Immune Reactions against Hepatitis B Viral Antigens Lead to the Rejection of Hepatocellular Carcinoma in BALB/c Mice

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ABSTRACT

Human hepatitis B virus (HBV) is closely associated with hepatocellular carcinoma. However, the mechanism of carcinogenesis and the immune responses to HBV infection and hepatocellular carcinoma are not clearly understood. Recently, we established BALB/c mouse liver (ML) cell lines and demonstrated that transfection of ML cell lines with HBV dimer DNA resulted in the expression of HBV antigens. The HBV-transfected ML cells and the parental ML cells showed similar tumorigenicity in nude mice. However, the HBV-transfected cells had much lower tumorigenicity in BALB/c mice. Similar results were also obtained in two cloned ML cell lines, ML-I.1 and ML-I.2, transfected with plasmid DNA containing HBs, HBC, or HBx gene. Furthermore, adoptive transfer of spleen cells from BALB/c mice immunized with HBsAg- or HBCAg-expressing ML-I.1 cells caused regression of tumor cells expressing the corresponding antigens in nude mice. In addition, transfer of spleen cells from BALB/c mice immunized with purified HBsAg or HBCAg also caused tumor regression. These results demonstrate that HBsAg and HBCAg can induce immunity which leads to the rejection of hepatocellular carcinoma in vivo.

INTRODUCTION

Human HBV is a partially double-stranded DNA virus that causes acute and chronic hepatitis (2-4). The pathogenic mechanism responsible for hepatocellular injury in HBV infection is not clearly defined. Previous studies suggested that HBV was probably not directly cytopathic for the infected hepatocytes and the immune response may play an important role in the pathogenesis of hepatitis (5, 6). However, direct evidence for a pathogenic role of immune response is lacking. The slow progress on the study of the pathogenetic mechanisms responsible for injury in HBV infection is mainly due to the difficulty of culturing human hepatocytes, the narrow host ranges of HBV, and the major histocompatibility complex restriction of T-cell-mediated immune responses. Recently, several interesting approaches have been designed to overcome these difficulties. Using HBV envelope transgenic mouse model system, Chisari et al. (7) showed that the immune response against HBsAg could induce hepatic injury (7). Other studies included the identification and cloning of HBsAg- and HBCAg-specific CTL from hepatitis B patients using lymphoblastoid cell lines as target cells (8-10).

In addition to hepatitis, considerable evidence indicate that HBV also induces HCC (2, 3), which is the leading cause of cancer death in some countries. The immune responses against HCC are also poorly understood. Whether the antigens encoded by HBV such as HBsAg and HBCAg play a role in the induction of tumor immunity remains to be analyzed. Recently, we established and characterized several well differentiated ML cell lines from BALB/c mice (1). Transfection of these cells with HBV DNA leads to the integration of HBV genome into the hepatocytes and the secretion of HBV antigens or HBV-like particles into the medium. This system serves as a model to study the immune response against HCC expressing HBV antigens. In this study, we report that immune responses against HBsAg or HBCAg lead to the rejection of HCC.

MATERIALS AND METHODS

Cell Lines and Plasmids. Mouse liver cell line ML-1HBxEn was originally derived from BALB/c mouse hepatocytes and later designated as ML-1 for convenience (1). At passage 35, ML-1 cells were cloned by micromanipulation and two cloned cell lines, ML-1.1 and ML-1.2, were established. Six plasmids which contained different HBV ORF or neomycin-resistant gene were used in this study. pSHH2.1 contained the HBV tandem dimer DNA (11). pMTLS was a deletion product of the HindIII-BglII fragment from pMH3/3097 (12), with the HBV large surface antigen being driven by the metallothionein promoter. This plasmid also generated the transcript for ORF X using its endogenous promoter. pMT2 (13) was a deletion product of the BamHI fragment (nucleotides 2906-1402) of pMH3/3091 (12) and contained a complete ORF for HBCAg driven by a metallothionein promoter. pSV, HBxEn contained HBV DNA from HpaI to BglII site (nucleotides 963 to 1982), and the HBx gene product was transcribed from SV40 early promoter (1). pXB22 also contained HBV dimer DNA, but a HindIII linker was inserted at the BamHI site 1402, causing a frame-shift in the reading frame of X gene. It expressed HBsAg and HBCAg but not HBV X antigen (14). pSV, Neo contained SV40 early promoter and the neomycin-resistant gene (15). ML-1 (HBV), the ML-1 cells transfected with pSHH2.1, expressed HBV antigen (1). ML-1 (HBV) cells were further cloned and the secreted HBsAg was measured by HBsAg enzyme-linked immunosorbent assay kits (EverNew, Taiwan, Republic of China). The P/N ratio of HBsAg secreted by each clone was calculated. The subcloned cells were divided into three groups: "++" denoting cells secreting high levels of HBsAg (P/N > 50); "++" denoting cells secreting medium levels of HBsAg (P/N ~ 10); and "--" denoting cells without detectable HBsAg secretion.

DNA Transfection and Selection of Clones. ML-1 cells were cultured in Dulbecco’s modified Eagle’s medium as described previously (1). Subconfluent cultures were cotransfected with 13.6 μg of pSHH2.1, pSV, HBxEn, pXB22, pMTC, or pMTLS DNA and 1.4 μg of pSV, Neo DNA by using the calcium phosphate/DNA coprecipitation procedure (1). The culture medium was replaced by fresh medium after incubation at 37°C for 2 h. Thereafter, medium was replaced by fresh medium containing 900 μg G418/ml (GIBCO, Grand Island, NY). Resistant colonies were obtained approximately 1 month later. The stable transfectants were isolated and designated according to the transfected HBV gene(s): ML-1.1 (HBV) containing pSHH2.1; ML-1.1 (S, X) containing pSV, HBxEn; ML-1.1 (C) containing pMTC; ML-1.1 (S, X) containing pMTLS; and ML-1.1 (S, C) containing pXB22. Control cells, ML-1.1 (Neo), were obtained by transfecting pSV, Neo DNA alone into ML-1.1 cells.

RNA Isolation and Northern Blot Analysis. Total RNA was extracted by the guanidinium/cesium chloride method (16). RNA was denatured by formamide, electrophoresed, transferred to nitrocellulose paper, and detected with 32P-labeled HBV-specific DNA probes as described previously (17).

Determination of Tumorigenicity in BALB/c and Nude Mice. BALB/c mice were obtained from the animal center, Veterans General Hospital, Taipei, Taiwan, Republic of China. Nude mice (BALB/c nu/nu) were purchased from the animal center of National Taiwan University, Taipei, Taiwan, Republic of China. For tumorigenicity test, each mouse was given an injection of 1 × 107 tumor cells s.c.

Adoptive Transfer. BALB/c mice were immunized with 1 × 107 tumor cells s.c. The spleen cells were collected after 11 days for adoptive transfer experiments. For immunization with soluble antigens, BALB/c mice were immunized with 30 μg of proteins emulsified with complete Freund’s adjuvant (DIFCO Laboratories, Detroit, MI) into the footpads. Two weeks later, mice were boosted twice with 10 μg of proteins in incomplete Freund’s adjuvant.
was kindly supplied by Life Guard Pharmacological, Inc., Hsin-Chu, Taiwan, to produce tumors in BALB/c mice. In contrast, the cells which did not express HBsAg formed tumors in BALB/c mice (Table 1). These results indicate that the expression of HBV antigens reduces the tumorigenicity of ML-1 cell lines in the immunocompetent host (BALB/c mice) but not in nude mice.

Analysis of HBV ORFs Responsible for the Reduction of Tumorigenicity. In order to know which ORF of HBV was responsible for the reduced tumorigenicity of ML-1 cells, two single cell clones, ML-1.1 and ML-1.2, were cotransfected with DNA of plasmids containing different HBV ORF and pSV2Neo. The expression of specific transcripts of transfected plasmids could be detected by Northern blot analysis (Fig. 1). The cells transfected with pSHH2.1 or pXB22 expressed three major transcripts, 3.5, 2.2, and 0.8 kilobases, as reported previously (11, 13). The transfected cells with pMTC revealed a transcript of 1.8 kilobases which encodes for HBCAg. The pSV2HBxEn-transfected cells expressed two transcripts, 1.5 and 0.8 kilobases. The 1.5-kilobase transcript was driven from the SV40 promoter while the 0.8-kilobase RNA was transcribed from the endogenous HBx promoter. The pMTLS-transfected cells had a 2.2-kilobase transcript for HBs and an 0.8-kilobase transcript for HBx. ML-1.1 and ML-1.2 cells expressing different HBV ORF were further tested for their tumorigenicity in BALB/c and nude mice. All the transfectants formed tumors in 100% of nude mice (data not shown), while cells expressing HBs, HBe, or HBx antigen had much lower tumorigenicity in BALB/c mice than ML-1.1, ML-1.2, or pSV2Neo-transfected cells (Table 2).

| Table 1 Tumorigenicity of ML-1 cell lines and their HBV transfectants in BALB/c and nude mice |
|-------------------------------------------------|-----------------|-----------------|
| Cell lines                                      | In BALB/c mice  | In nude mice    |
| ML-1                                            | 12/12           | 5/5             |
| ML-1 (HBV)                                      | 0/11            | 5/5             |
| Cloned cells                                     |                 |                 |
| ML-1 (HBV) ++ + b                               | 0/3             | 4/4             |
| ML-1 (HBV) ++ + b                               | 0/3             | 2/2             |
| ML-1 (HBV) ++ + b                               | 3/3             | 2/2             |

*Tumor cells, 10^7 were S.C. injected into BALB/c or nude mice. Tumorigenicity was expressed as numbers of tumor-bearing mice/numbers of mice inoculated with tumor cells and measured 1 month after inoculation. ML-1 (HBV) cells were cloned and the secreted HBsAg was measured by enzyme-linked immunosorbent assay. The P/N ratio was determined. ++ , cells secreting high levels of HBsAg (P/N >50); + +, cells secreting medium levels of HBsAg (P/N >10); -, undetectable secretion of HBsAg.

RESULTS

Reduced Tumorigenicity of ML-1 Cells Transfected with HBV. ML-1 cells formed tumors in both nude mice and BALB/c mice. However, ML-1(HBV) cells grew as tumors only in nude mice. None of the BALB/c mice had tumors when 10^7 ML-1(HBV) cells were injected (Table 1). Furthermore, all the cloned ML-1(HBV) cells formed tumors in nude mice but the “+++” and “++” clones did not produce tumors in BALB/c mice. In contrast, the “-” cells which did not express HBsAg formed tumors in BALB/c mice (Table 1). These results indicate that the expression of HBV antigens reduces the tumorigenicity of ML-1 cell lines in the immunocompetent host (BALB/c mice) but not in nude mice.

Inhibition of Tumor Growth by Adoptive Transfer of Spleen Cells from Mice Immunized with ML-1.1 Cells Expressing Different ORF of HBV. Adoptive transfer experiments were performed in order to investigate whether the reduced tumorigenicity of ML-1.1 cells expressing HBV antigens was due to immune responses of the BALB/c mice. Spleenocytes from BALB/c mice which were immunized with ML-1.1(HBV) or ML-1.1(Neo) cells were injected i.v. into nude mice. The same day, the nude mice were inoculated s.c. with ML-1.1 (HBV) cells. As shown in Fig. 2, transfer of spleen cells from mice immunized with ML-1.1(HBV) cells could significantly inhibit the growth of ML-1.1(HBV) cells as compared with the transfer of spleen cells from mice immunized with ML-1.1(Neo) cells (P < 0.01). To analyze which ORF of HBV was responsible for the inhibition of tumor growth, spleen cells from BALB/c mice immunized with ML-1.1 cells expressing different ORF of HBV were transferred to nude mice. Spleen cells from mice immunized with ML-1.1(S,X) or ML-1.1(S,C) cells could significantly reduce the growth of ML-1.1(HBV) or ML-1.1(S,X) cells as compared with the transfer of spleen cells from mice immunized with ML-1.1(Neo) cells (P < 0.01) (Fig. 3). These results suggested that HBsAg induced immune responses which inhibited the growth of tumor cells expressing HBSAg. Similarly, when spleen cells from mice expressing ML-1.1(C) cells were transferred, the growth of ML-1.1(HBV) or ML-1.1(S,X) cells was inhibited significantly (P < 0.01) in contrast to those mice transferred with spleen cells sensitized with ML-1.1-(Neo) or ML-1.1(X) cells (Fig. 4). For the specificity control, adoptive transfer of spleen cells sensitized with ML-1.1(HBV), ML-1.1(S,X),
ML-1.1(X), ML-1.1(S,C), or ML-1.1(C) cells had no effect on the reduction of growth of ML-1.1(Neo) cells (Fig. 5).

Inhibition of Tumor Growth by Adoptive Transfer of Spleen Cells from Mice Immunized with Purified HBV Antigens. To further prove that the inhibition of tumor growth was induced by HBV gene products, purified HBsAg or HBcAg was used to immunize BALB/c mice. As shown in Fig. 6, specific inhibition of tumor growth was observed after adoptive transfer of spleen cells from mice immunized with specific antigens. Splenocytes from mice immunized with HBsAg could significantly reduce the growth of ML-1.1(HBV) or ML-1.1(S,X) cells which expressed HBsAg when compared to the splenocytes from mice immunized with ovalbumin as a control antigen (P < 0.01). Similar results were obtained when spleen cells from mice immunized with HBCAg were transferred. The growth of ML-1.1(C) cells which expressed HBcAg was inhibited as compared to those mice transferred with spleen cells sensitized with ovalbumin (P < 0.01). Finally, spleen cells from mice immunized with purified HBsAg or HBCAg did not inhibit the growth of tumor cells not expressing the corresponding HBsAg or HBCAg, respectively (data not shown). These results indicate that the inhibition of tumor growth is mediated by the immunity induced by HBsAg or HBCAg.

DISCUSSION

HBV has been reported to be strongly associated with HCC. The occurrence of HCC, however, is observed only after a long period of time following HBV infection. This long delay reflects the complexity of tumor formation, i.e., initiation, promotion, and progression, etc. Immune responses seem to be involved in the development of HCC, similar to other viral induced tumors. HBsAg may serve as a target molecule on infected hepatocytes for cytotoxic cells. Barnaba et al. (8) identified liver-infiltrating CTL to be pre-S2 specific in chronic hepatitis B patients. Moriyama et al. (7)
sensitized spleen cells from mice immunized with HBsAg to transgenic mice for HBV envelope gene and observed hepatocyte injury in vivo. Other studies (9, 10) suggested that HBcAg can serve as a target antigen for CTL. Using recently developed ML-1 cell lines, we examined the possible in vivo role of HBV antigens in the immunological surveillance against HCC. In BALB/c mice, we demonstrated that ML-1 cells expressing HBsAg or HBcAg had greatly reduced tumorigenicity as compared with parental cells without HBV antigens. In contrast, ML-1 cells with or without HBV antigens were equally tumorigenic in nude mice (Tables 1 and 2). We also demonstrated (Table 2) that cells expressing any one of HBV antigens (S, C, or X) were less tumorigenic than cells not expressing HBV antigens. In other words, any of HBV antigens could elicit immune responses against transfected cells in vivo.

The different tumorigenicity of ML-1 (HBV) cells in immunocompetent and immunodeficient hosts (BALB/c and nude mice) strongly suggested the involvement of immune responses. Adoptive transfer experiments further demonstrated that spleen cells from BALB/c mice immunized with specific HBV antigens significantly reduced tumor sizes in nude mice inoculated with tumor cells expressing corresponding antigens. These results indicate that immune responses against HBV antigens are responsible, at least in part, for the inhibition of tumor growth.

Our results strongly imply that HCC cells expressing HBV antigens will encounter strong immunological surveillance and hence will not be able to grow in the immunocompetent host. In small HCC, Hsu et al. (19) reported that 42% of cells expressed HBsAg and 24% of cells expressed HBcAg. Interestingly, when large HCC were analyzed, HBsAg-positive cells decreased to 24% and HBcAg-positive cells reduced to 10.5%. These data together with the results presented in this article suggest the following scenario. HBV infection plays an important, probably essential, role in the induction of HCC. The original HCC cells probably express HBV antigens. As the tumor gets larger, immune responses against HBV antigens are elicited and react with HCC cells bearing HBV antigens. During this process, inactivation of viral gene expression through HBV gene rearrangement or excision of HBV genome may occur. HCC cells not expressing HBV antigens will escape from the immune surveillance. Thus, as the tumor increases in size, an enrichment of cells not expressing HBV antigens is observed. However, immune selection may not be the only explanation for the growth of HBV antigen-negative HCC. In HCC developed in transgenic mice overproducing the HBV large S antigen (20), there was no evidence of major rearrangement of transgene HBV DNA. However, the tumor cells were transcriptionally altered. The tumors displayed a major decrease in the steady-state level of HBV envelope-specific transcripts. Furthermore, no HBV X-specific transcripts were detectable in the tumors (20). Since the transgenic mice were immunologically tolerant to the HBV gene products, these cells could not be the result of immune selection. Thus, mechanism(s) other than immune selection is also involved in the nonexpression of HBV antigens in HCC.

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