雷公藤甲素对病毒性心肌炎细胞凋亡及 Fas/FasL 蛋白表达的研究 *

曹 蕾[1] 林 佳[1] 王 臻[2] 邢朝斌[1] 刘爱华[3] 何 冰[1] 王 洋[1]

(1 河北联合大学生命科学学院 河北 唐山 063009;2 复旦大学附属肿瘤医院实验研究中心 上海 200032;
3 河北联合大学基础医学院 河北 唐山 063009)

摘要 目的:研究雷公藤甲素对柯萨奇病毒 B3 病毒(CVB3)感染的病毒性心肌炎小鼠心肌细胞凋亡和 Fas/FasL 蛋白表达的抑制作用,探讨 TP 治疗病毒性心肌炎的作用机制。方法:将 Balb/c 小鼠随机分成 4 组作为动物模型,分别为对照组、模型组、利巴韦林组和 TP 组。对照组腹腔注射生理盐水,其余三组腹腔注射 CVB3,利巴韦林组和 TP 组小鼠分别予以相应的药物治疗后,测定各组小鼠存活率及心肌病变积分,采用末端转移酶标记技术(TUNEL 法)检测小鼠心肌细胞凋亡,免疫组化法检测 Fas/FasL 蛋白阳性表达。结果:空白对照组心肌无病变,利巴韦林组、TP 组与模型组相比有显著性差异(P <0.01)。正常组鲜见心肌细胞凋亡,模型组细胞凋亡率较正常组显著增加(P <0.01),治疗组利巴韦林组和 TP 组凋亡率比模型组明显降低(P<0.05, P<0.01)。模型组 Fas/FasL 表达比正常组显著增多(P <0.01),治疗组利巴韦林组和 TP 组较模型组显著降低(P <0.01)。结论:雷公藤甲素具有通过抑制 Fas/FasL 蛋白的表达,减缓心肌细胞凋亡,达到抑制病毒性心肌炎从而保护心肌细胞的作用。

关键词:雷公藤甲素;病毒性心肌炎;细胞凋亡;Fas/FasL

中图分类号:Q95-33,542.21 **文献标识码**:A **文章编号**:1673-6273(2012)09-1636-03

Influence of Triptolide on Apoptosis and the Expression of Fas/fasL in in Murine Model With the Coxsackievirus B3-Induced Viral Myocarditis*

CAO Lei[1], LIN Jia[1], WANG Zhen[2], XING Chao-bin[1], LIU Ai-hua[3], HE Bing[1], WANG Yang[1]

(1 College of Life Science, Hebei United University, Tangshan 063009;

2 Experiment Research Center of Tumour Hospital Affiliated to Fudan University, Shanghai 200032;

3 College of Basic Medical, Hebei United University, Tangshan 063009)

ABSTRACT Objective: To explore the effects of triptolide on apoptosis and expression of Fas/FasL in myocardial cells of mice with Coxsackievirus B3(CVB3) infected viral myocarditis. Methods: The Balb/c mice were randomly divided into four groups as animal models, namely the control group, model group, ribavirin group and TP group. Control group were injected intraperitoneally with normal saline, and other three groups were injected of CVB3. After treatment of ribavirin group and TP group, the survival rate of mice and myocardial histopathologic scores were determined. Terminal transferase labeling(TUNEL method) was used to detect mouse cardiomyocyte apoptosis, and immunohistochemistry to detect expression of Fas/FasL. Results: Cardiomyopathy of ribavirin group and TP group were significantly different compared with the model group (P <0.01). Compared with normal group, apoptosis rate of the model group was significantly increased (P <0.01). Apoptosis rate of ribavirin treatment group and TP group rate was significantly lower than model group (P <0.05, P <0.01). Expression of Fas/FasL in model group was significantly increased than the control group (P <0.01), and ribavirin treatment group and TP group was significantly lower than model group (P <0.01). Conclusion: Triptolide could inhibit apoptosis of cardiocytes through regulating the expression of Fas/FasL in order to inhibit viral myocarditis and protect myocardial cells.

Key word: Triptolide; Viral myocarditis; Apoptosis; Fas/FasL

Chinese Library Classification(CLC): Q95-33, 542.21 Document code: A

Article ID:1673-6273(2012)09-1636-03

前言

病毒性心肌炎(viral myocarditis,VM)是一种病毒感染引起的弥漫性炎症心肌疾病,是最常见的感染性心肌炎。近年来随着检测技术的提高,发现多种病毒可引起心肌炎,其发病率呈逐年增高趋势,是遍及全球的常见病和多发病[1-4]。柯萨奇病毒 B3(Coxsackie Virus B type 3,CVB3)属肠道病毒,它感染小鼠引起 VM 的病理改变与人类似,是研究 VM 的常用动物模型[5,6]。Fas 及其配体 FasL 是近年来研究得最为深入的有关细胞凋亡的膜表面分子,Fas/FasL 即死亡受体转导通路是引起细胞凋亡的重要信号转导路径之一[7]。研究表明,Fas/FasL 通过引起心肌细胞凋亡参与病毒性心肌炎的发病[8,9]。雷公藤甲素(triptolide,以下简称 TP),又称雷公藤内酯醇、雷公藤内酯,是从卫矛科雷公藤属雷公藤(Tripterygium wilfordiiHook1 fl)中分离得到的活性最高的环氧化二萜内酯化合物,是雷公藤中的主要有效成分之一[10]。TP 的生理活性强,具有显著的抗炎、抗肿瘤、抗生育

* 基金项目:河北省教育厅课题(2009151)

作者简介:曹蕾(1977-),女,讲师,研究方向:分子生物学;E-mail: cao_lei2006@163.com

(收稿日期:2011-12-04 接受日期:2011-12-27)

及免疫调节作用，在临床上得到了广泛的应用[11-14]。本研究就TP对VM小鼠心肌细胞凋亡的影响及Fas/FasL蛋白的表达做了初步探讨。

1 材料与方法

1.1 材料

雷公藤甲素，由上海雅吉生物科技有限公司生产，CAS号38748-32-2，纯度大于98%，20mg/支。利巴韦林注射液，石家庄神威药业生产，规格为1ml∶100mg。CVB3病毒Nancy株由河北联合大学B2实验室保存。实验动物采用3~6周龄Balb/c小鼠，雄性，体重(15± 3) g，由河北联合大学动物实验中心提供。随机选取20只小鼠腹腔注射生理盐水0.1ml归为未感染病毒的正常对照组，其余60只每只腹腔注射100TCID50 CVB3病毒稀释液0.1ml，常规饲养，成活72 h以上小鼠为病毒性心肌炎模型。将病毒感染并成活小鼠随机分为3组 模型组(20只)、利巴韦林组(20只)、TP组(20只)。治疗组利巴韦林组腹腔给要剂量为50mg/kg，TP组按照0.8mg/kg剂量给药[15]。

1.2 方法

1.2.1 检测小鼠心肌组织病变积分 心肌组织病理检查及结果半定量：心肌石蜡标本组织连续切片，HE染色后光镜下观察心肌组织的病理改变，计算每个视野中心肌病变积分，即在每张切片上随机选取5个高倍视野，计算每个视野中炎性细胞浸润及坏死区域面积与整个视野心肌面积之比，无病变计0分，比值25%计1分，> 25%~50%计2分，> 50%~75%计3分，> 75%计4分[16]。

1.2.2 TUNEL法检测细胞凋亡 按照TUNEL细胞凋亡检测试剂盒(碧云天生物技术研究所生产)使用说明操作，样品脱蜡后加蛋白酶K，20-37℃作用30分钟，PBS洗涤3次之后将蛋白酶K洗涤干净。样品加入配置好的TUNEL检测液50μl，37℃避光孵育60分钟。PBS洗涤之后荧光显微镜下观察，按TUNEL试剂盒说明设立阳性和阴性对照。

1.2.3 免疫组化检测心肌组织Fas/FasL蛋白表达 将小鼠心肌组织脱蜡和水化，3%甲醇H_2O_2，37℃孵育15min。蒸馏水洗净甲醇，高压热修复7分钟，蒸馏水洗2次，置于0.01mPBS浸洗2次，每次5min。封闭，加入一抗工作液，4 ℃孵育过夜。加入二抗，室温孵育1h。加苏木精衬染3min，蒸馏水冲洗，苏木精复染，脱水，透明，中性树胶封片。用Bandscan5.0软件进行图像分析，检测阳性染色光密度值，每张切片随机检测5个视野，计算平均积分光密度值[17]。

1.2.4 统计学方法进行结果分析 计量资料用均数± 标准差($\bar{x}\pm s$)。运用统计学软件SPSS 13.0进行数据处理，结果均以均数和标准差的形式显示，两组比较采用t检验，多组比较采用单因素方差分析。

2 结果

2.1 小鼠心肌病变积分

正常对照组心肌无病变，模型组、利巴韦林组、TP组心肌病变积分和正常组相比有所增加，利巴韦林组、TP组与未经治疗组即模型组相比有显著性差异(P<0.01)，但两组治疗组之间并无显著性差异(图1)。

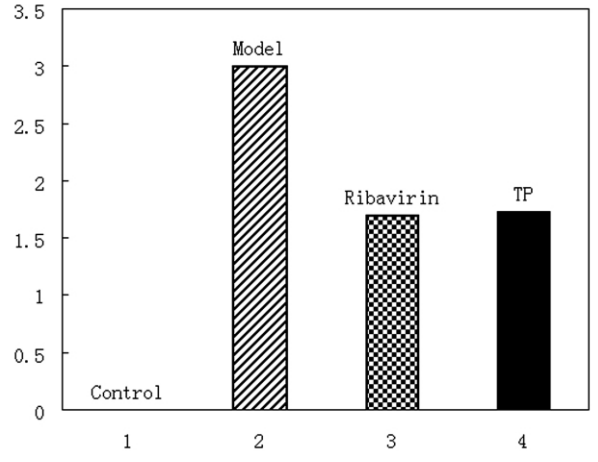

图1 TP对小鼠心肌病变积分的影响

Fig.1 Effects of TP on cardiomyopathy credits of mice

2.2 小鼠心肌细胞凋亡

用TUNEL法分别检测第7天、14天和21天心肌凋亡细胞，光镜下凋亡细胞核呈棕黄色，非凋亡细胞核呈蓝绿色。正常对照组心肌中鲜见凋亡细胞，模型组、利巴韦林组、TP组视野内均可见大量凋亡的心肌细胞。表1明确表示了正常对照组、模型组和治疗组心肌细胞凋亡指数，各组之间的比较具有统计学意义。感染病毒7天后，模型组凋亡细胞较正常组有显著增加 (P<0.01)，药物治疗组凋亡细胞数明显下降(P<0.01，P<0.05)。接受药物注射第7天、14天和21天，治疗组细胞凋亡率和模型组相比均有所下降，说明利巴韦林和TP均具有抑制病毒性心肌炎细胞凋亡的作用。两治疗组之间无显著差异(P>0.05)。

表1 个组小鼠组心肌细胞凋亡指数比较($\bar{x}\pm s$)

Table 1 Comparison of myocardial apoptosis rate ($\bar{x}\pm s$)

Group	Apoptotic index %		
	7d	14d	21d
Control group	1.0 ± 0.3	1.2 ± 0.7	1.6 ± 0.4
Model group	63.5 ± 1.8 *	27.6 ± 2.2 *	16.4 ± 0.4 *
Ribavirin group	21.5± 1.6 # **	9.3 ± 0.3 # **	6.2 ± 1.1 # **
TP group	23.4 ± 0.3 # **	10.9 ± 0.9 # **	3.8 ± 1.3 # **

Note : *P<0.01, # P<0.05 compared with control group; ** P<0.01 compared with model group.

control group, model group, ribavirin group and TP group

2.3 Fas/FasL 蛋白的表达

Fas/FasL 蛋白阳性染色成棕黄色颗粒，位于细胞膜或胞浆内，在正常组和模型组心肌组织内均可检测到 Fas/FasL 蛋白的阳性表达。表 2 列出用药 30 天后各组细胞 Fas/FasL 蛋白的阳性表达率。模型组 Fas/FasL 阳性表达较正常组显著增加，治疗组阳性细胞表达明显下降（P<0.01），TP 组和利巴韦林组之间无明显差异（P>0.05），说明 TP 和利巴韦林均能通过抑制 Fas/FasL 的阳性表达进而抑制病毒性心肌炎。

表 2 各组小鼠心肌细胞 Fas/FasL 蛋白阳性表达率（$\bar{x}\pm s$）

Table 2 Comparison of myocardial expression of Fas/FasL

Group	Fas	Fasl
Control group	0.052 ± 0.006	0.038 ± 0.005
Model group	0.090 ± 0.007 *	0.081 ± 0.009 *
Ribavirin group	0.063 ± 0.009 #	0.056 ± 0.005 #
TP group	0.068 ± 0.011 #	0.060 ± 0.006 #

Note : *P<0.01 compared with control group; # P<0.01 compared with model group

3 讨论

已经证实 CVB3 能够损害健康小鼠心肌细胞，导致心肌细胞凋亡。CVB3 病毒感染会导致心肌细胞凋亡从而使心肌细胞减少，引起病毒性心肌炎进而形成心功能障碍的重要机制。细胞凋亡目的是去除机体组织内异常、受损细胞，是细胞应激下的状态正常反应。Fas/FasL 被认为是与细胞凋亡密切相关的基因，细胞膜表面 FasL 蛋白阳性细胞可与 Fas 蛋白阳性的细胞相互作用，将死亡信号传递给 Fas 蛋白阳性细胞并激活细胞的凋亡程序而诱发细胞凋亡[18-21]。

本研究将感染小鼠建立为病毒性心肌炎动物模型，从分子水平上比较研究 TP 对病毒性心肌炎小鼠心肌细胞凋亡及调控基因的影响，尤其是对 Fas/FasL 蛋白表达的影响。心肌细胞凋亡结果显示正常组偶见微量凋亡细胞，模型组细胞凋亡较之显著增加，表明病毒性心肌炎疾病的发展过程出现了心肌细胞凋亡。治疗组较模型组心肌细胞凋亡明显减少，凋亡细胞多为散在分布，主要集中在病灶及其周边，提示 TP 和利巴韦林皆具有抑制心肌细胞凋亡、保护心肌细胞的作用。

免疫组化法显示模型组 Fas/FasL 阳性表达增加，研究提示 Fas/FasL 蛋白阳性的细胞，可传递并激活该细胞的凋亡过程。本研究发现，Fas/FasL 的分布与心肌凋亡细胞的分布趋势一致，更验证了前述 Fas/FasL 蛋白表达与心肌细胞凋亡的密切联系，说明病毒性心肌炎心肌细胞凋亡中检测 Fas/FasL 的阳性表达具有重要的指导作用。

病毒性心肌炎的发展过程伴随了心肌细胞的凋亡和 Fas/FasL 的阳性表达，结果显示，两组治疗组小鼠 利巴韦林组和 TP 组心肌细胞凋亡数目较未治疗组的数目显著减少，且治疗组的 Fas/FasL 蛋白表达程度皆有显著下降，表明药物利巴韦林和 TP 均有降低 Fas/FasL 阳性表达的作用。综上所述，雷公藤甲素和常用治疗病毒性心肌炎利巴韦林一样，具有减少病毒性心肌炎小鼠心肌细胞凋亡的能力，推测其原理可能为减弱 Fas/FasL 蛋白的表达和凋亡细胞的信号转导，其分子调控的详细机制尚需进一步验证。

参 考 文 献 (References)

[1] Antoniak S, Boltzen U, Riad A, et al. Viral myocarditis and coagulopathy: increased tissue factor expression and plasma thrombogenicity [J]. J Mol Cell Cardiol, 2008, 45(1):118-126

[2] Kuhl U, Schultheiss HP. Viral myocarditis: diagnosis, aetiology and management[J]. Drugs, 2009, 69(10): 1287-1302

[3] Ellis CR, Di ST. Myocarditis: basic and clinical aspects [J]. Cardiol Rev, 2007, 15(4): 170-177

[4] Dennert R, Crijns HJ, Heymans S. Acute viral myocarditis [J]. Eur Heart J, 2008, 29(17):2073-2082

[5] Zhang MX, Zhang YM, Esther J, et al. Effects of yiqi huoxue recipe and Coxsackie virus B type 3 on the expression of ribosomal protein S20 in rat cardiac myocytes [J]. Chin J Integr Med, 2011, 17(5): 376-380

[6] Fujioka S, Kitaura Y. Coxsackie B virus infection in idiopathic dilated cardiomyopathy: clinical and pharmacological implications [J]. Bio-Drugs, 2001, 15(12):791-799

[7] Nomura J, Matsumoto K, Iguchi-Ariga SM, et al. Mitochondria-independent induction of Fas-mediated apoptosis by MSSP[J]. Oncol Rep, 2005, 14(5):1305-1309

[8] Ristic T, Djordjevic VB, Deljanin-Ilic M, et al. Serum Fas/FasL levels in dependence on clinical presentations of coronary disease and their relationship with risk factors [J]. Vojnosanit Pregl, 2010, 67(7): 537-542

[9] Satoh M, Hiramori K, Tamura G, et al. Apoptosis in the process from viral myocarditis to dilated cardiomyopathy [J]. Nihon Rinsho, 1996,54(7):1982-1985

[10] Liu Q. Triptolide and its expanding multiple pharmacological functions[J]. Int Immunopharmacol, 2011, 11(3):377-383

[11] 曹蕾，林佳.雷公藤甲素药理作用研究进展及临床应用[J].中国生化药物杂志，2011，32(6)：502-504
Cao Lei, Lin Jia. Research and clinical application of Triptolide Pharmacological effects [J]. Chinese Journal of Biochemical Pharmaceutics, 2011, 32(6): 502-504(In Chinese)

[12] 王雷，王超，赵延栋. 雷公藤甲素的药理作用研究进展 [J]. 湖南中医药大学学报，2010，30(2):37-38，41
Wang Lei, Wang Chao, Zhao Yan-dong, et al. Triptolide pharmacological research[J]. Journal of Traditional Chinese Medicine University of Hunan, 2010, 30(2):37-38, 41(In Chinese)

（下转第 1676 页）

参 考 文 献 (References)

[1] Kibbe MR,Rhee RY,et al.Heparin-induced thrombocytopenia: Pathophysiology [J].Semin Vasc Surg, 1996, 9(1):284-291

[2] Kelton JG.Heparin-induced thrombocytopenia: An overview [J].Blood Rev, 2002, 16(1):77-80

[3] Blakeman B. Management of heparin-induced thrombocyt openia: a cardiovascular surgeon's perspective [J]. Semin Hematol, 1999, 36 (Suppl 1): 37-47

[4] Amiral J. Antigen involved in heparin-induced thrombocyt openia[J]. Semin Hematol, 1999, 36(Suppl 1): 7-11

[5] Robinson JB, Lewis BE. Plasmapheresis in the management of heparin-induced thrombocytopenia. Semin Hematol, 1999, 36 (Suppl 1): 29-32

[6] Untch B, Ahmad S, Jeske WP, et al. Prevalence, isotype and functionality of antihepatin-platelet factor 4 antibodies in patients treated with heparin and clinically suspected for heparin-induced thrombocytopenia.The pathogenic role of IgG [J]. Thromb Res, 2002, 105 (2): 117-123

[7] Amiral J, Wole M, Fischer AM, et al. Pathogenicity of IgA and/ or IgM antibodies to heparin-PF4 complexes in patient s with heparin-induced thrombocytopenia [J]. Br J Haematol, 1996, 92 (4): 954-959

[8] Carisson LE, Santoso S, Bauricht er G, et al. Heparin-induced thrombocytopenia: new insights into the impact of the Fcγ RIIα-R-H131 polymorphism. Blood, 1998, 92(5): 1526-1531

[9] Kappers Klunne MC, Bvon DMS, Hopw CJ, et al. Heparin-induced thrombocytopenia and thrombosis, a prospective analysis of the incidence in patients with heart and cerebrovascular diseases [J]. Br J Haematol, 1997, 96(3): 442-446

[10] Kelton JG. The clinical management of heparin-induced thrombocytopenia[J]. Semin Hematol, 1999, 36(Suppl 1): 17-21

[11] Barsters A, Zawadzki C, Trillot N, et al. Labratory diagnosis of heparin-induced thrombocytopenia: comparison of the diagnosis value of platelet aggregation test and platelet fact or 4/ heparin complexes ELISA[J]. Br J Haematol, 1998, 102(1): 262

[12] Lunven C,Gauffeny C, Lecoffre C,et al.Inhibition by Argatroban,a specific thromb in inhibitor of platelet activation by fibrin clot-associated thrombin[J]. Thromb Haemost, 1996, 75(1): 154-160

[13] Cohen M. Heparin-induced thrombocytopenia and the clinical use of low molecular weight heparin in acute coronary syndromes[J]. Semin Hematol, 1999, 36(Suppl 1): 33

[14] Lewis BE, Matthai WH Jr, Cohen M, et a1. Argatroban anticoagulation during percutanceous coronary intervention in patients with heparin-induced thrombocytopenia [J]. Cathete Cardiovasc Interv, 2002, 57(2):177-184

(上接第 1638 页)

[13] 赵湘,孙建实,金李君,等. 雷公藤内酯醇作用机制研究进展[J]. 中国中医药科技,2006,13(3):200-204
Zhao Xiang, Sun Jian-shi, Jin Li-jun, et al. Progress in research on triptolide [J]. Chinese Journal of Traditional Medical Science and Technology, 2006, 13(3):200-204(In Chinese)

[14] 陈江飞, 胡毅坚, 苗彩云. 雷公藤甲素药理作用的研究进展 [J]. 中国医药工业杂志, 2007,38(8):604-607
Chen Jiang-fei, Hu Yi-jian, Miao Cai-yun. Progress of Main Pharmacological Actions of Triptolide [J]. Chinese Journal of Pharmaceuticals, 2007, 38(8):604-607(In Chinese)

[15] 丁虹,吴建元,童静,等.雷公藤甲素急性毒性及其机制研究[J].中药材,2004,27(2) :115-118
Ding Hong, Wu Jian-yuan, Tong Jing, etc. Acute toxicity and mechanism of triptolide[J]. Chinese herbal medicines, 2004, 27(2): 115-118 (In Chinese)

[16] Kloner RA, Hale S, Alker K, et al. The effects of acute and chronic cocaine use on the heart[J]. Circulation, 1992, 85(2): 407-419

[17] Kumamoto H. Detection of apoptosis-related factors and apoptotic cells in ameloblastomas: analysis by immunohistochemistry and an in situ DNA nick end-labelling method [J]. J Oral Pathol Med, 1997, 26 (9):419-425

[18] Bossowska A, Bossowski A, Galar B. Analysis of apoptotic markers Fas/FasL (CD95/CD95L) expression on the lymphocytes in patients with acute coronary syndrome [J]. Kardiol Pol, 2007, 65(8):883-839, discussion, 890-892

[19] Bossowski A, Czarnocka B, Stasiak-Barmuta A, et al. Analysis of Fas, FasL and Caspase-8 expression in thyroid gland in young patients with immune and non-immune thyroid diseases [J]. Endokrynol Pol, 2007, 58(4):303-313

[20] Yin XC, Peng YH, Xiao ZX. Mechanism of Fas/FasL signal transduction pathway in K562 cell apoptosis induced by diallyl disulfide [J]. Journal of Experimental Hematology, 2011, 19(3):634-637

[21] 周丽华. Fas/FasL 系统与心肌细胞凋亡 [J]. 中国地方病学杂志, 2003, 22(4):370-372
Zhou Li-hua. Fas/FasL system and myocardial apoptosis [J]. Chinese Journal of Endemiology, 2003, 22(4):370-372