the patient sera or normal sera reacted with the n-AFP (Fig. 1A). The lack of reactivity with increasing concentrations of n-AFP (up to 2.5 μg/lane) confirmed the inability of serum immunoglobulins to detect the native protein and ruled out the dose-dependency of immunoreactivity. Spec-

Table 1 Clinicopathological characteristics of patients

<table>
<thead>
<tr>
<th>Disease (No. of patients)</th>
<th>Age mean (range)</th>
<th>Etiology</th>
<th>Child-Pugh stage</th>
<th>Clip scores</th>
<th>AFP levels (ng/dl) mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocellular carcinomas (60)</td>
<td>65.5 (48–79)</td>
<td>HCV* HBV Alcohol</td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Liver cirrhosis (15)</td>
<td>62.6 (48–79)</td>
<td>12</td>
<td>1</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Chronic hepatitis (15)</td>
<td>50.3 (33–64)</td>
<td>14</td>
<td>1</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

* HCV, hepatitis C virus; HBV, hepatitis B virus.

b Five patients had both HCV and alcohol; in four patients, the etiology was undetermined.

Fig. 1. Detection and characterization of antibodies to AFP cryptic epitopes in patient sera. Western blotting (A) and immunoprecipitation (B) analyses using n-AFP and d-AFP were performed using HCC (P.1, P.4, P.5, P.6, and P.7) and LC (P.8 and P.9) patient sera, mAb C3 and the rabbit polyclonal anti-AFP as positive controls, mAb D612 and the anti-CD69 as negative controls, and normal donor serum (N) as indicated. Western blotting analysis (C) of DG-AFP and d-AFP using HCC (P.3, LC (P.10), and CH (P.11) patient sera and normal donor serum (N) was performed as indicated. Size is given in kilodaltons.
specific antibodies for d-AFP were detected in 14 of 60 HCC (23.3%), 3 of 15 LC (20%), and 1 of 15 CH (6.6%) patient sera, whereas none of the sera from 40 normal individuals was immunoreactive (Table 2).

To confirm the restricted recognition of d-AFP, all sera were analyzed for their ability to immunoprecipitate n-AFP and d-AFP (Fig. 1B). Goat anti-AFP antiserum was used as a positive control to immunoprecipitate n-AFP and d-AFP and then was detected in Western blotting by mAb C3. None of the patient sera was capable of immunoprecipitate n-AFP and d-AFP and then was detected in Western blotting by mAb C3. None of the patient sera was capable of immunoprecipitating the AFP in its native conformation, whereas the immunoprecipitation of d-AFP was confirmed for all Western blotting-positive sera (Fig. 1B and Table 2). In addition, the specific recognition of AFP linear epitopes was demonstrated by the lack of reactivity with the structurally related human serum albumin (d-Hu-SA) and with purified carcinoembryonic antigens in denaturing conditions (data not shown).

Although specific antibody titers varied, detectable anti-d-AFP antibodies were observed up to a 1:250 dilution in all positive patients and up to 1:2,500 serum dilution in one HCC patient (patient 5, P.5). Overall, the highest antibody titers were seen in 3 HCC (range, 139–135 D.U at 1:25) and 1 LC (111 D.U at 1:25) patient sera. Specific anti-AFP immunoglobulins of the IgG class were detectable in all positive sera, suggesting T-helper cell involvement in the anti-AFP antibody response (13). Moreover, IgM-specific anti-AFP immunoglobulins were detected in the sera of one HCC and one LC patient, indicating more recent immunostimulatory events in these patients (Table 2).

To investigate whether the anti-d-AFP antibodies recognized proteic or carbohydrate epitopes, AFP was deglycosylated with PNGase F to remove N-linked carbohydrate moieties. Fig. 1C shows representative sera reactivity with DG-AFP. As shown in Table 2, all positive patient sera reacted with DG-AFP.

These results demonstrate that liver disease patients spontaneously develop antibodies to AFP. The specific reactivity with denatured and DG-AFP indicates that patient immunoglobulins recognize exclusively structural determinants not accessible on n-AFP and located on the proteic backbone of the antigen.

Correlation between the Presence of AFP Antibodies and Clinicopathological Variables. Spontaneous immune responses to AFP were found to correlate with the level of liver diseases. The highest percentage of anti-AFP antibodies was detected in HCC (23.3%; \( P = 0.0006 \)) and LC (20%; \( P = 0.0173 \)) patients (Table 2). Correlation with the clinicopathological parameters reported in Table 1 was also determined. No significant association between the presence of anti-AFP antibodies and age, sex, Child-Pugh, or Clip scores was found. Anti-AFP immunoglobulins were detected both in early (Clip scores 0–2) and late stages of disease (Clip scores 3–5), with a higher percentage of antibody-positive early stages (31% versus 12%).

HCC patients had higher AFP serum levels (mean, 4002 ng/dl) than LC (mean, 26.5 ng/dl) or CH (mean, 4.9 ng/dl) patients (Table 1). Eight of the 14 antibody-positive HCC patients and 2 of the 3 antibody-positive LC patients had increased AFP serum levels, suggesting an immunostimulatory role of antigen overexpression. However, no statistically significant correlation was found between antibody presence and antigen levels.

Discussion

Several studies have demonstrated the existence of immune responses to antigens expressed by both malignant and normal cells, suggesting that in pathological conditions, the human immune system may recognize self-antigens (5–9). Different mechanisms, including tissue overexpression and exposure of sequestered antigens, may explain tolerance circumvention (5–9). The detection of spontaneous immune responses indicates that some antigens could be used to enhance immune responses against tumor cells by boosting immunizations. In addition, the detection of antibody responses may be an indicator of tumor progression and can be used in patient monitoring.

In this study, we provide evidence of a significant spontaneous anti-AFP humoral response in liver disease patients. AFP represents one of the most useful markers for hepatocarcinomas and teratocarcinomas and for the monitoring of the patient response to therapy (1). We detected high titers of anti-AFP immunoglobulins in HCC (14/60), LC (3/15), and CH (1/15) patients but not in 40 healthy individuals. Patient anti-AFP immunoglobulins selectively reacted with denatured and DG-AFP, indicating specific recognition of structural proteic epitopes that are cryptic on n-AFP. The reactivity with hidden epitopes is not surprising because anti-p53 antibodies, which are found in cancer patient sera, also reacted with a linear peptide that is cryptic in the native p53 and accessible only on the denatured or mutant protein (6). Cryptic epitopes may become exposed to the immune system secondary to events, i.e., trauma and necrosis, which may synergistically act causing conformational changes of the protein and/or changes in the processing machinery (14). Inflammatory status and enhancement of lysosomal cathepsin B and L blood levels in liver diseases patients (15) may contribute to unveil hidden epitopes, thus stimulating ex novo immune responses to conformationally modified AFP. It is worth noting that all of the anti-AFP-positive patients observed in our study had a positive serum test for hepatitis C and that this infection is often associated with autoimmune diseases and with microsomal autoantibodies (16).

AFP is highly immunogenic in xenogenic animals, whereas it does not elicit immune responses in the species of origin (17, 18). In this respect, our study suggests that the exposure of hidden epitope(s) on AFP may result in a natural appearance of self-reactive antibodies, indicating lymphocyte activation of otherwise eliminated clones (19).

The presence of anti-AFP antibodies was significantly associated to HCC (\( P = 0.0006 \)) and LC (\( P = 0.0173 \)) patients. The occurrence of anti-AFP antibodies in HCC and LC patients may be a useful tool to

### Table 2 Detection of antibodies to α-fetoprotein in serum of patients with liver diseases

<table>
<thead>
<tr>
<th>Serum origin</th>
<th>No. of cases</th>
<th>Reactivity with n-AFP</th>
<th>Reactivity with d-AFP</th>
<th>Reactivity with DG-AFP</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy donors</td>
<td>40</td>
<td>0/3</td>
<td>0/40</td>
<td>ND</td>
<td>IgM</td>
</tr>
<tr>
<td>Liver diseases</td>
<td>90</td>
<td>0/90</td>
<td>18/90</td>
<td>18/18</td>
<td>IgM</td>
</tr>
<tr>
<td>Hepatocellular carcinomas</td>
<td>60</td>
<td>0/60</td>
<td>14/60</td>
<td>14/14</td>
<td>IgM</td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>15</td>
<td>0/15</td>
<td>3/15</td>
<td>3/3</td>
<td>IgG</td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>15</td>
<td>0/15</td>
<td>1/15</td>
<td>1/1</td>
<td>IgG</td>
</tr>
</tbody>
</table>

\( ^* \text{ND, not determined.} \)

\( ^{\text{a}} P = 0.0006 \) (Fisher’s exact test).

\( ^{\text{b}} P = 0.0006 \) (Fisher’s exact test).

\( ^{\text{c}} P = 0.0173 \) (Fisher’s exact test).

\( ^{\text{d}} P = \text{not significant (Fisher’s exact test).} \)

\( ^{\text{e}} \text{anti-d-AFP-positive sera.} \)

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monitor and understand the role of the specific immune responses in disease progression and may be used as a prognostic/diagnostic marker in addition to the AFP determination. However, a prospective study evaluating a large cohort of patients would help to elucidate the biological and prognostic role of these antibodies.

The presence of the circulating antigen might interfere with the detection of specific antibodies. However, we detected antibodies in patients with both low and high levels of circulating AFP (up to 2075 ng/dl). Therefore, it appears that high AFP serum levels do not influence the detection of those anti-AFP antibodies recognizing epitopes that are not accessible on the native antigen. The lack of increased serum levels of AFP in six antibody-positive patients does not exclude the expression of AFP at the tumor tissue level because some tumors do not secrete the protein (20). Alternatively, the presence of anti-AFP immunoglobulins in AFP-negative liver disease patients indicates the possibility of an active immunoselection for AFP-negative tumor cell populations after elimination of AFP-positive tumor cells (9).

Our results suggest that the ex novo appearance or exposure of cryptic epitopes on AFP may be a key event to circumvent immunological tolerance to this self-antigen and highlight the need to use screening methods involving both native and denatured proteins to detect self-reacting antibodies. The identification of the in vivo immunogenic cryptic epitope(s) might bear relevance for potential immunotherapeutic strategies targeting AFP.

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References

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