HBeAg阴性小鼠模型的研究现状与展望

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摘要 乙型肝炎病毒(hepatitis B virus, HBV)感染是全球肝病的重要原因之一, 全球约有2.5亿患者感染HBV. 其中, 慢性HBV感染将导致肝纤维化(liver fibrosis), 肝硬化(liver cirrhosis, LC)甚至肝细胞癌(hepatocellular carcinoma, HCC). HBV感染的临床进程可分为乙型肝炎e抗原(hepatitis B e antigen, HBeAg)阳性和HBeAg阴性两个阶段. 而长期携带和难以清除的共价闭合环状DNA(covalently closed circular DNA, cccDNA)是两个阶段共同的固有特征. 慢性HBV感染不能完全治愈, 其致命原因是没有有效药物能彻底清除cccDNA; 严重瓶颈是无理想的实验动物模型, 尤其是针对HBeAg阴性cccDNA小鼠模型. 目前, HBeAg阴性慢性乙型肝炎患者在全球范围内呈逐年上升趋势, 本文就cccDNA的重要性、HBeAg基因结构及HBeAg转阴机理、HBeAg阴性cccDNA小鼠模型的研究现状与未来的应用前景进行简要述评.

关键词 HBV, 慢性乙型肝炎, HBeAg阴性, cccDNA, 小鼠模型

据世界卫生组织最近统计, 全球有超过2.5亿人携带乙型肝炎病毒(hepatitis B virus, HBV), 东南亚与非洲部分地区仍为“重灾区”, 其中我国约有8600万以上^[1]. HBV感染是全球肝病包括肝纤维化、肝硬化、肝功能衰竭和肝细胞癌等疾病发生的重要原因^[2]. 尽管已有最新的基因工程HBV疫苗和多种抗HBV的药物开发和应用, 但HBV感染仍然是全球面临的重大健康问题之一. 根据普遍接受的HBV感染的自然发病史, 临床上可分为HBeAg阳性(包括免疫耐受与免疫清除期)和HBeAg阴性(非复制与免疫再激活期)两个阶段^[3]. 迄今, 已有众多HBeAg阳性动物模型的研究报道, 在开展HBeAg阳性相关发病机理及药物评价中显示了重要的作用. 随着HBeAg阴性患者发病率逐年上升, HBeAg的生物学功能、阴性毒株的产生机制、以阴性毒株建立慢性模

型等研究成为全球关注的新焦点^[4]. 本文对HBeAg阴性cccDNA小鼠模型的研究现状与展望进行如下综述.

1 HBV cccDNA的重要性

HBV是特异感染人类最小的嗜肝DNA病毒, 基因组全长约3200 bp, 其进入细胞前为不完全闭合的松弛环状(relaxed circular DNA, rcDNA), 正链较短, 长度不定, 与完整的负链基因组相比有较大的缺口. 含有rcDNA的成熟的病毒颗粒具有较高的感染力, 首先通过特异性受体进入细胞质, 再脱去包膜后基因组进入细胞核, 在病毒多聚酶蛋白的作用下, 形成以共价键紧密连接的完全闭合的环状DNA分子(covalently closed circular DNA, cccDNA)^[5]. cccDNA以超螺旋的小型染色体的形式, 长期稳定地存留在肝细胞核内, 作为病毒

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复制周期的总模板, 长期持续地产生新的病毒颗粒, 并通过产生的新的rcDNA再进入细胞核对自身进行循环式的更新和补充^[6-8]. 因此, cccDNA既是慢性乙肝长期持续迁延的根本原因, 也是现有药物难以根除病毒以及停药后复燃的症结所在, 是慢性乙肝的固有标志.

2 HBeAg基因结构及HBeAg转阴机理

2.1 HBeAg编码基因结构

HBV具有S、C、P和X 4个重叠的开放阅读框(ORF), HBV C区包括前C基因和C基因编码区, 分别编码病毒HBeAg及核心抗原(hepatitis B core antigen, HBcAg). 前C基因位于nt1814~2452, 编码HBeAg前体, 含212个氨基酸(25 kD). 氨基端是含有19个氨基酸残基的非结构信号肽, 能使HBeAg前体进入内质网, 羧基端是精氨酸富集区, 含33个氨基酸残基. 在内质网, 其氨基端的信号肽和羧基端残基分别被切除和水解, 由于水解后羧基端暴露半胱氨酸残基, 使其不能进行病毒颗粒组装, 最终形成可溶性的HBeAg(15~18 kD), 由高尔基复合体转运并分泌到血液中^[9](图1).

2.2 HBeAg转阴机理

临床慢性乙型肝炎(CHB)常见有两种血清学模式: HBeAg阳性和HBeAg阴性^[10]. HBeAg阴性的慢性乙型肝炎目前在世界范围内呈逐年上升趋势, 其在流行病学、发病机理、自然病程和抗病毒治疗等方面与HBeAg阳性慢性乙型肝炎存在很大不同^[11-13]. 在一项关于慢性乙型肝炎感染自然史的研究中, 纳入的患者中约94.1%(206/219)为HBeAg阴性慢性乙型肝炎^[14]. 在服用替诺福韦8年后, HBeAg阳性的患者中表面抗原

(hepatitis B surface antigen, HBsAg)转阴率约10%, 而HBeAg阴性的患者HBsAg转阴不到1%^[15]. HBeAg阴性的慢性乙型肝炎患者总是需要核苷类似物终身治疗. 此外, 其病程较HBeAg阳性感染更为复杂, 也最难获得持续病毒学应答^[16].

HBeAg阴性慢性乙型肝炎形成的主要机制是HBV的高复制率、聚合酶缺乏校正功能、机体免疫压力及药物压力导致的两种基因变异: 一是转录水平的基本核心启动子(basic core promoter, BCP)突变, 典型位点为ntA1762T/G1764A联合突变^[17], 由于该突变影响preC mRNA的转录, 从而导致HBeAg表达显著降低^[18,19]; 二是翻译水平的前C区ntG1896A突变, 其突变导致28位TGG(色氨酸)转变为TAG(终止密码子), 从而使HBeAg合成终止^[20]. 此外, 前C区ntG1899A^[21]及C区ntC1914G, ntC2048T/G, C2049A, ntG2291C/A, C2292A/G, T2293C, ntC2304A, ntT2441C, C2442A也会导致HBeAg表达不同程度的降低^[20](表1).

3 HBeAg阴性慢性乙型肝炎小鼠模型的状态

目前虽然已有许多关于HBeAg阳性HBV感染的小鼠模型研究, 模型的建立方法主要有转基因技术^[22-25]、重组腺病毒(adeno virus, AV)及腺相关病毒(adeno-associated virus, AAV)载体介导方法^[26-30]、构建人源化小鼠的方法^[31,32], 及高压水动力注射方法^[33-35]等, 这些模型的建立在诠释HBV生活史及发病机制中彰显其重要作用^[22]. 然而, 即使现有建模方法可以借鉴于HBeAg阴性cccDNA慢性乙型肝炎小鼠模型的建立, 但迄今仅有少量HBeAg阴性小鼠模型报道^[36,37]. 其模型建立的基本流程、HBeAg阴性cccDNA

图1 (网络彩色版)HBV及HBeAg编码基因结构

Figure 1 (Color online) The genetic structure of HBV and HBeAg

表 1 HBV BCP区、前C区和C区变异及对HBeAg形成的影响

Table 1 The variation in HBV BCP, Pre-C, core region and its influence on formative of HBeAg

基因区	变异		影响	文献
	核苷酸	氨基酸		
BCP (1743~1849) Pre C (nt1814~1900)	ntA1762T, G1764A	K130M, V131I	HBeAg-reduce	[18]
	ntG1896A	W28 stop	HBeAg-negative	[20]
	ntG1899A	G29D	HBeAg-reduce	[21]
C (nt1901~2452)	ntC1914G	P5R	HBeAg-reduce	[20]
	ntC2048T/G, C2049A	S64A, P50Y/H/A	HBeAg-reduce	[20]
	ntG2291C/A, C2292A/G, T2293C	A131P/N/G	HBeAg-reduce	[20]
	ntC2304A	P135Q	HBeAg-reduce	[20]
	ntT2441C, C2442A	S181P/H	HBeAg-reduce	[20]

图 2 HBeAg阴性cccDNA小鼠模型建立基本流程

Figure 2 The basic procedure about the HBeAg negative mouse model with cccDNA

制备、模型鉴定指标、意义及其检测方法和模型表型如下。

3.1 基本流程

首先, 制备、鉴定以及纯化HBeAg阴性cccDNA模板。然后, 选择6~8周龄雄性的受体小鼠。最后, 建立、鉴定小鼠模型(图2)。

3.2 HBeAg阴性cccDNA制备

HBeAg阴性cccDNA的制备模板来源于的HBV PreC ntG1896A/ntG1899A及BCP ntA1762T/G1764A位点联合突变的患者血清样本。提取血清样本DNA, 采用PCR扩增HBV全长基因, 并将其序列克隆构建HBeAg

阴性HBV质粒, 采用BspQ I酶切、胶回收、连接、在体外环化制备cccDNA并进行酶切和测序验证^[36]。通过上述方法制备得到的HBeAg阴性HBV cccDNA, 没有碱基插入、缺失或者突变, 接近于感染HBV患者体内cccDNA状态。此外, 有研究组以Cre-loxp介导位点特异性DNA重组技术^[38,39]以及微环技术^[40,41]产生重组cccDNA(rcccDNA)。

3.3 模型的表型

Wang等人^[36]以C57BL/6J小鼠为受体小鼠, 采用尾静脉水动力注射PreC/BCP联合突变cccDNA, 建立了cccDNA慢性乙型肝炎小鼠模型。该模型特征: HBeAg阴性, 在注射PreC/BCP联合突变cccDNA5周内, 小鼠HBV感染率为100%, 且60%的小鼠HBV感染可持续达10周左右, 血清中HBsAg持续表达, HBsAg阳性的小鼠血清中检测到HBV DNA拷贝数可达 10^7 拷贝/mL; 肝脏组织中可检测到HBsAg, HBeAg的表达及慢性乙肝固有标志cccDNA的存在, HBV DNA拷贝数可达 10^8 拷贝/g; 肝脏组织中发现有多个单核细胞浸润, 整个感染周期血清ALT水平无明显波动。Cao等人^[37]进一步对Wang等人^[36]所用的PreC/BCP联合突变cccDNA基因序列进行了分析, 结果表明, 除存在典型BCP区ntA1762T/G1764A及前C区ntG1896A/ntG1899A联合突变外, 该血清基因型为B型, 基因亚型为B2, 血清亚型为adw2, 无耐药位点和免疫逃逸位点。随后, Cao等人^[37]再以CBA/CaJ小鼠为受体小鼠, 采用Wang等人^[36]所用方法注射PreC/BCP联合突变的cccDNA建立了HBeAg阴性cccDNA慢性乙型肝炎小鼠模型, 其血清标志物HBsAg在小鼠体内能携带长达26周(半年)以上。

HBeAg阴性cccDNA慢性乙型肝炎小鼠模型的建立, 对进一步进行HBeAg阴性发病机制研究、开展

HBeAg阴性新药创制研究以及在更高更深的层次上进行系统、精准的评价基因编辑, 根除cccDNA的新策略^[42]等具有重要意义。

3.4 模型鉴定指标、意义及其检测方法

血清标志物包括HBV DNA, HBsAg, HBeAg和HBcAg (hepatitis B core-related antigen), 其能很好反映病毒感染状态及复制活跃程度。肝组织标志物包括cccDNA, HBV DNA, HBsAg和HBcAg。cccDNA是病毒在肝脏中进行复制和增殖的直接模板, 其决定了HBV的复制水平; HBV DNA则是病毒复制最直接的标志, 肝脏中HBsAg与血清中HBsAg呈正相关。HBcAg与HBeAg约有四分之三的氨基酸序列相同, 肝脏中检测出HBcAg和血清中检测出HBeAg皆能反映病毒复制活跃。血清生化包括转氨酶ALT及AST, ALT和AST能反映肝细胞炎症坏死程度, 其水平能作为HBV感染肝组织炎症活性的一个指标。肝脏病理情况包括观察肝脏炎症反应、是否发生肝纤维化、肝细胞癌变, 能反映感染模型的病理状态。

检测方法: 血清病毒抗原可通过酶联免疫吸附测定(enzyme linked immunosorbent assay, ELISA)方法、放射免疫法对其表达水平进行定量分析; 血清ALT及AST可通过血液生化仪及酶联免疫吸附测定方法对其进行定量分析; 肝组织病毒抗原可通过免疫组化或免疫荧光检测HBsAg, HBcAg在肝脏中表达; 肝脏炎症病理变化及纤维化病变可分别通过苏木精-伊红(hematoxylin-eosin, HE)染色法、天狼星红(Sirius Red)染色和Fibrotest进行评价。

cccDNA作为慢性乙肝固有标志, 由于其在细胞内拷贝数较低, 检测较难, 所以在HBeAg阴性cccDNA小鼠模型的指标评价中, cccDNA的检测方法至关重要。Southern blot作为广泛接受的一种检测方法, 其可信度高, 可重复性好, 但是其灵敏度低, 操作复杂, 不适用于大量的药物筛选研究。定量PCR(qPCR)其灵敏度高, 检测简便快速, 适用于药物筛选, 但其特异性不强, 结果

易出现假阳性。原位杂交技术特异性强, 能很好区分不同形式的HBV DNA和RNA, 能对单个细胞内的cccDNA进行细胞定位, 有利于药物研究过程中对cccDNA进行实时动态跟踪^[43]。但其操作较复杂, 有待进一步优化。闭环扩增联合原位PCR(rolling circle amplification combined with *in situ* PCR, RCA-IS-PCR)能对cccDNA的存在进行直接检测, 同时对细胞核内原位的cccDNA水平进行直观、敏感和特异的“终极”鉴定, 有望成为cccDNA理想的检测方法^[44]。

4 模型的应用前景

随着HBeAg阴性HBV感染逐年上升, 进行HBeAg阴性慢性乙型肝炎发病机制以及抗病毒药物研究日趋重要。而cccDNA是现有药物难以根除病毒以及停药后复燃的症结所在, 清除肝细胞核内cccDNA是抗病毒治疗的终极目标^[45]。因此, 建立能够长期携带cccDNA且能模拟HBeAg阴性慢性乙型肝炎的小鼠模型具有非常大的应用前景。

现有HBeAg阴性cccDNA慢性乙型肝炎小鼠模型具有以下特点: 建立方法简单、快速和方便; 可以持续携带慢性乙肝固有标志(cccDNA); 免疫功能健全; 病毒株DNA来源于临床。模型可以广泛运用于抗病毒药物、基因治疗(如基因编辑技术)及免疫治疗(如淋巴毒素受体激动剂BS1和CBE11)^[46]评价; 病毒与宿主相关的免疫机制研究; 可为乙型肝炎病人的个体化和精准治疗奠定基础。

在现有基础上, 可以通过分子生物学方法回复突变PreC/BCP联合突变基因序列, 以同样的方法制备HBeAg阳性cccDNA, 同时建立HBeAg阳性、HBeAg阴性cccDNA慢性乙型肝炎小鼠模型。通过对比分析, 可进一步精准探讨HBeAg在HBV慢性感染过程中的生物学功能, HBeAg与HBV持久性感染的免疫机制, 两种血清学模式下HBV携带时限、病毒标志物表达水平差异。综上, 以期新型抗HBV药物对两种不同血清学模式下药效评估提供通用模型。

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Summary for “HBeAg阴性小鼠模型的研究现状与展望”

The present situation and prospect of research on HBeAg-negative mouse model

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Hepatitis B virus (HBV) infection is one of the important causes of global liver disease. There are about 250 million patients infected with hepatitis B virus in the world. Chronic HBV infection will lead to liver fibrosis, liver cirrhosis (LC) and even hepatocellular carcinoma (HCC). HBV is a partially double-stranded circular 3.2 kb DNA virus containing four overlapping open reading frames that encode HBsAg, HBeAg, HBcAg, HBV polymerase and HBx protein, respectively. Once HBV particles interacting with sodium taurocholate cotransporting polypeptide (NTCP) on the cell membrane, the nucleocapsid is released into cytoplasm. Subsequently, relaxed circular DNA (rcDNA) is converted to covalently closed circular DNA (cccDNA) in the nucleus, then forms stable minichromosome in the existence of histone and nonhistone proteins. The nucleocapsid can either assemble with viral envelope and released as mature HBV particles or transport back to the nucleus to refill the cccDNA pool. HBV cccDNA serves as a template for viral mRNA synthesis. Persistence of cccDNA is the major course that HBV is hard to eliminate and relapses after drug withdrawal. Though current approved drugs have made huge progress in suppressing viral replication, completely eliminating HBV remains a challenge. The natural history of chronic hepatitis B (CHB) may go through four phases, including the immune tolerant, the immune clearance, the inactive or non-replicative and the re-activation or immune escape phases. During the first two phases the patient is hepatitis B e antigen (HBeAg) positive. On the contrary, during the last two phases the patient is HBeAg-negative. Most infected patients that are HBeAg-negative is usually associated with HBV gene dual mutations of ntA1762T/G1764A and mutation of ntG1896A leading to reduce or HBeAg-negative in basic core promoter (BCP) and PreC region, respectively. The prevalence of HBeAg-negative patients is likely to vary across geographic areas. In recent years, HBeAg-negative patients are increasing globally. Compared with HBeAg-positive patients, HBeAg-negative patients have the following clinic characteristics: More complex course of disease, lower sustained response rate to antiviral drugs and more likely to develop into end-stage liver disease. The mechanism research and drug treatment on HBeAg-negative infection are seriously hampered by the absence of ideal animal model: Simple and efficient transduction method, long-term cccDNA, and model animal with normal immunological function. So far, there have been numerous studies on mouse models of CHB, but few reports about HBeAg-negative mouse models. It is of great significance to study disease mechanisms and develop therapeutic drugs of HBeAg-negative CHB infection using HBeAg-negative CHB mouse model with cccDNA and normal immunological function. In this paper, we have briefly reviewed the research status and application prospect of the model.

hepatitis B virus, chronic hepatitis B, HBeAg-negative, covalently closed circular DNA, mouse model

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