肿瘤坏死因子α诱导人髓核细胞凋亡的作用途径*★

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Effect of tumor necrosis factor alpha on apoptosis of human nucleus pulposus cells

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Abstract

BACKGROUND: Tumor necrosis factor (TNF) superfamily plays an important role in apoptosis. However, the role of TNF on nucleus pulposus cells remains poorly understood.

OBJECTIVE: To investigate the effects of TNF-α on apoptosis of human nucleus pulposus cell from pathways of P38MAPK and stress-activated protein kinase/c-Jun NH2-terminal kinase (JNK/SAPK).

METHODS: The human nucleus pulposus cells were cultured in vitro and randomly divided into 4 groups: TNF-α, P38MAPK inhibition, P-JNK/SAPK inhibition and control groups. The apoptosis of nucleus pulposus cells was detected by TUNEL; the expression and location of P38MAPK and P-JNK/SAPK were determined by immumofluorescence method; and the expression of P38MAPK, JNK/SAPK, and their phosphorylations were measured by Western Blot.

RESULTS AND CONCLUSION: TUNEL results showed that there was higher density of apoptotic nucleus pulposus cells in the TNF-α group than that of the other groups ($P < 0.01$). Immunofluorescence showed that, compared with inhibition groups and control group, the expressions of phosphorylations of P38MAPK and JNK / SAPK were increased after treatment with TNF-α ($P < 0.01$). Western Blot analysis also demonstrated that P38MAPK and P-JNK / SAPK were expressed and distributed mainly in cytoplasmic and nuclear, however, there was only P-JNK / SAPK expression in the TNF–α group, but no expression could be found in the inhibition groups. TNF-α induces the apoptosis of human nucleus pulposus cell via P38MAPK and JNK / SAPK pathway.

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摘要

背景：在与细胞凋亡有关的众多因素中，肿瘤坏死因子(tumor necrosis factor，TNF)超家族成员发挥了重要的作用。但TNF是通过何种途径诱导椎间盘髓核细胞凋亡的机制尚未阐明。

目的：探讨TNF-α激活后，丝裂原活化蛋白激酶(mitogen-activated protein kinases，MAPKs)信号转导通路中P38MAPK和应激活化蛋白激酶/c-Jun氨基末端激酶(stress-activated protein kinase/c-Jun NH2-terminal kinase，JNK/SAPK)两条途径对人髓核细胞凋亡的作用。

方法：体外培养人髓核细胞，将细胞随机分成4组：TNF-α刺激组，P38MAPK阻断组，P-JNK/SAPK阻断组和对照组。采用TUNEL法检测髓核细胞凋亡情况，免疫荧光法检测P-P38MAPK和P-JNK/SAPK的表达及定位；Western Blot法检测P38MAPK，JNK/SAPK及其磷酸化形式的表达。

结果与结论：TUNEL法检测凋亡结果中，TNF-α刺激组较其他各组的凋亡细胞密度大($P < 0.01$)；免疫荧光结果显示TNF-α刺激组P-P38MAPK和JNK/SAPK在细胞质和细胞核的表达高于各阻断组和对照组($P < 0.01$)；Western Blot结果显示P38MAPK，P-JNK/SAPK在各组髓核细胞内均有表达，但无活化形式，TNF-α刺激组可见P-P38MAPK，P-JNK/SAPK表达，但相应阻断组无表达。结果表明，外源性TNF-α可通过P38MAPK和P-JNK/SAPK途径导致人髓核细胞凋亡。

关键词：人髓核细胞；信号传导；肿瘤坏死因子α；P38丝裂原活化蛋白激酶；P-应激活化蛋白激酶/ c-Jun氨基末端激酶；细胞凋亡
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0 引言

髓核细胞凋亡导致的细胞数量减少是椎间盘退变的主要形态学改变之一[1]，但目前引起其凋亡的机制尚未阐明。在与细胞凋亡有关的众多因素中，肿瘤坏死因子超家族成员发挥了重要的作用。目前已发现了3种诱发细胞凋亡的死亡因子即FasL、肿瘤坏死因子α(tumor necrosis factor，TNF-α)及TRAIL，它们可通过凋亡诱导信号途径来促使细胞凋亡。研究表明，在退变椎间盘组织中，已经检测到了其分泌的众多炎性因子，如白细胞介素1β、白细胞介素6、白细胞介素8、TNF-α、一氧化氮和前列腺素E2等[2-5]。但炎性因子是通过何种途径诱导椎间盘髓核细胞凋亡的机制尚未明确。

以往在生物力学和生物化学方面进行了很多研究[6-12]，随着研究的进一步深入，丝裂原活化蛋白激酶(mitogen-activated protein kinases，MAPKs)信号转导通路逐渐受到重视，其存在于大多数细胞内，对细胞增殖、分化、转化及凋亡起着至关重要的作用[13-15]。其中P38 MAPK和

应激活化蛋白激酶/c-Jun氨基末端激酶(stress-activated protein kinase/c-Jun NH2-terminalkinase，JNK/SAPK)两条途径均可被炎性应激激活，主要功能为介导应激反应中的细胞凋亡[16]，但其在椎间盘细胞退变中的作用尤其是对髓核细胞凋亡影响的研究鲜有报道。因此，实验应用细胞生物学的方法，观察TNF-α激活后P38MAPK和JNK/SAPK途径对人髓核细胞凋亡过程中发挥的作用，探讨炎性因子诱导椎间盘髓核细胞凋亡的可能机制。

1 材料和方法

设计：体外培养，细胞学对照实验。

时间及地点：实验于2009-02/12在青岛大学医学院附属医院中心实验室完成。

材料：人髓核细胞购自上海沪峰生物科技有限公司，进口于美国标准菌库。

主要试剂及仪器：

试剂及仪器	来源
DMEM/F12 细胞培养液	美国 hyclone 公司
胎牛血清，胰蛋白酶(1∶250)	美国 Gibco 公司
TNF-α	美国 Sigma 公司
P38MAPK 特异性阻断剂(SB203580)，JNK/SAPK 特异性阻断剂(SP600125)，小鼠抗人 P38MAPK 单克隆抗体，小鼠抗人 P-P38 MAPK 单克隆抗体，小鼠抗人 P-JNK/SAPK 单克隆抗体，兔抗人 JNK/SAPK 单克隆抗体，辣根过氧化物酶标记的羊抗小鼠 IgG，辣根过氧化物酶标记的羊抗兔 IgG，FITC 标记的兔抗小鼠 IgG，TUNEL 凋亡检测试剂盒	上海碧云天生物技术有限公司

实验方法：

人髓核细胞培养及处理：用含体积分数10%胎牛血清的DMEM/F12培养液(pH 7.2)，置于37 ℃、体积分数5% CO_2的培养箱中培养。待生长到80%~90%融合时用0.25%的胰蛋白酶消化传代。

细胞刺激模型及阻断模型的建立：将细胞培养于6孔板中，随即分成4组：TNF-α刺激组，P38MAPK阻断组，P-JNK/SAPK阻断组和对照组。每个培养孔为1组，TNF-α刺激组，每孔分别加入0.5 mg/L TNF-α，重复3个孔，细胞继续培养24 h。P38MAPK阻断组和P-JNK/SAPK阻断组每孔先分别给予20 μmol/L特异性P38MAPK阻断剂(SB203580)和JNK/SAPK阻断剂(SP600125)，20 min后再给予0.5 mg/L TNF-α，重复3个孔。37 ℃、体积分数5% CO_2的培养箱中继续培养24 h后检测相关指标。同时以无血清培养基培养的细胞为对照组。

TUNEL法检测髓核细胞凋亡：各组细胞经过处理达到实验要求后，弃反应液后用40 g/L多聚甲醛固定细胞，再加入0.1% Triton X-100的PBS冰浴孵育2 min，按TUNEL凋亡试剂盒说明操作后荧光显微镜下观察。在装有目镜网格测微尺(130 μm×130 μm)的200倍视野下，分别计数6个网格内的凋亡髓核细胞数，随机选取10个视野，取平均值，计算凋亡细胞密度。

免疫荧光检测P-P38MAPK和P-JNK/SAPK在细胞内的定位及表达：各组细胞经过处理达到实验要求后，多聚甲醛室温固定。用含有山羊血清的封闭液37 ℃湿盒内封闭60 min，分别加入1∶100稀释的小鼠抗人P-P38MAPK或小鼠抗人P-JNK/SAPK抗体，37 ℃湿盒内孵育 1 h。再加入FITC标记的兔抗小鼠IgG(1∶100)的二抗，37 ℃湿盒内避光孵育1 h。封固后荧光显微镜观察结果，随机选取10个视野拍照并用Image Pro Plus V6.0软件分析图片。

Western Blot检测P38MAPK、P-P38MAPK及JNK/SAPK、P-JNK/SAPK的表达：分别以P38MAPK和JNK/SAPK作为对照，测定其活化形式P-P38MAPK及P-JNK/SAPK的表达。细胞处理达到实验要求后，RIPA裂解法提取蛋白，考马斯亮蓝法测定总蛋白浓度。样品经琼脂糖凝胶电泳后，转移至PVDF 膜。然后加入封闭液室温下摇床上孵育60 min，一抗分别采用1∶1 000稀释的小鼠抗人P-P38 MAPK、P-JNK/SAPK、P38 MAPK或兔抗人JNK/SAPK的单克隆抗体，再分别用1∶1 000稀释的辣根过氧化物酶标记的羊抗小鼠和羊抗兔IgG室温摆摇床上孵育60 min，ECL法显影，胶片拍照。

主要观察指标：TUNEL法检测各组细胞的凋亡密度；免疫荧光以及蛋白免疫印迹法检测P38MAPK和JNK/SAPK的活化形式；Western Blot法检测P38MAPK、P-P38MAPK及JNK/SAPK、P-JNK/SAPK的表达。

设计、实施、评估者：本实验由第二作者设计并评估，由第一作者实施，均受过正规培训。

统计学分析：采用SPSS17.0统计软件对多组均数间比较采用单因素方差分析，所有数据均用$\bar{x}\pm s$表示，$P < 0.05$为差异有显著性意义。

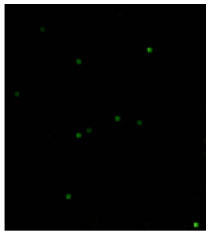
2 结果

2.1 各组髓核细胞凋亡情况 见表1，图1。

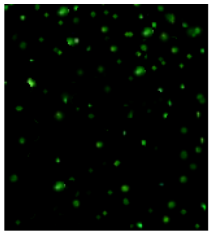
表1 各组TUNEL法检测髓核细胞凋亡结果
Table 1 The density of apoptotic nucleus pulposus cells detected by TUNEL ($\bar{x}\pm s$, cell/mm^2)

Group	Density of apoptotic cells
TNF-α	32.48±2.10[a]
P38MAPK inhibition	6.32±0.95
JNK/SAP inhibition	8.19±1.67
Control	1.77±0.46

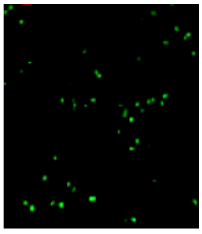
TNF-α: tumor necrosis factor α; MAPK: mitogen-activated protein kinase; JNK/SAPK: stress-activated protein kinase/c-Jun NH2-terminal kinase; [a]$P < 0.01$, *vs.* control group



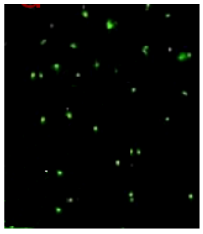
a: Control group



b: Tumor necrosis factor α group



c: P38 mitogen-activated protein kinaseinhibitor group



d: Stress-activated protein kinase/c-Jun NH2-terminal kinase inhibitor group

Figure 1 The density of apoptotic nucleus pulposus cells detected by TUNEL (×200)

图 1 TUNEL 方法检测人髓核细胞凋亡的结果(×200)

TNF-α刺激组髓核细胞的凋亡细胞密度较对照组高($P < 0.01$)。在P38MAPK阻断组及JNK/SAPK阻断组中可见少量凋亡细胞。

2.2 各组髓核细胞内P-P38MAPK和P-JNK/SAPK在细胞内的定位及表达 见表2，3，图2。

表 2 各组 P-P38 丝裂原活化蛋白激酶表达总荧光密度结果

Table 2 Results of P-P38 mitogen-activated protein kinase total fluorescent density ($\bar{x}\pm s$)

Group	Total fluorescent density
Control	943.12±88.14
TNF-α (×10⁴)	546.27±19.98
P38MAPK inhibition	4 515.65±264.57[a]

TNF-α: tumor necrosis factor; MAPK: mitogen-activated protein kinase; [a]$P < 0.01$, *vs.* TNF-α group

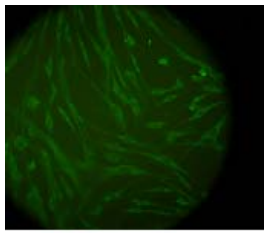
表 3 各组 P-应激活化蛋白激酶/c-Jun 氨基末端激酶表达总荧光密度结果

Table 3 Results of total fluorescent density of P-stress-activated protein kinase/c-Jun NH2-terminal kinase ($\bar{x}\pm s$)

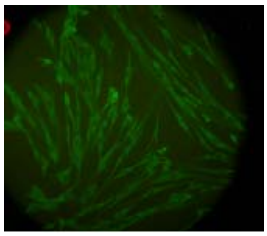
Group	Total fluorescent density
Control	862.04±95.32
TNF-α (×10⁴)	456.92±31.75
JNK/SAPK inhibition	4 841.81±296.74[a]

TNF-α: tumor necrosis factor α; JNK/SAPK: stress-activated protein kinase/c-Jun NH2-terminal kinase; [a]$P < 0.01$, *vs.* TNF-α group

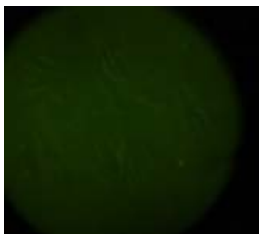
TNF-α刺激组P-P38MAPK在细胞质和细胞核内均有表达，P-JNK/SAPK同样在细胞质和细胞核内均有表达；P38MAPK阻断组及JNK/SAPK阻断组在仅少量细胞胞浆及细胞核内可见P-P38MAPK和P-JNK/SAPK的表达，总荧光密度显著低于TNF-α刺激组($P < 0.01$)；对照组P-P38MAPK和P-JNK/SAPK仅可见极少量表达。



a: p-P38 mitogen-activated protein kinase (MAPK) in tumor necrosis factor (TNF)-α group



b: p-stress-activated protein kinase/c-Jun NH2-terminal kinase (JNK/SAPK) in TNF-α group



c: p-P38MAPK in P38MAPK inhibitor group

d: p-JNK/SAPK in JNK/SAPK inhibitor group

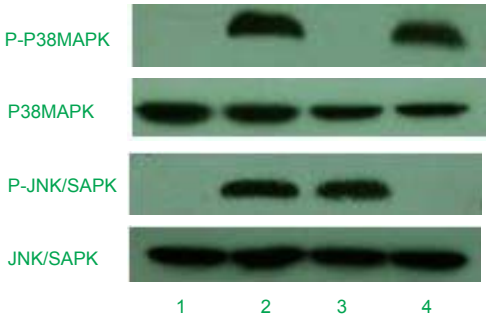
e: p-P38MAPK in control group

f: p-JNK/SAPK in control group

Figure 2 Expression of phosphorylations of P38 mitogen-activated protein kinase or stress-activated protein kinase/c-Jun NH2-terminal kinase in human nucleus pulposus cells (×200)

图 2 P-P38 丝裂原活化蛋白激酶及 P-应激活化蛋白激酶/c-Jun 氨基末端激酶在细胞中的表达(×200)

2.3 各组髓核细胞内P38MAPK、P-P38MAPK及JNK/SAPK、P-JNK/SAPK蛋白的表达 见图3。



1: control group; 2: tumor necrosis factor-α group; 3: P38 mitogen-activated protein kinase inhibition group; 4: stress-activated protein kinase/c-Jun NH2-terminal kinase inhibition group

Figure 3 Results of P38 mitogen-activated protein kinase, P-P38MAPK, stress-activated protein kinase/c-Jun and P-JNK/SAPK expression

图 3 各组 P38 丝裂原活化蛋白激酶(MARK)、P-P38 及应激活化蛋白激酶(JNK)/c-Jun 氨基末端激酶、P-JNK MARK/c-Jun 氨基末端激酶的蛋白表达

正常髓核细胞内存在P38 MAPK和JNK/SAPK的表达，但无活化形式。经TNF-α刺激后，可检测到P38 MAPK和JNK/SAPK的活化形式，即P-P38MAPK及P-JNK/SAPK的表达。而用P38MAPK阻断剂和特异性JNK/SAPK阻断剂预处理后，再用TNF-α刺激髓核细胞，P38MAPK或JNK/SAPK激活被显著抑制。

3 讨论

退变的椎间盘与正常椎间盘相比在结构和功能上都发生了很大的变化，椎间盘的细胞数量大幅度减少，其中又以髓核细胞减少最多。髓核细胞的凋亡与此有着密切的关系[17-18]。在凋亡众多的诱发因素中，炎症因子有着重要地位[19-20]。退变椎间盘中的髓核细胞可以分泌TNF-α等众多炎症因子，并对其产生反应[21]。作为其中主要的炎性因子TNF-α在生物力学不稳的脊柱节段的椎间盘及退变椎间盘中的含量明显高于正常椎间盘。TNF-α被认为是椎间盘退变的关键因子[22-23]。TNF-α通过诱导炎性反应，激活免疫系统在椎间盘退变过程中发挥作用。本实验中使用TNF-α作为刺激因子，在TUNEL细胞凋亡检测中，TNF-α刺激组与对照组相比有着较高的细胞凋亡密度，说明TNF-α可以诱导人髓核细胞的凋亡。

MAPKs是细胞内的一类丝氨酸/苏氨酸蛋白激酶。其由3层激活酶体系，即MAPKs，MAP2Ks和MAP3Ks所介导，通过连续的蛋白质磷酸化诱发瀑布式级联反应而发挥作用。激活的P38MAPK和JNK/SAPK途径通过磷酸化转录因子和其他目的因子来调节基因转录，进而诱发细胞凋亡[24-25]。

本实验结果显示各组髓核细胞中既有P38MAPK的表达又有JNK/SAPK的表达。可见，这两条信号转导途径均存在于髓核细胞中。进一步检测P-P38MAPK和P-JNK/SAPK的表达，结果显示在TNF-α刺激组中存在着磷酸化P-P38MAPK和P-JNK/SAPK，而P38MAPK和JNK/SAPK阻断组中均未见P-P38MAPK和P-JNK/SAPK表达，在免疫荧光结果中仅有少量表达。由此推论人髓核细胞中存在着P38MAPK和JNK/SAPK信号转导途径，且可以被外源性TNF-α所激活，并诱导髓核细胞的凋亡。

综上所述，外源性TNF-α可通过P38MAPK和JNK/SAPK途径导致人髓核细胞凋亡，炎性因子可能是诱导椎间盘髓核细胞凋亡的机制之一。

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