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胆管上皮细胞 TLR3 内源性活化在原发性胆汁性肝硬化中的作用

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摘要 目的:探讨胆管上皮细胞中 Toll 样受体 3(TLR3)内源性活化对原发性胆汁性肝硬化损伤的影响。**方法:**体外培养人肝内胆管上皮细胞(HiBEC),并通过冻融处理制备坏死 HiBEC 样本。将死亡样本与活样本混合培养 6 h,以制备 TLR3 内源性活化样本,将其标记为观察组;以核酸酶处理坏死细胞及活细胞,并混合培养 6 h,以制备对照样本,将其标记为对照组。检测 2 组细胞凋亡率、TLR3 及β干扰素 Toll 样受体结构域衔接蛋白(TRIF)表达水平、Caspase-3 活性。**结果:**观察组凋亡率明显高于对照组,TLR3 及 TRIF 相对表达量明显高于对照组,细胞 Caspase-3 平均表达量明显高于对照组,上述差异均有统计学意义(P＜0.05)。**结论:**TLR3 的内源性活化可导致 HiBEC 凋亡,在原发性胆汁性肝硬化的发生及发展中可能有一定促进作用。

关键词: Toll 样受体 3;胆管上皮细胞;原发性胆汁性肝硬化;内源性活化

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Role of Endogenous TLR3 Activation in Primary Biliary Cirrhosis

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ABSTRACT Objective: To investigate the effect of Toll like receptor 3 (TLR3) on the injury of primary biliary cirrhosis in bile duct epithelial cells. **Methods:** The human intrahepatic bile duct epithelial cells (HiBEC) were cultured in vitro, and HiBEC samples were prepared by freezing and thawing treatment. Death samples and the live samples were mixed and cultured for 6h to prepare TLR3 endogenous activated samples, labeled as observation group. After treated with nuclease, the necrotic cells and live cells were mixed and cultured for 6h to prepare a control sample, labeled as control group. The apoptosis rate, the expression level of TLR3 and the expression level of β interferon-Toll-like receptor domain adaptor protein (TRIF) and the Caspase-3 activity were detected in the two groups. **Results:** The apoptosis rate of the observation group was significantly higher than that of the control group. The TLR3 and TRIF relative expression of the observation group was also significantly higher than that of the control group. Moreover, the caspase-3 average expression of the observation group was obviously higher than that of the control group. All the above mentioned differences were statistically significant (P<0.05). **Conclusion:** The endogenous activation of TLR3 may lead to HiBEC apoptosis. It may promote the occurrence and development of primary biliary cirrhosis.

Key words: Toll like receptor 3; Bile duct epithelial cells; Primary biliary cirrhosis; Endogenous activation

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前言

HiBEC 细胞的炎性细胞浸润及细胞凋亡是原发性胆汁性肝硬化的早期病理改变之一[1],探明此炎性反应的过程,有助于明确其该病的机制。研究证实 T 细胞、NK 细胞介导的炎性反应能够导致 HiBEC 凋亡[2],但上述研究忽视了 HiBEC 直接参与免疫调节的能力,因此存在一定局限性。报道指出,HiBEC 细胞能够通过自身表达 TLR 及多种粘附分子等途径，直接参与炎症反应[3],以 TLR3 为例,其能够受坏死细胞释放的 RNA 影响,出现内源性活化,可能也参与了原发性胆汁性肝硬化的形成及发展。本研究采用体外实验，重点探讨了胆管上皮细胞 TLR3 内源性活化在原发性胆汁性肝硬化中的作用机制。

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1 材料与方法

1.1 材料及仪器

主要材料:HiBEC，由美国 Sciencell 提供;1640 培养基及胎牛血清,由美国 Hyclone 提供;核酸酶,由美国 Sigma 提供;Caspase-3 抗体，由美国 BD bioscince 提供;RNA 提取试剂盒,由美国 Qiagen 提供。主要仪器:FACSCalibur 流式细胞仪,由美国 BD 提供;PCR 分析仪,由美国 ABI 提供。

1.2 方法

样本制备：⓪ 体外培养 HiBEC：在 37℃、5% CO_2、RPMI 1640 完全培养基条件下培养。取适量 HiBEC,通过反复冻融制备死亡细胞。按照 2:1 的比例混合死细胞与活体细胞,4℃条件下孵育 6 h 后作为观察组;按照 2:1 的比例混合死细胞与活体

细胞，并经核酸酶处理，在4℃条件下孵育6 h，作为对照组；同条件下培养36 h。

检测指标：① 采用流式细胞术检测HiBEC凋亡率：收集细胞样本，加入缓冲液（由三羟甲基氨基甲烷50 mmol/L、NaCl 100 mmol/L、BSA 1%，叠氮化钠0.02%构成，调整pH为7.4），调节密度至每毫升50× 10^5个细胞，取195 μL悬液加入5 μL Annexin-V，常温静置10min，再行洗涤，并加入缓冲液190 μL，最后加入20 μg/mL PI 10 μL，应用流式细胞仪检测细胞凋亡率，统计早期凋亡率（Annexin-V^+/PI^-区域）、晚期凋亡率（Annexin-V^+/PI^+区域）及总凋亡率（=早期凋亡率+晚期凋亡率）；② 采用荧光定量PCR技术检测TLR3 mRNA、TRIF mRNA水平：收集细胞并提取总RNA，利用基因库相关数据设计TLR3 mRNA、TRIF mRNA序列合成引物，将总RNA反转录为cDNA，应用定量PCR扩增，读取阈循环值（Ct），根据△Ct=目的基因Ct-内参基因Ct及△△Ct=研究组△Ct-对照组△Ct，计算$2^{-\triangle\triangle Ct}$。③ 以流式细胞术检测Caspase-3活性：计算Perm/Wash缓冲液及抗体用量，制备Perm/Wash缓冲液，收集细胞后首先以预冷PBS缓冲液洗涤2次，并调整细胞密度至1× 10^6/0.5mL，冰浴20 min后离心弃上清，在室温下以Perm/Wash缓冲液洗涤2次，最后应用流式细胞仪检测Caspase-3活性。

1.3 统计学方法

计量资料按（$\bar{x}\pm s$）表示，组间比较采用t检验，以$P<0.05$为差异有统计学意义。

2 结果

2.1 两组HiBEC凋亡率对比

观察组早期凋亡率、晚期凋亡率及总凋亡率均明显高于对照组，差异有统计学意义（$P<0.05$）（表1与图1）。

表1 两组HiBEC凋亡率对比（$\bar{x}\pm s$，%）

Table 1 Comparison of the HiBEC apoptosis rate between the two groups（$\bar{x}\pm s$，%）

Groups（n）	early apoptosis rate	later period apoptosis rate	total apoptosis rate
Observation group（3）	15.81± 2.05	18.17± 2.01	33.98± 2.39
Control group（3）	1.13± 0.27	1.75± 0.33	2.88± 0.58
t	12.297	13.962	21.903
P	0.000	0.000	0.000

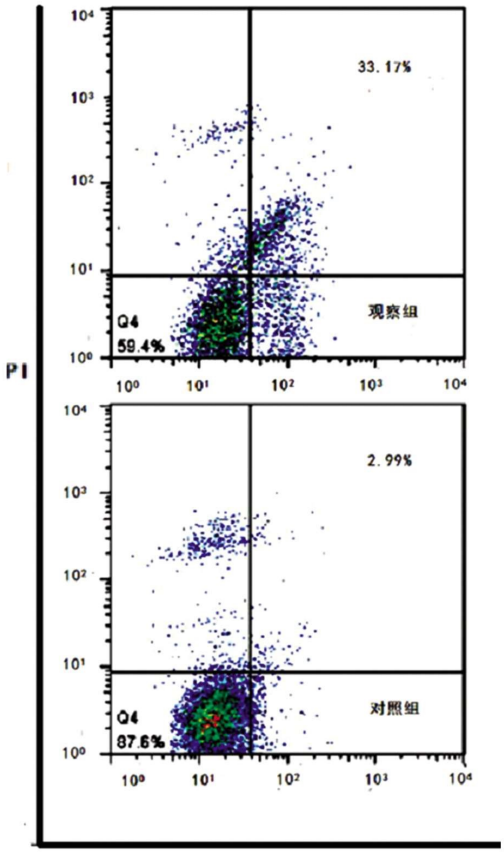

图1 两组细胞凋亡率对比

Fig. 1 Comparison of the cell apoptosis rate between the two groups

2.2 两组TLR3 mRNA及TRIF mRNA表达水平对比

观察组TLR3 mRNA及TRIF mRNA表达水平均明显高于对照组，差异有统计学意义（$P<0.05$）（表2）。

表2 两组TLR3 mRNA及TRIF mRNA表达水平对比（$\bar{x}\pm s$）

Table 2 Comparison of the mRNA expression levels of TLR3 and TRIF between two groups（$\bar{x}\pm s$）

Groups（n）	TLR3 mRNA	TRIF mRNA
Observation group（3）	3.77± 0.25	4.17± 0.18
Control group（3）	0.93± 0.07	1.05± 0.07
t	18.947	27.981
P	0.0000	0.0000

2.3 两组细胞Caspase-3活性对比

观察组Caspase-3阳性率（27.7± 3.1）%，明显高于对照组（8.2± 1.8）%，组间差异有统计学意义（t=9.422；$P<0.001$）。

3 讨论

原发性胆汁性肝硬化为常见自身免疫性疾病，此类疾病的直接病因多为自身免疫系统对自身细胞、组织及器官的免疫耐受丧失，使得免疫反应直接对自身造成损伤[4,5]，目前尚未完全探明此类疾病发病机制。临床研究[6]显示，原发性胆汁性肝硬化的发病，不仅与适应性免疫有关，还与固有免疫有关，固有免疫可能加速了该疾病的发展。

TLR3在固有免疫中发挥了关键作用，费允云等[7]报道显示TLR3介导的信号通路刺激IRF-3产生1型干扰素，进而诱导疾病的发生，但此研究主要通过polyI:C活化TLR3信号通路，并未涉及TLR3的内源性活化；赵立宇等[8-10]报道也显示TLR3作用下，NK细胞对HiBEC的杀伤能力有一定上升，但尚未明确TLR3与HiBEC主动参与炎性反应的关系。以既往研究为

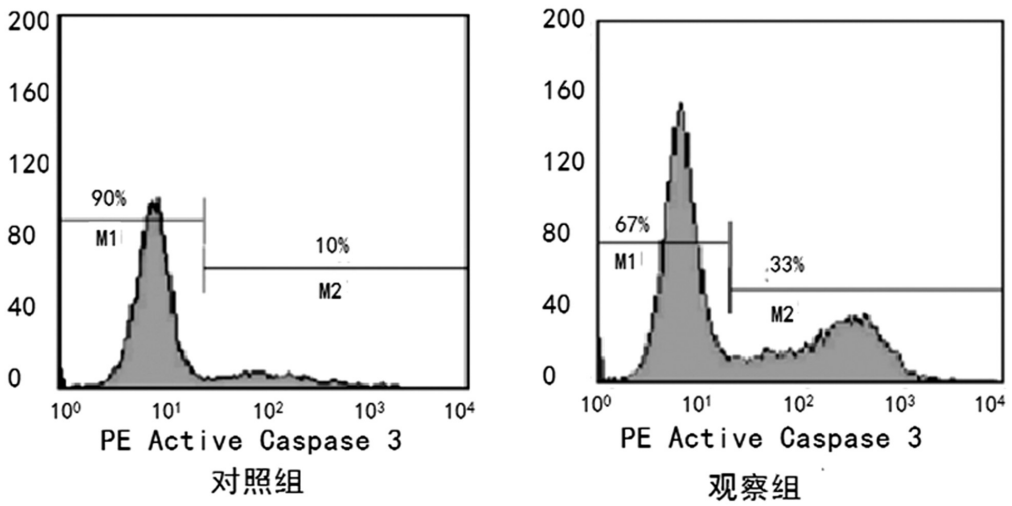

图 2 两组细胞 Caspase-3 活性对比

Fig.2 Comparison of the Caspase 3 activity between the two groups

基础，本研究重点分析了 TLR3 的内源性活化是否与也与 HiBEC 的炎性反应有关,及该内源性活化的具体机制。

本研究利用坏死 HiBEC 与活体 HiBEC 共同培养，制备 TLR3 内源性活化 HiBEC 样本，其原理在于 HiBEC 坏死所释放的内源性 RNA 能够通过 TLR3 途径，活化周边正常上皮细胞[11]。观察组细胞凋亡率明显高于对照组,提示在原发性胆汁性肝硬化早期,少量 HiBEC 细胞受炎性反应杀伤后,其释放的内源性 RNA 能够进一步活化周边细胞,导致损伤的迅速扩散,从而加速 HiBEC 的病理性损伤,上述结果提示,在早期阻断内源性 RNA 对 TLR3 途径的活化,可能有助于控制疾病,但具体的阻断方案,可能仍需探讨。

TLR3 活化与 HiBEC 细胞损伤的关系与其信号通路有关[12-14]。TLR3 的信号通路主要有 2 条,其一为髓样细胞分化因子 88 依赖性信号通路，该通路下游为丝裂原活化蛋白激酶和 Nk-κB,下游受到激活后,释放 TNF-α 及 IL-6 等细胞因子,可以参与并加剧炎性反应,从而导致 HiBEC 细胞损伤[15-17];TLR3 还存在一条非髓样细胞分化因子 88 依赖性信号通路，该通路主要能活化 IFN 调节因子 3,诱导产生 IFN-β,从而直接诱导细胞凋亡,以此损伤 HiBEC 细胞[18-20]。本研究观察到观察组 TLR3 和 TRIF mRNA 表达量明显升高,提示 HiBEC 的进展性凋亡过程中,TLR3 通路处于激活状态,且由于 TRIF 主要参与非髓样细胞分化因子 88 依赖性信号通路,故 HiBEC 的进展性损伤主要与非髓样细胞分化因子 88 依赖性信号通路的激活有关,HiBEC 的自发性凋亡在原发性胆汁性肝硬化中有一定作用。观察组 Caspase-3 的表达量上升，则提示 TLR3 内源性活化所致的细胞凋亡,与 Caspase-3 途径有关,这有助于明确凋亡发生的信号途径。上述结果提示,阻断非髓样细胞分化因子 88 依赖性信号通路的激活,可能有助于控制疾病,但本研究并未深入得出具体的阻断方案,可能仍需后续研究补充。

综上所述，本研究证实原发性胆汁性肝硬化早期,HiBEC 细胞的局部性损伤可导致内源性 RNA 的释放，进而活化周边正常细胞 TLR3,导致炎症反应的扩大及细胞凋亡率的上升。其中细胞凋亡可能与非髓样细胞分化因子 88 依赖性信号通路有关,这有助于解释疾病的发生及发展机制。明确上述机制,有助于为控制疾病提供靶向的干预方式,这还有待后续研究补充。

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