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CD95配体分子诱导人肝癌细胞凋亡的作用

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cDNA cloning and expression of human CD95 ligand and its role in apoptosis of HepG$_2$ cell lines

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Abstract

AIM: To investigate CD95 ligand and its physiological function in liver neoplasms.

METHODS: The levels of soluble Fas ligand (sFasL) were evaluated in a group of patients affected by hepatitis B virus (HBV)-induced chronic hepatitis, HBV-positive liver cirrhosis and hepatocellular carcinoma (HCC). To further study, we constructed recombinant eukaryotic expression vector pcDNA3.1 hisB-CD95L, which was then tranfected into human hepatoma cell line HepG2 by lipofection. After stained by annexin V and propidium iodine, HepG2 cells were detected by flow cytometer.

RESULTS: s CD95L levels were significantly decreased in patients with HCC when compared to the patients with hepatitis or liver cirrhosis. The correct recombinant pcDNA3.1hisB-CD95L was selected by PCR and restriction endonuclease digestion and confirmed by DNA sequencing respectively. Subsequently a significant proportion of cells became apoptotic, as evidenced by positive annexin staining.

CONCLUSION: CD95-CD95 ligand system can induce apoptosis of hepatoma cells.

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摘要

目的: 探讨CD95 L在肝癌细胞凋亡过程中的作用.

方法: ELISA法对慢性乙型肝炎、肝炎肝硬化与肝癌患者血清可溶性CD95 L(sCD95L)水平进行了初步检测, 构建了人CD95 L的重组真核表达体pcDNA3.1hisB-CD95 L, 将pcDNA3.1hisB-CD95 L转染至人肝癌细胞株HepG2细胞, 采用Annexin V/PI双染后双变量流式细胞仪检测细胞凋亡率.

结果: sCD95L在肝癌患者明显低于肝炎及肝硬患者, 构建的表达重组体pcDNA3.1hisB-CD95 L经菌落PCR和限制性酶切消化有预期的目的片段出现, DNA序列分析证实CD95 L完整、正确插入, 转染后的HepG2细胞细胞凋亡率为36.30%; 未转染CD95L的对照组细胞凋亡率11.53%.

结论: CD95L可使肝癌细胞凋亡.

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0 引言

CD95分子及其配体CD95配体(CD95 ligand)在细胞凋亡的信号传导过程中有重要作用. CD95配体通过与靶细胞CD95分子结合, 传导凋亡信号, 诱导靶细胞凋亡. 在肝癌细胞发生发展和转移过程中, CD95配体的作用尚不清楚. 拟比较可溶性CD95配体在慢性乙型肝炎、肝炎肝硬化、肝癌患者血清中的差异, 将CD95配体cDNA克隆, 构建pcDNA3.1hisB表达载体, 转染至人肝癌细胞HepG2, 观察CD95配体对该细胞株细胞凋亡的影响.

1 材料和方法

1.1 材料 人CD95配体定量EIA检测试剂盒购自MBL公司, Trizol试剂、cDNA第一链合成试剂盒、Lifefectamine regent、RPMI1640培养基购自Gibco公司, Qiagen mini kit, Qia quick gel extration kit购自Qiagen公司, plus SV minipreps DNA purification reagent system, *Eco*RI、*Bam*H I内切酶、T4DNA连接酶、Taq酶、胎牛血清购自Promega公司, FITC-Annexin V试剂盒购自北京宝灵曼公司, 兔抗人CD95(即用型)及SP-超敏免疫组化检测试剂盒购自福建迈新公司, 其余试剂为本室自备. HepG2人肝癌细胞株购自中南大学湘雅医学院细胞中心 HepG2.2.15人肝癌细胞株由第一军医大学传染病研究所馈赠, PCR pGEM-T easy vector为Promega公

司产品，pcDNA3.1hisB为Invitrogen公司产品. 选用2003-01/02中南大学湘雅二医院传染科住院患者22例，其中慢性重度病毒性肝炎(乙型)12例，肝炎肝硬化(活动期)6例，诊断标准遵照2000-09西安中华医学会传染病与寄生虫病学分会、肝病学分会联合修订《病毒性肝炎防治方案》. 选用2002-06/2003-01中南大学湘雅二医院肝胆外科住院患者16例，经病检确诊为肝细胞癌. 另选6名正常志愿者为对照.

1.2 方法 采用双抗体夹心EIA检测慢性肝炎、肝硬化、肝癌患者血清的可溶性CD95配体水平，酶标仪采用Lab Systems，Wellscan MK2全自动酶标仪. 无菌采取一例慢性乙肝患者前臂静脉血10 mL，进行PBMC分离，ConA活化后培养6 h，采用Trizol RNA提取细胞总RNA. 将CD95配体基因编码区的cDNA克隆pGEM-T easy克隆载体，根据文献报道的人CD95L cDNA基因序列，按照引物设计要求，该对引物含有*Eco*RI和*Bam*HI两个酶切位点设计引物序列如下: Primer1:5'-GAC *GGA TCC* CCT CTA CAG GAC TGA GAA GAA G-3'; Primer2: 5'-GAC *GAA TTC* CAA CAT TCT CGG TGC CTG TAA C-3'.采用RT-PCR合成第一链cDNA，然后采用标准PCR反应体系扩增其编码区，采用Qia quick gel extration kit回收目的片段. 按照PCR产物:pGEM-T easy vector(摩尔数)为3∶1的比例，用T4连接酶于4 ℃连接过夜，将连接产物转化大肠杆菌TG1，挑选白色转化菌落小量培养，采用Qiagen mini kit提取质粒DNA，酶切鉴定插入片段大小，PCR筛选阳性克隆后，ABI377自动测序仪测序证实. 构建pcDNA3.1 hisB/CD95配体表达载体 用适量*Eco*RI/*Bam*H I双酶切闭环pcDNA3.1hisB和pGEM-T easy vector-CD95配体重组质粒，分别进行目的片段的再切胶回收，将回收CD95配体基因目的片段与线性pcDNA3.1hisB用T4连接酶于4 ℃连接过夜，将连接产物转化大肠杆菌TG1，将连接产物转化大肠杆菌TG1，铺于Am(+)平板中，挑选白色转化菌落小量培养，提取质粒DNA，*Eco*RI/*Bam*H I双酶切鉴定插入片段大小，PCR筛选阳性克隆后，ABI377自动测序仪测序证实. pcDNA3.1 hisB/CD95配体表达重组质粒转染HepG2细胞: 常规复苏HepG2细胞，根据细胞生长情况传代，收获生长良好细胞，在6孔板中，每孔接种3×10^5个细胞于完全培养液中，待细胞生长至80%汇合期，稀释pcDNA3.1hisB/CD95配体表达重组质粒为2，4，6，8 μg及pcDNA3.1hisB质粒6 μg，分别加入5个无血清培养基的EP管; 另设第6管为阴性对照，仅加入无血清培养基100 μL. 将10 μL脂质体加入上述稀释液中，轻轻摇匀，室温放置20 min，加无血清培养基800 μL于复合物中，混匀后，小心滴加细胞中，37 ℃，50 mL/L CO_2培养箱培养24 h，取生长有细胞的盖玻片，PBS洗3次，950 mL/L酒精固定后，加入1抗兔抗人CD95，然后采用S-P超敏试剂盒进行免疫组化检测，收集细胞培养液上清采用EIA进行可溶性CD95配体检测. 将已转染CD95配体的HepG2细胞与HepG2.2.15细胞共同培养24 h后收集细胞，按FITC-Annexin V试剂盒及PI染色细胞，进行流式细胞仪分析及激光共聚焦显微镜观察.

统计学处理 采用SPSS10.0统计软件处理数据，计算采用*t*检验.

2 结果

统计学分析表明，肝癌患者血清的可溶性CD95L水平(2.8±0.4 pg/L)与慢性肝炎(3.2±0.4 pg/L)、肝硬化患者(3.8±1.1 pg/L)及正常对照组(3.5±0.7 pg/L)比较，均存在显著性差异($P<0.05$).

2.1 pcDNA3.1hisB/CD95 L表达重组质粒的鉴定 重组质粒pGEM-T easy vector-CD95 L重组质粒、pcDNA3.1 hisB-CD95 L表达重组质粒酶切及PCR扩增后，鉴定含有预期目的片段(图1，2)，测序后GenBank Blast检测，与人CD95序列一致. nBLAST FAQs nTaxonomy reports nDistribution of 150 Blast Hits on the Query Sequence nAlignments n>gi|601892|dbj|D38122.1|HUMHPC Human mRNA for Fas ligand, complete cds Length = 1890 Score = 499 bits (1083), Expect = e-139 Identities = 205/206 (99%), Positives = 205/206 (99%) Frame = -3/+1. 转染后细胞CD95 L的表达(见图3).

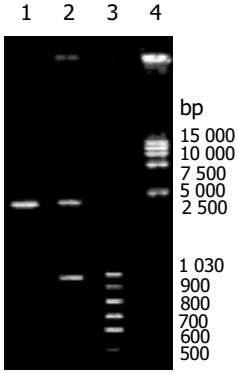

图1 质粒pGEM-FasLEcoRI酶切结果. lane1: 未酶切质粒; lane2: EcoRI酶切结果; lane3:1030分子量marker; lane4: 15 000分子量marker.

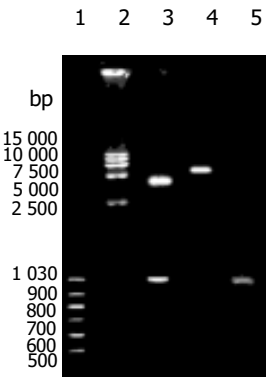

图2 pcDNA3.1hisB-FasLEcoRI和BamHI酶切及PCR结果. lane1: 1 030分子量marker; lane2: 15 000分子量marker; lane3: EcoRI和BamHI酶切; lane4: 未酶切质粒; lane5: PCR结果.

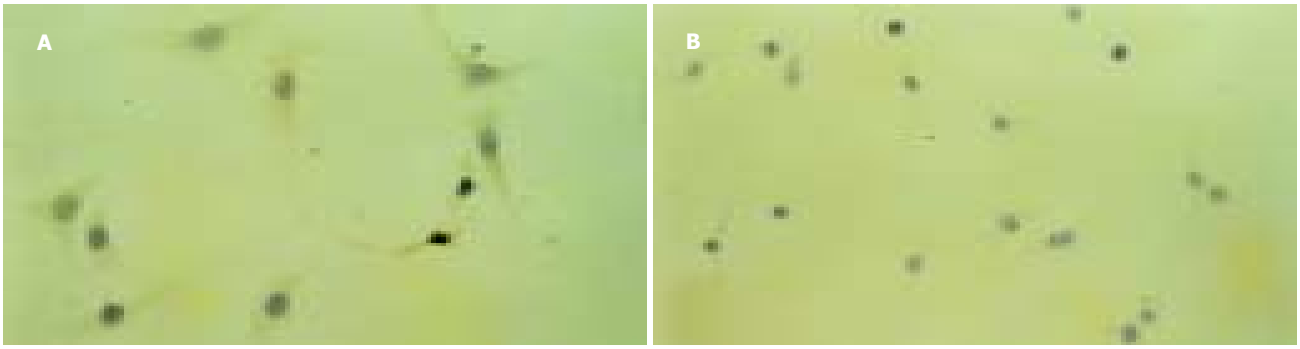

图3 HepG2 苏木素复染, DAB 染色, × 200. A: 转染后细胞质呈棕褐色; B: 未转染细胞质呈蓝色.

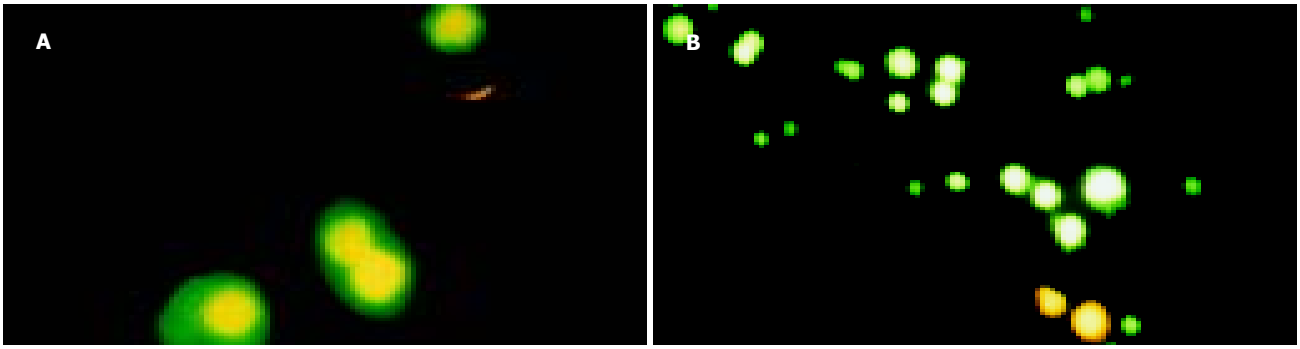

图4 凋亡细胞. A:中晚期, Annexin V/PI 双染, × 400 倍; B: 早、中、晚期, × 100 倍.

2.2 细胞凋亡情况 Annexin V/PI 双染阴性为活细胞; 死细胞为 PI 染色阳性, Annexin V 阴性; 早期凋亡细胞 PI 染色阴性, Annexin V 阳性; 晚期凋亡细胞 Annexin V/PI 双染阳性. 在本试验中, 有各种不同时期的细胞(图 4). 流式细胞仪结果表明转染 CD95 L 的 HepG2 细胞与 HepG2.2.15 细胞凋亡率为 36.3%, 未转染 CD95 L 的对照组细胞凋亡率为 11.5%.

3 讨论

CD95 /CD95 L 是研究较为广泛的细胞凋亡分子, 是传导细胞凋亡信号的重要途径之一[1-5]. 目前对 CD95 配体在肝癌的发生、增生和转移过程中的作用尚不明确, 方法仅限于一些肝组织原位检测方法如免疫组化、原位杂交等. 初步证实肝癌患者 CD95/CD95L 及其 mRNA 表达在癌周正常组织明显高于癌组织, 且恶性程度愈高, 在分化很差的癌细胞, 难以发现 CD95/CD95L 及其 mRNA 表达表达量愈低[6-10]. 在肝癌细胞转移过程中, Kupffer 细胞可释放 TNFa, 诱导肝癌细胞表达 CD95/CD95L导致肝癌细胞凋亡, 依此阻止肝癌细胞的转移[11]. sCD95L 以溶解状态存在于体液中, 可与靶细胞 CD95 结合, 启动凋亡信号的传导[12-17]. 我们通过对血清 sCD95L 检测表明, 肝癌患者血清 sCD95L 明显低于慢性肝炎及肝炎肝硬化患者, 提示在肝癌的发生中, sCD95L 有一定的作用, 有进一步研究价值.

真核表达载体 pcDNA3.1hisB 在多克隆位点的上游和下游分别带有 CMV 的启动子和 BGH 的 polyA 尾, 这种强有力的巨细胞病毒的增强启动子序列, 能高效表达插入的目的基因, 并且可在范围广泛的宿主细胞中工作. 该载体带有筛选标志 Neo 基因, 在 Neo 基因的上游和下游分别带有 SV40 的启动子和 polyA 尾, 保证了 Neo 基因的有效转录[18-24]. 为研究 FasL 的生物学效应, 我们选择了 pcDNA3.1hisB 作为表达载体, 将 CD95LcDNA 转染入功能接近正常肝细胞肝癌细胞系 HepG2, 免疫组化显示 CD95L 已在细胞膜得以表达, 而且在培养液上清也检测到了 sCD95L 的表达, 表明 CD95LcDNA 转染后的 HepG2 细胞已具备表达 CD95L 的能力. 在细胞凋亡检测手段上, 采用检测细胞膜成分变化的 Annexin V 联合 PI 法, 其原理磷脂酰丝氨酸(phosphatidylserine, PS)正常位于细胞膜的内侧, 但在细胞凋亡期, PS 可从细胞膜的内侧翻转到细胞膜的表面, 暴露在细胞外环境中. Annexin-V 能与 PS 高亲和力特异性结合. 碘化丙啶(propidine iodide, PI)是一种核酸染料, 他不能透过完整的细胞膜, 但在凋亡中晚期和坏死期的细胞, PI 能够透过细胞膜而使细胞核红染. 因此将 Annexin-V 与 PI 匹配使用, 就可以将凋亡早晚期的细胞以及坏死细胞区分开来. 采用流式细胞仪测量细胞悬液中细胞荧光强度来区分正常细胞、坏死细胞和凋亡细胞[25-32]. 转染了 CD95L 的 HepG2 细胞与转染了 HBV DNA 的 HepG2 细胞(HepG2.2.15)的共同培养时, 可使 HepG2 细胞出现细胞凋亡. 由此表明, 肝癌细胞可通过 CD95/CD95L 途径凋亡.

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