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乙型肝炎病毒核壳蛋白变异株在$HepG_2$细胞的HLA-Ⅰ表达

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Expression of HLA-I on $HepG_2$ cells by hepatitis B virus nucleocapsid mutants

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Abstract

AIM: To study the expression of HLA-I/antigen peptide complex on $HepG_2$ cells transfected with HBV (adr) wild type and nucleocapsid protein mutants.

METHODS: The site-directed mutation was performed to introduce nucleocapsid protein point mutations V60 and L97 into 1.2 copies of HBV genome plasmid p3.8 Ⅱ. After identification of DNA sequence and biological activities, the plasmid p3.8 Ⅱ and mutant plasmid constructs were subcloned respectively into EB virus based vector EBO-plpp for stable expression. The vector constructed EBO-wild type, EBO-V60, and EBO-L97 were analyzed by restriction enzyme digestion and DNA sequenceing, then transfected into $HepG_2$ cells via the liposome technique, respectively. HBV antigen in their culture supernatants was quantified by Abbott kits. The cells were stained with murine monoclonal antibody anti-HLA-ABC conjugated directly to FITC, and expression of HLA-I on their membrane was analyzed by flow-cytometry.

RESULTS: Restriction enzyme digestion of 3 vector constructs showed two bands similar to HBV 1.2 copies genome and EBO vector, respectively. Analysis of DNA sequence confirmed the mutated nucleotides of EBO-V60 and EBO-L97 (i.e nt2078 C→G, nt2189 A→C). The expression of HBeAg S/CO in culture supernatant of EBO-wild type was much higher than that of mutant EBO-V60 and EBO-L97, while the expression of HBsAg S/N in three constructs had similar level, indicating similar transfecting rate in this experiment. The expression of HLA-I on $HepG_2$ cells transfected with EBO empty vector was at low level. Fluorescence intensity of HLA-I expression of transfected cells was elevated by EBO-wild type (18.2), while that of L97 was increased to 34.5 and V60 declined to 3.4.

CONCLUSION: HBV might enhance the expression of HLA-I/antigen peptide complex on $HepG_2$ cells. Hot-spot mutations of HBV nucleocapsid protein L97 and V60 could influence the expression level of HLA-I on host cells.

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摘要

目的: 研究HBV adr亚型野生株和核壳蛋白变异株在$HepG_2$细胞表面的HLA-I/抗原肽复合物的表达.

方法: 通过定点突变技术将1.2拷贝HBV野生型质粒p3.8Ⅱ构建成核壳蛋白变异株V60和L97. 经序列测定和生物学活性检测后,野生株和变异株重组质粒分别亚克隆入EB病毒表达载体EBO-plpp以稳定表达. 重组载体EBO-野生株、EBO-V60和EBO-L97分别作内切酶双酶切和序列测定鉴定, 再用脂质体介导转染$HepG_2$细胞, ELISA(Abbott)试剂盒定量检测各株培养上清HBV抗原, 转染细胞用FITC标记的鼠抗HLA-ABC单抗染色, 流式细胞术分析细胞表面HLA-I表达.

结果: 3株重组载体经内切酶消化, 电泳后显示2条区带, 分别与1.2拷贝HBV基因组和EBO载体大小相同. 测序结果证实EBO-V60和EBO-L97分别在nt2078 C→G和nt2189 A→C, 保持原定点突变. EBO-野生株的培养上清HBeAg定量S/CO值明显高于变异株V60和L97, 3株HBsAg定量S/N值接近, HBsAg表达相近表明实验的转染效率相当. EBO空载体转染的$HepG_2$细胞HLA-I轻微表达, 3株重组载体转染细胞HLA-I的荧光强度不同,野生株增强为18.2, L97明显升高至34.5, 而V60降低至3.4.

结论: HBV能增强$HepG_2$细胞表面HLA-I/抗原肽复合物的表达, 核壳蛋白热点变异V60和L97可使宿主细胞HLA-I表达发生变化.

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0 引言

细胞表面的人类白细胞抗原(human leukocyte antigen, HLA)是激发并调节机体 T 细胞免疫应答的关键分子, HLA 与 HBV 的感染、病毒诱导的免疫应答和免疫病理的相关性已受到广泛关注[1-9]. HBV C基因编码的核壳蛋白是免疫应答的主要靶抗原, 免疫效应细胞 CTL 通过TCR识别并结合HLA-I/病毒肽复合物实施细胞毒作用. 慢性感染者的HBV C基因长期处于免疫选择压力下常发生聚集性变异, 其中 AA97 的点突变占错义变异 45 % 以上[10-12]. 我国 HBV 主要流行亚型 adr 型的 C 基因变异中以 AA97 的异亮氨酸 ATC →亮氨酸 CTC(L97)为最常见, AA60 亮氨酸 CTG →缬氨酸 GTG(V60)为另一热点变异. 我们构建 HBV(adr)全基因野生株及 L97, V60 变异株EBO真核表达载体, 体外实验分析各株诱导 $HepG_2$ 细胞表面 HLA-I/ 抗原肽复合物表达的特性, 探讨核壳蛋白热点变异对 HLA-I 表达的影响.

1 材料和方法

1.1 材料 含 1.2 拷贝 HBV adr 亚型基因组质粒 p3.8 Ⅱ 由中国科学院生物化学研究所汪垣教授惠赠, 为野生株 3.2 kb 基因组 3'端重叠 587 bp(nt1 402-1 987)片段插入 pBS^+ 的重组质粒. EB病毒稳定真核表达载体EBO-plpp 由美国 Scripps 研究所 Francis V. Chisari 博士惠赠. 根据Gan *et al* (Zhongguo Kexue B Gi 1986;1:55-65)报道的我国 HBV adr 核苷酸序列设计 PCR 引物(表 1).

表 1 PCR 引物核苷酸序列

引物		序列(5'→3')
L97 点突变引物	L1	GGCCTAAAAC<u>T</u>CAGACAACTA
	L2	TAGTTGTCTGA<u>G</u>TTTTAGGCC
V60 点突变引物	V1	AGGCAAGCTATT<u>G</u>TGTGTTGGGGTG
	V2	CACCCCAACACA<u>C</u>AATAGCTTGCCT
测序引物	Pee2	GCGGGTACCCTAACATTGAGATTCCCG
亚克隆引物	P1	GCT<u>GTCGAC</u>CGCTTGATGCCT *Sal* Ⅰ
	P2	CCTTCTC<u>GGTACC</u>GGGGATCTA *Kpn* Ⅰ

1.2 方法 p3.8 Ⅱ变异株重组质粒的构建采用 Quik Chang™ 定点突变试剂盒(Stratagene), 以质粒 p3.8 Ⅱ DNA 为模板, 分别加入 L97 及 V60 点突变引物 L_1, L_2 及 V_1, V_2 进行定点突变, 构建成 p3.8 Ⅱ L97 和 p3.8 Ⅱ V60 重组质粒. 变异株重组质粒用 Pee_2 引物扩增后进行全自动测序(上海基康生物公司). 将 p3.8 Ⅱ野生株(WT)及变异株重组质粒以脂质体(Gibco)介导分别转染 $HepG_2$ 细胞, 用含 100 mL/L 胎牛血清的 DMEM 培养液(Gibco)培养 96 h, 收集细胞和培养上清, 用 Southern 印迹杂交(HBV 地高辛标记探针)分析细胞内 HBV DNA 的复制, ELISA 法测定上清中 HBV 抗原, 以检测其生物活性. EBO-野生株及变异株重组载体的亚克隆构建以 p3.8 Ⅱ WT, p3.8 Ⅱ L97 及 p3.8 Ⅱ V60 质粒 DNA 为模板, 各自加入 5'端带 *Sal* Ⅰ及 *Kpn* Ⅰ酶切位点的引物 P_1 及 P_2, 用 Z-*Taq* DNA 聚合酶(TaKaRa)进行 PCR 扩增, 各株 PCR 产物及 EBO-plpp 载体分别用 *Sal* Ⅰ及 *Kpn* Ⅰ(Promega)双酶切, 电泳后凝胶回收, 用 T_4DNA 连接酶(Promega)在 16 ℃连接过夜, 转化感受态细菌 XL-1, 筛选阳性克隆. 各株重组载体阳性克隆用 *Sal* Ⅰ及 *Kpn* Ⅰ双酶切, 进行凝胶电泳. 以 Pee_2 引物 PCR 扩增各株阳性克隆, 再次作自动化测序(上海基康生物公司). 将 EBO-WT, EBO-L97, EBO-V60 重组载体及 EBO 空载体 DNA 各 2 μg, 经脂质体 10 μL 介导转染 $HepG_2$ 细胞, 用潮霉素 200 mg/L 筛选, 每 2 d 定量更换培养液, 建立各株稳定表达的细胞. 转染实验重复进行3次. 用ELISA(Abbott)试剂盒测定各株细胞培养上清中 HBsAg 和 HBeAg 的含量, 分别以 S/N 值和 S/CO 值表示. 收集各株转染的 $HepG_2$ 细胞 2×10^6 个, 以 FITC 标记的鼠抗 HLA-ABC 单克隆抗体(Phar Mingen)室温避光染色 30 min, PBS 洗 2 次, 40 mL/L 多聚甲醛固定, 以未转染的 $HepG_2$ 细胞作阴性对照, 用流式细胞仪检测, 分析 5×10^4 个细胞的荧光强度.

2 结果

2.1 p3.8Ⅱ变异株重组质粒序列测定及生物活性检测 变异株质粒 DNA 测序结果, p3.8 Ⅱ L97 的 nt2189 A→C, p3.8 Ⅱ V60 的 nt 2 078 C→G, 证实定点突变. p3.8 Ⅱ变异株及 p3.8 Ⅱ野生株转染细胞的 Southern 印迹杂交显示在3.8 kb处呈现一明显杂交带, 符合目的基因组大小, 表明所构建的重组质粒均能在细胞内复制, 细胞培养上清中均测出 HBsAg 和 HBeAg , 有 HBV 抗原表达.

2.2 EBO 重组载体的酶切鉴定及序列测定 所构建的 EBO-WT, EBO-97 及 EBO-V60 重组载体 DNA 各经 *Sal* Ⅰ及 *Kpn* Ⅰ内切酶双酶切, 电泳后均出现二条区带, 分别与 HBV 目的基因组及 EBO 表达载体的大小相符. EBO-L97 及 EBO-V60 DNA 测序结果显示均保持原定的点突变.

2.3 EBO 重组载体转染细胞培养上清 HBsAg 和 HBeAg 定量检测 3 次转染实验的细胞培养上清中 HBV 抗原平均含量, 3 株表达的 HBeAg S/CO 值不同, EBO-L97 及 EBO-V60 均明显低于 EBO-WT, 3 株上清的 HBsAg S/N 值接近, 表达水平相似(表 2).

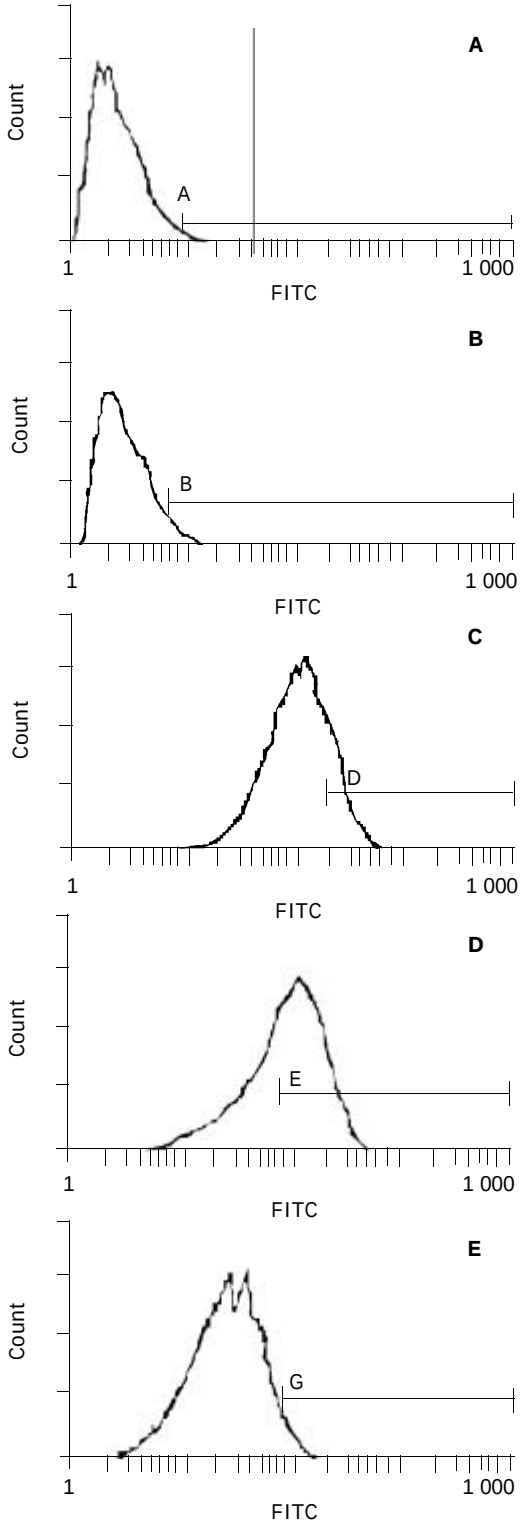
表 2 转染细胞培养上清 HBV 抗原含量

HBV 抗原	EBO-WT	EBO-L97	EBO-V60
HBsAg(S/N)	4.34	3.96	4.18
HBeAg(S/CO)	3.30	0.98	1.52

HBsAg 含量 S/N 参考值<2.00 HBeAg 含量 S/CO 参考值<1.00.

2.4 宿主细胞表面 HLA-I 表达 流式细胞术分析测定 3 批转染实验的 $HepG_2$ 细胞. 未转染的及EBO空载体转染

的 $HepG_2$ 细胞表面的 HLA-I 为轻微表达，其荧光强度为 1.8 和 2.2. 3 株重组载体转染细胞的 HLA-I 表达水平各不相同，野生株的 HLA-I 荧光强度增强为 18.2，L97 变异株升高至34.5，而V60变异株表达的荧光强度仅为 3.4 (图 1).



A: 空白未转染; B: EBO 空载体转染;
C: EBO-WT 转染; D: EBO-L97 转染; E: EBO-V60 转染
图 1 流式细胞仪对 $HepG_2$ 细胞表面 HLA-I 表达的检测.

3 讨论

HBV 并非直接导致肝细胞病变，而是其编码蛋白与机体免疫应答相互作用后，引起肝组织发生免疫病理损伤，同时抑制和清除细胞内外病毒，在免疫应答过程中 HLA-I 类分子限制的特异性 CD_8^+ T 细胞的细胞毒作用十分重要[13-18]. 正常肝细胞表面不表达或仅微量表达 HLA-I 类分子. 研究已表明 HBV 感染者肝细胞膜上 HLA-I 表达强度与肝组织病变活动性及损伤程度往往呈正相关，急性重型肝炎患者为强表达，慢性感染者在免疫耐受期为弱表达，免疫激活期 HLA-I 表达增强，肝组织炎症活动，部分病毒被清除[4,6,19]. 机体内细胞膜上 HLA-I 的表达受到病毒及免疫细胞激活后产生的细胞因子及其他诸多方面的诱导和调节[20,21]，建立细胞模型可探讨病毒本身与宿主细胞间的相互作用. 由于 HBV 基因组结构复杂，编码基因相互重叠，基因内部有调节序列[10,22,23]，单一基因片段的体外表达有局限性，用全基因重组质粒转染细胞更接近病毒在体内的复制、表达、加工和分泌情况. 我们用 HBV adr 亚型野生株含 1.2 拷贝基因组质粒 p3.8 Ⅱ，对核壳蛋白热点变异 97 位氨基酸及 60 位氨基酸分别进行定点突变，再双酶切定向亚克隆入 EB 病毒真核表达载体 EBO-plpp，能在转染的 $HepG_2$ 细胞中稳定表达，$HepG_2$ 细胞的 HLA 抗原型别以 HLA-A2 为主，HLA-A2 在亚洲人群中携带频率最高，因而本实验采用了较合理的细胞模型[24-26]，进行核壳蛋白密码子点突变的 HLA-I 表达的研究. 实验结果显示，HBV 野生株重组载体 EBO-WT 较 EBO-plpp 空白载体转染和未转染的 $HepG_2$ 细胞表面 HLA-I 表达明显增强，与国外(Zhou *et al* 1990， Takehara *et al* 1992) 体外表达的研究结果相同，初步表明 HBV 病毒自身可增强细胞膜上 HLA-I 表达. 其他病毒可上调感染细胞 HLA-I 表达的实验研究报道也不少，如黄热病病毒家族、呼吸道合胞病毒、单纯疱疹病毒、人副流感病毒 3 型等[27-31].

EBO-WT， EBO-L97 及 EBO-V60 重组载体转染 $HepG_2$ 细胞稳定传代后，培养上清中 HBV 抗原定量检测结果，3 株表达 HBeAg 的水平不同，L97 及 V60 变异株的 HBeAg S/CO 值明显较野生株为低，3 株的 HBsAg S/N 值接近，HBsAg 表达水平相近，表明重组载体转染率及检测结果相当，因而其转染细胞膜上 HLA-I 表达分析有一定可比性. 流式细胞术分析 3 株宿主细胞 HLA-I 表达，L97 变异株 HLA-I 荧光强度高于野生株，而 V60 变异株表达强度低于野生株，表明 HBV 核壳蛋白单个错义点突变可使宿主细胞表面 HLA-I 表达发生差异. Yuan *et al* [32-34]用 HBV adr 亚型双拷贝基因组的核壳蛋白变异株做体外实验，发现 L97(I97L)及 V60(L60V) 变异在 $HepG_2$ 细胞内的复制水平与野生株相近，但 L97 分泌未成熟的(含 ssDNA)Dane 颗粒，且颗粒数量明显增加，V60 则分泌成熟的 Dane 颗粒，其量下降 3-6 倍，充分说明 HBV 核壳蛋白上述密码子错义突变能引起病毒的表达、加工、分泌等生物学功能的变化. 我国 HBV 感染率高[35-40]，关于 HBV 转染细胞中内源性细胞因子对

HLA –Ⅰ表达的影响以及核壳蛋白 L97 及 V60 变异导致宿主细胞 HLA-Ⅰ表达变化的机制，均有待深入研究探讨.

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