Failure of the Lamivudine-Resistant rtM204I Hepatitis B Virus Mutants To Efficiently Support Hepatitis Delta Virus Secretion

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Received 26 August 2004/Accepted 14 January 2005

Hepatitis delta virus (HDV) is encapsidated by the envelope proteins of hepatitis B virus (HBV). The major HBV lamivudine (LMV)-resistant mutations in the polymerase gene within the reverse transcriptase (rt) region at rtM204V or rtM204I are associated with changes in the overlapping envelope gene products, in particular, the gene encoding small envelope protein (s) at sI195M or sW196I/S/Stop. We have demonstrated that the LMV resistance mutations corresponding to sW196I/S inhibited secretion of HDV particles, while changes corresponding to sI195M did not affect secretion. Differential efficiencies of HBsAg proteins expressed by LMV-resistant HBV to support HDV secretion may have consequences for clinical prognosis as coinfected patients are treated with antiviral agents.

The hepatitis delta virus (HDV) can cause severe chronic and acute hepatitis in humans (11). HDV coexists with hepatitis B virus (HBV) as it is dependent on the HBV envelope proteins for virus assembly. Inside the HBV envelope, the HDV virion contains a 1.7-kb circular RNA complexed with two HDV-encoded proteins, the large (214-amino-acid) and small (195-amino-acid) forms of hepatitis delta antigen (HDAg-L and HDAg-S, respectively) (22, 28). HDAg-L is crucial for virus assembly and interaction with the HBV envelope proteins (6). The other viral protein, HDAg-S, is essential for viral RNA replication (17).

The HBV genome is approximately 3.2 kb and contains a number of overlapping reading frames. The HBV envelope consists of three surface proteins, small (hepatitis B surface antigen; HBsAg), middle, and large. All three HBV envelope proteins are encoded by a single open reading frame. The mRNA transcripts have different in-phase translational start codons and share a common stop codon, resulting in proteins with identical C-terminal ends and different N-terminal sequences. The HBsAg protein can form subviral particles that are secreted in excess in comparison to the HBV virion. The presence of HBsAg alone is sufficient for HDV assembly, although as with HBV, the large hepatitis B surface protein is required for infectivity. The envelope genes overlap the polymerase, and mutations may result in concomitant changes in both reading frames (21).

The nucleoside analogue lamivudine (LMV) has been used for the treatment of hepatitis B infection (12, 19, 20). Lau and coworkers (18) demonstrated that although LMV is a potent inhibitor of HBV DNA replication, it did not inhibit HDV RNA replication or improve disease activity. Nevertheless, LMV is being used to treat hepatitis B infection in patients that are coinfected with HDV (4, 20, 29, 30). The major problem with LMV treatment has been the selection of HBV-resistant strains. In a study of long-term LMV treatment, HBV resistance was detected in 67% of patients after 4 years and 69% of patients after 5 years (19, 20). Resistance to LMV is associated with a mutation in the reverse transcriptase (rt) region of the polymerase gene at the M204V/I codon (rtM204V/I), with or without an accompanying rtL180M mutation (according to the nomenclature by Stuyver and coworkers [24]) (Table 1). The rtL180M change does not result in an amino acid change within the envelope protein. The concomitant HBsAg mutations selected by the antiviral agents are located towards the C-terminal region of the envelope protein. The mutation at the rtM204V/I codon results in a change in the envelope gene at the sI195M codon, and the rtM204I/I mutation results in changes to the sW196I/S/Stop codon. In addition to these changes, the rtV173L mutation occurs in 9 to 22% of patients with LMV resistance (8, 9). This mutation results in a change in the envelope gene at the sE164D codon. Recently, Torrisi and coworkers (26) investigated the antigenicity of HBsAg proteins expressed by HBV vaccine escape mutants and LMV-resistant mutants. HBsAg proteins containing mutations in the major immunological determinant designated “a” (vaccine escape mutations sG145R and sD144E/sG145R), as well as HBsAg proteins selected during LMV treatment (those with mutations sE164D, sI195 M, sW196S, and sE164D/sI195M), showed reduced binding to anti-HBsAg antibodies, depending partly on the anti-HBs antibodies used and the assay applied. Jenna and Sureau (15) have demonstrated that certain amino acid sequences in the C-terminal domain of the S protein are essential for the assembly of HDV particles. In particular, they examined the region between amino acid residues 163 and 224 of HBsAg and showed that an sW196F mutation severely reduced the assembly of HDV. However, this previous study did not examine the effect of the LMV-resistant mutations at residue 196 (i.e., sW196I/S/Stop) on HDV secretion. Thus, it is very important to determine if these clinically significant HBV mutants have an effect on the secretion of HDV. The aim of this study was to investigate the effect of HBsAg proteins encoded by common LMV-resistant HBV variants on HDV secretion and to assess whether these clinically signifi-
cantly HBV mutants have an effect on HDV secretion. HDV genotype I was used in this study due to its worldwide distribution. In addition, HDV genotype I is more often associated with grave outcomes than is HDV genotype II (31), and HDV genotype III is associated with HDV infection in northern South America (5).

**In vitro analysis of LMV-resistant HBsAg mutants cotransfected with plasmid-expressing HDV.** A series of HBV envelope mutations associated with LMV resistance were constructed to determine the effect of these mutations on HDV secretion. For this study, HuH-7 cells were cotransfected with HBsAg-expressing plasmids and replication-competent HDV cDNA. HBsAg alone is sufficient for HDV secretion and replication-competent HBV is not required. HDV depends only on the presence of the HBV envelope protein and not on any other factors/proteins provided by HBV (27). The cDNA encoding HBsAg genotype A was subcloned from the infectious clone pHBV1.5 into the expression vector pCI, and site-directed mutagenesis was used to construct the mutants (sI195M and sW196S) as previously described by Torresi and coworkers (2, 26). The additional mutations sW196F and sW196L used in this study were created by site-directed mutagenesis with Pfu polymerase (Promega) by use of the following sets of oligonucleotides (Micromon, Monash University): P1, 5’-GCTTTCAGCTATATTCATGATG TGG-3’; P2, 5’-CCACATCATGAATATAGCTGAAAGC-3’; P3, 5’-GCTTTTCAGCTATATTTGATGAGTGG-3’; and P4, 5’-CCACATCATGATGATGCTGAAGC-3’. The plasmid pSVLD3 with inserted replication-competent HDV cDNA was described previously by Kuo and coworkers (17). To analyze HBsAg expression and HDV secretion, the appropriate plasmids were cotransfected into the human hepatoma cell line HuH-7 (23). Briefly, the HuH-7 cells were grown in Dulbecco’s modified Eagle’s medium (CSL) supplemented with GlutaMax-1 (Gibco-BRL), 10% fetal calf serum, penicillin, and streptomycin. The cells were transfected using the Ca phosphate method, as described previously (13). The transfections were performed in the presence of the plasmid pSEAP expressing secreted alkaline phosphatase (SEAP) (1). Variation in SEAP activity was less than twofold, indicating similar transfection efficiencies with all the constructs (Table 1). The plasmid pCI-HBsAg expressing wild-type HBsAg was used as a positive control, and cells transfected with (i) pSVLD3 and pCI vector without insert and (ii) untransfected cells were used as negative controls.

The amount of HBsAg produced was measured at days 6, 9, and 11 posttransfection by a chemiluminescence HBsAg assay (Abbott Prism, Abbott Diagnostics). The resulting light counts were in the same range for each time point for each construct (Fig. 1), indicating that the anti-HBs antibody identifies various proteins efficiently. All the HBsAg mutant polypeptides studied were secreted. Studies with antibodies to LMV-specific mutant HBsAg polypeptides present in vaccinee sera demonstrated that antibody binding was reduced to various degrees relative to that of wild-type HBsAg (26). Therefore, we cannot exclude the possibility that some mutant HBsAg proteins were identified with reduced efficiency, resulting in an underestimation of the calculated HBsAg concentration (Table 1). Nevertheless, the various HBsAg proteins were clearly detected in the cell culture supernatant by this assay and investigated with regard to their potential to support secretion of HDV particles.

**Effect of HBsAg mutants on secretion of HDV antigens.** To determine if there was an effect of the HBV mutations on

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**TABLE 1. Virological markers in cell culture supernatant and cell lysate**

<table>
<thead>
<tr>
<th>Mutation in polymerase gene</th>
<th>Mutation in S gene product</th>
<th>HBsAg protein (ng/ml in culture fluid)</th>
<th>HDAg&lt;sup&gt;a&lt;/sup&gt; in:</th>
<th>HDV RNA (pg/ml of culture fluid)</th>
<th>Ratio of HDV RNA standardized to the corresponding HBsAg</th>
<th>SEAP expression (enzyme reactivity/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type polymerase</td>
<td>Wild-type HBsAg</td>
<td>74</td>
<td>284</td>
<td>191</td>
<td>312</td>
<td>201</td>
</tr>
<tr>
<td>rtV173L</td>
<td>sE164D</td>
<td>23</td>
<td>260</td>
<td>182</td>
<td>112</td>
<td>85</td>
</tr>
<tr>
<td>rtV173L/rtL180M/rtM204V</td>
<td>sE164D/sI195M</td>
<td>69</td>
<td>206</td>
<td>195</td>
<td>220</td>
<td>177</td>
</tr>
<tr>
<td>rtM204V</td>
<td>si195M</td>
<td>89</td>
<td>161</td>
<td>205</td>
<td>364</td>
<td>265</td>
</tr>
<tr>
<td>rtM204I</td>
<td>sW196S</td>
<td>96</td>
<td>224</td>
<td>207</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>rtM204I</td>
<td>sW196L</td>
<td>106</td>
<td>256</td>
<td>219</td>
<td>54</td>
<td>16</td>
</tr>
<tr>
<td>rtD205H</td>
<td>sW196F</td>
<td>144</td>
<td>243</td>
<td>265</td>
<td>211</td>
<td>63</td>
</tr>
<tr>
<td>Empty pCI vector</td>
<td>Not applicable</td>
<td>0</td>
<td>203</td>
<td>144</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Untransfected cells</td>
<td>Not applicable</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Relative total intensity of the band determined by using a PhosphoImager.

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**FIG. 1. Measurement of HBsAg in the cell culture supernatant (Abbott prism assay; Abbott Diagnostics) done on days 1 to 6 (black bars), days 7 to 9 (grey bars), and days 10 to 12 (white bars). The mutations of the corresponding HBsAg proteins are indicated on the x axis, and the arbitrary light counts as an HBsAg-specific measure are indicated on the y axis. p.t, posttransfection.
HDV secretion competence, the relative amounts of HDV RNA and HDAg in the cell culture supernatant derived from cotransfected cells were analyzed. For the detection of HDAg proteins, the cellular fraction of transfected cells and the corresponding cell culture supernatants were analyzed. HuH-7 cells were solubilized by boiling them in loading buffer (250 mM Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate, 0.4% bromophenol blue, 40% glycerol, and 20% 2-mercaptoethanol). Virus particles in the cell culture supernatant were concentrated over a 20% sucrose cushion in STE buffer (100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA) and centrifuged for 4 h (TH641 rotor, Sorvall, 39,000 rpm), and the pellet was resuspended in loading buffer. The proteins were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a Hybond-P membrane (Amersham Pharmacia Biotech). The membrane was blocked, incubated with human anti-HDAg antibody, washed, and then incubated with a rabbit anti-human immunoglobulin G antibody conjugated to horseradish peroxidase (1:10,000 dilution; Dako Corporation). The membrane was incubated with ECL Plus Western blotting detection substrate (Amersham Pharmacia Biotech) and then exposed to Kodak BioMax MS film.

Densitometry of the bands showed similar levels of HDAg-L and HDAg-S in the cell lysate (Fig. 2A and Table 1). The presence of HDAg-L and HDAg-S in the cell culture supernatant varied, with almost no HDAg protein detectable in the presence of the clinically important HBsAg sW196S and sW196L mutants. HDAg-L was secreted at least six times less efficiently in the presence of the HBsAg sW196S and sW196L mutants than in the presence of wild-type HBsAg (Fig. 2B, and Table 1). HBsAg polypeptides containing the sW196F mutation, which is not associated with LMV resistance, also showed a reduced capacity to support HDAg protein secretion.

Effect of HBsAg mutants on HDV RNA secretion. To determine the effect of the HBsAg mutations on the secretion of HDV RNA, we analyzed the amount of HDV RNA in both the cell lysate and the supernatant of cotransfected cells (Fig. 3 and Table 1). Total RNA was extracted from HuH-7 cells using an RNeasy minikit (QIAGEN) according to the manufacturer’s instructions. HDV particles in the cell culture supernatant were concentrated by centrifugation over a 20% sucrose cushion, and HDV RNA was extracted from particles with a QIAamp viral RNA minikit (QIAGEN). The isolated RNA was glyoxylated as described by Chen and coworkers (7). A pGEM plasmid with an HDV cDNA insert was used to generate a 32P-labeled riboprobe to detect HDV genomic RNA. Unincorporated nucleotides were removed from the probe mixture with an RNase-free P30 spin column (Bio-Rad). After prehybridization and hybridization steps, the membrane was washed and exposed to Kodak BioMax MR film.

Genomic RNA was detected in all cellular lysates derived from samples transfected with pSVLD3 (Fig. 3A). Consistent with the outcome of the Western blot analysis for the presence of HDAg proteins (Fig. 2), the Northern analysis demonstrated that the presence of the HBsAg-S proteins with the sW196S or sW196L changes did not efficiently support secretion of HDV particles. HDV RNA was not detected in the cell culture supernatant (Table 1 and Fig. 3B). The previously described sW196F mutation (14) was used as a control, and its presence resulted in reduced HDV secretion by a factor of 5, as measured by densitometry, compared to that of wild-type HBsAg (Table 1).

HBV variants with mutations in the polymerase protein and concomitant sI195M or sE164D and sI195M mutations in HBsAg open reading frame products are associated with enhanced HBV replication and virion production (9). The HBsAg
gnotype 1 in this study, it should be noted that there is genetic variability among the three HDV genotypes, and the effect of HBsAg carrying the LMV resistance mutations on the secretion of HDV genotypes II and III has yet to be determined.
protein containing the clinically important mutation s195M was able to support HDV secretion (Fig. 2B and 3B). The HBsAg with the sE164D and s195M double mutation also supported HDV RNA secretion (Fig. 3A).

These experiments confirmed the previous data of Jenna and Sureau (14) and demonstrated that codon 196 is critical for HDV packaging. But, importantly, the data demonstrated that the key clinically relevant mutants currently being selected during antiviral therapy have a differential HDV packaging profile. In a recent study of HBV LMV resistance, HDV coinfection was reported to be associated with the early development of LMV resistance (H. L. Tillmann et al., International Symposium on Viral Hepatitis and Liver Disease, Sydney, Australia, p. 115, 2003). In this study, 3/15 coinfected patients who had selected for LMV resistance did not show any clinical improvement or further deterioration after the emergence of resistance. However, Joh and coworkers reported severe flare-ups during LMV treatment in an HDV-coinfected individual (16). In both reports, a correlation between the specific LMV-selected HBV mutation(s) and the clinical response was not presented. These studies demonstrate that coinfected patients with LMV resistance can have a variety of clinical prognoses, and specific mutations selected for during antiviral therapy may have an effect on direct virus-virus interactions.

Although there are now HDV-specific antiviral agents (3, 10, 25), LMV is being used to treat HBV infection in patients that are coinfected with HDV (4, 20, 29, 30). An understanding of virus interactions and how newly selected, virus-encoding resistance mutation after these interactions is important for the treatment of these coinfected patients. Both HDAg secretion and HDV RNA secretion were affected in the presence of HBsAg proteins expressed by LMV-resistant HBV mutants. The HBV LMV-resistant mutant (rtM204I) also carrying the sW196S or sW196L change may indirectly abrogate an underlying HDV infection, while the HBV LMV-resistant mutant carrying the rtM204V change, which encodes HBsAg, as well as the s195M change is expected to have a minimal effect on HDV packaging and release.

This work was supported by an NIMRC research grant (no. 210232) and the Dora Lush NIMRC Biomedical Research Scholarship.

REFERENCES