P.O.Box 2345 Beijing 100023, China
Fax: +86-10-85381893
Email: wcjd@wjgnet.com www.wjgnet.com

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aFGF 和 genistein 对大肠癌细胞株 CCL229 PKC 及 ERK 活性的影响

尚 海, 张 颐, 单吉贤

尚海, 辽宁省肿瘤医院肝胆胰外科 辽宁省沈阳市 110042
张颐, 中国医科大学附属第一医院妇科 辽宁省沈阳市 110001
单吉贤, 中国医科大学附属第一医院肿瘤外科 辽宁省沈阳市 110001
尚海, 男, 1968-01-26生, 辽宁省沈阳市人, 汉族. 肿瘤学博士, 讲师. 主要从事消化道肿瘤的研究.
项目负责人: 尚海,110042, 辽宁省沈阳市, 辽宁省肿瘤医院肝胆胰外科. syzi@163.com
电话: 024-22711682
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Effects of aFGF and genistein on PKC and ERK activity in human colorectal cancer cell line CCL229

Hai Shang, Yi Zhang, Ji-Xian Shan

Hai Shang, Department of Hepatobiliary Surgery, Liaoning Provincial Tumor Hospital, Shenyang 110042, Liaoning Province, China
Yi Zhang, Department of Gynecology, First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China
Ji-Xian Shan, Department of Oncology, First Affiliated Hospital of China Medical University , Shenyang 110001, Liaoning Province ,China
Correspondence to: Hai Shang, Liaoning Provincial Tumor Hospital, Shenyang 110042, Liaoning Province, China. syzi@163.com.cn
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Abstract

AIM: To observe the effects of aFGF and TPK inhibitor genistein on intracellular PKC and ERK activity in CCL229 cell line.

METHODS: The activities of PKC and ERK in cells induced by different concentrations of aFGF (0.15 mg/L, 0.30 mg/L, 0.60 mg/L, 1.20 mg/L) and genistein (6.00 mg/L, 12.00 mg/L, 24.00 mg/L, 48.00 mg/L) were detected by incorporation of [γ-^{32}P]-ATP into exogenous substrates.

RESULTS: The intracellular PKC and ERK activity increased with aFGF in a dose dependent manner (P <0.05). When the concentration of aFGF was 1.20 mg/L , the activity of PKC in cytosol and PKC in membrane and ERK was 2.60, 2.79, 1.77 times higher than control group. Genistein suppressed the intracellular PKC and ERK activity also in a dose dependent manner (P <0.05). When the concentration of genistein was 48.00 mg/L, the activity of PKC in cytosol and PKC in membrane and ERK was 0.41,0.36,0.50 times higher than that in control group, The activity of PKC and ERK decreased apparently when the cells were treated with aFGF.

CONCLUSION: aFGF receptor in human colorectal cancer cell line CCL229 possesses TPK activity. Tyrosine-specific protein phosphorylation may initiate a cascade of biochemical events, which may increase the intracellular PKC and ERK activity.

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摘要

目的: 观察 aFGF 及 TPK 抑制剂 genistein 对大肠癌细胞株 CCL229 细胞内 PKC 及 ERK 活性的影响，探讨其信号传导途径.

方法: 以不同浓度的 aFGF (0.15 mg/L，0.30 mg/L，0.60 mg/L，1.20 mg/L) 和 genistein (6.00 mg/L，12.00 mg/L，24.00 mg/L，48.00 mg/L) 诱导 CCL229 细胞，利用[γ-^{32}P]ATP 掺入外源性底物的方法，液体闪烁测定 PKC 及 ERK 活性.

结果: 随着 aFGF 浓度的增加，PKC 及 ERK 活性随之升高，与aFGF浓度呈显著正相关(P<0.05). 当aFGF浓度为1.20 mg/L时，PKC (胞质)，PKC (胞膜)和 ERK 活性分别为对照组的 2.60，2.79，1.77 倍. genistein 抑制细胞内 PKC 及 ERK 活性，且与 genistein 浓度呈剂量依赖效应(P <0.05). 当 genistein 浓度为 48.00 mg/L 时，PKC (胞质)，PKC (胞膜)和 ERK 活性分别为对照组的 0.41，0.36，0.50 倍. genistein 对 aFGF 诱导的 PKC 及 ERK 活性抑制更显著.

结论: 大肠癌细胞株 CCL229 中 aFGF 受体具有 TPK 活性，TPK 激活后促进蛋白质和酶磷酸化，导致 PKC 和 ERK 活性升高,进一步证明PKC及ERK确是TPK的下游信号分子.

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0 引言

酸性成纤维细胞生长因子(acidic fibroblast growth factor，aFGF)是一种多肽生长因子，能刺激血管生长，与肿瘤的生长密切相关. 目前 aFGF 在实体瘤细胞内的信号传导途径尚不清楚. 我们观察 aFGF 对大肠癌细胞株 CCL229 细胞蛋白激酶 C (protein kinase C，PKC)和细胞外信号调节激酶(extracellular regulated kinase，ERK)活性的影响，以及用酪氨酸蛋白激酶(tyrosine protein kinase，TPK)抑制剂 genistein 处理后 PKC 及 ERK 的变化趋势，进一步认识肿瘤细胞的信号传导机制.

1 材料和方法

1.1 材料 人大肠癌细胞系CCL229，由中国医科大学细胞生物教研室提供; aFGF购自北京邦定科技有限公司; Genistein，DMEM培养基、鱼精蛋白(protamine)、髓鞘碱性蛋白(myelin basic protein，MBP)均购自Sigma公司; [γ-^{32}P]ATP购自北京亚辉生物制品公司; 液闪仪(美国Beckman 1801型)，紫外分光光度计(UV310型).

1.2 方法 CCL229细胞在含100 mL/L小牛血清、100 kU/L青霉素，100 g/L链霉素的DMEM培养基中贴壁生长，于37 ℃，50 mL/L CO_2，950 mL/L空气培养箱中传代培养. 将培养的CCL229细胞随机分为: 空白对照组; aFGF组(0.15 mg/L，0.3 mg/L，0.6 mg/L，1.2 mg/L); Gen组(6 mg/L，12 mg/L，24 mg/L，48 mg/L); aFGF+genistein组. 当细胞达到亚融合状态时，吸出旧培养液，每瓶加入无血清培养液2 mL，12 h后吸出旧培养液，按分组要求分别加入肝素15 μL (40 μg/L)，不同量的aFGF，Genistein及培养液，使各瓶终体积均为2 mL. aFGF+genistein组，细胞与Genistein温浴30 min后加入aFGF，3 h后测定PKC及ERK活性.

1.2.1 PKC活性的测定 按改良Takai法. 将处理的细胞于粉碎缓冲液(1 mmol/L EDTA，1 mmol/L EGTA，10 mmol/L Tris-HCl pH7.5，100 mmol/L NaCl，50 mmol/L NaF，1 mmol/L PMSF，0.01 μg/L亮肽素，0.01 μg/L抑肽酶，0.01 μg/L胃酶抑素，50 mmol/L β-磷酸甘油，1 mmol/L二硫苏糖醇，0.9 g/L Brig35)超声粉碎，离心(100 000 g，1 h，4 ℃)，上清为胞质蛋白提取液. 沉淀部分加入溶膜液(2 mmol/L EDTA，10 mmol/L EGTA，20 mmol/L Tris-Cl pH7.5，0.25 mol/L蔗糖)，悬起后超声粉碎，4 ℃过夜，离心(100 000 g，1 h，4 ℃)，上清为膜蛋白提取液. PKC活性测定，以PKC使[γ-^{32}P]ATP掺入外源性底物鱼精蛋白的磷酸放射活性为PKC活性标志. 取胞质及胞膜提取液，每个样品取3个平行管，每管20 μL，同加有[γ-^{32}P]ATP的底物混合液30 μl (25 mmol/L醋酸镁10 μL，2.5 × 10^{-4} mol/L ATP 10 μl，10 g/L protamine 2 μL，1 mol/L Tris-HCl pH7.5 1 μl，H_2O 7 μL)，30 ℃反应8 min，反应完成后取25 μL点在Whatman强阳离子交换滤纸上，在75 mmol/L磷酸溶液中洗3次，每次3 h，装入液闪瓶，液体闪烁测定cpm数.

1.2.2 ERK活性测定 取胞质提取液20 μL (3个平行管)，同10 μL加有[γ-^{32}P]ATP底物混合液(5 mmol/L $MgCl_2$，2.5 × 10^{-4} mol/L ATP，MBP，20 mmol/L Tris-HCl pH7.5，H_2O)混合，30^{0}C反应30 min，其余同测PKC活性.

统计学处理 数据以$\bar{x} \pm s$标准差表示. 数据分析采用统计程序软件包(SPSS8.0 for Windows)进行方差分析和多重比较. 应用Student't检验来判断差异的统计学意义，P<0.05具有显著性.

2 结果

2.1 aFGF诱导大肠癌细胞株CCL229后，PKC及ERK活性均增高，且与aFGF呈剂量依赖效应. 与对照组比较，有显著差异(P <0.05). 对比之下，胞膜PKC活性比胞质PKC活性升高更为明显(表1).

表1 aFGF诱导后CCL229细胞PKC及ERK活性变化($\bar{x} \pm s$, nkat/L)

aFGF(mg/L)	PKC(胞质)	PKC(胞膜)	ERK
0.00	6.30 ± 1.56	6.61 ± 1.27	0.22 ± 0.04
0.15	6.63 ± 1.69	7.30 ± 2.23	0.24 ± 0.03
0.30	9.44 ± 2.20[a]	10.46 ± 2.09[a]	0.30 ± 0.06[a]
0.60	11.39 ± 2.12[a]	13.32 ± 1.81[a]	0.34 ± 0.05[a]
1.20	16.38 ± 1.93[a]	18.42 ± 2.87[a]	0.39 ± 0.07[a]

[a]P <0.05 *vs* 对照组.

2.2 Genistein诱导作用 加入抑制剂Genistein后，PKC及ERK活性与对照组相比，明显受抑制(P <0.05，表2). 其抑制程度与Genistein浓度呈剂量依赖关系.

表2 Genistein诱导后CCL229细胞PKC及ERK活性变化($\bar{x} \pm s$, nkat/L)

Genistein (mg/L)	PKC(胞质)	PKC(胞膜)	ERK
0.00	6.30 ± 1.56	6.61 ± 1.27	0.22 ± 0.04
6.00	5.96 ± 1.84	6.05 ± 1.64	0.20 ± 0.05
12.00	5.04 ± 1.44[a]	5.19 ± 2.02[a]	0.17 ± 0.03[a]
24.00	3.56 ± 1.46[a]	3.40 ± 1.21[a]	0.15 ± 0.03[a]
48.00	2.61 ± 1.20[a]	2.40 ± 1.08[a]	0.11 ± 0.02[a]

[a]P <0.05 *vs* 对照组.

2.3 Genistein及aFGF作用 Gen组与aFGF+gen组比较可见，Genistein对aFGF+Gen组细胞的PKC及ERK抑制作用更明显(P<0.05，表3).

表3 aFGF+Gen组PKC及ERK活性变化($\bar{x} \pm s$, nkat/L)

aFGF(mg/L)	Genistein(mg/L)	PKC(胞质)	PKC(胞膜)	ERK
0.60	0.00	11.39 ± 2.12	13.32 ± 1.81	0.34 ± 0.05
0.60	6.00	10.25 ± 1.99	11.60 ± 1.51	0.30 ± 0.03
0.60	12.00	8.20 ± 2.06[a]	10.26 ± 2.10[a]	0.24 ± 0.01[a]
0.60	24.00	5.47 ± 1.56[a]	5.33 ± 1.38[a]	0.14 ± 0.02[a]
0.60	48.00	4.10 ± 1.78[a]	3.46 ± 1.52[a]	0.07 ± 0.01[a]

[a]P <0.05 *vs* 对照组.

3 讨论

aFGF是一种强有力的细胞分裂促进因子，对成纤维细胞在内的多种细胞具有促进增生和分化的功能，与肿瘤生长密切相关[1-13]. 我们观察aFGF诱导大肠癌细胞株CCL229细胞PKC及ERK活性变化，并用TPK抑制剂genistein作用细胞，进一步认识aFGF诱导肿瘤细胞分裂增生的细胞内信号传导机制. PKC是一种丝/苏氨酸蛋白激酶，广泛分布于真核细胞，在跨膜信息传递、细胞

增生分化及肿瘤侵袭转移等方面均发挥重要作用[14-24]. ERK 则是有丝分裂原活化蛋白激酶(Mitogen-activated protein kinase，MAPK)家族的成员之一，可被生长因子、激素、神经递质等激活，在细胞生长、发育、分裂、死亡及恶性转化等过程中起重要作用[25-27]. 我们发现不同浓度的 aFGF 作用于细胞后，可导致该细胞内 PKC 及 ERK 活性明显升高，且其升高程度与 aFGF 浓度均呈显著正相关. 说明 aFGF 在一定浓度范围内，可激活该细胞株 PKC 及 ERK. 随 aFGF 浓度升高，PKC 及 ERK 的活性变化趋势基本一致. 说明 PKC 及 ERK 两个途径不是孤立的，aFGF 对该细胞株的影响在这两个系统中持续循环放大. 胞质PKC及胞膜PKC活性均显著升高，尤其胞膜 PKC 活性升高更明显，提示 PKC 激活时可能发生膜转移现象.

VEGF 诱导内皮细胞的促有丝分裂作用通过激活 PKC 途径，酪氨酸蛋白激酶抑制剂可阻断此反应，从而说明 PKC 是 TPK 的下游信号分子. 本实验用 TPK 的特异性抑制剂 Genistein 作用细胞，发现细胞内 PKC 及 ERK 活性均明显受抑制，且抑制程度与 Genistein 浓度呈剂量依赖效应. Genistein对aFGF+Gen组PKC及ERK的活性的抑制作用明显强于 Gen 组. 提示 aFGF 对 CCL229 细胞 PKC 及 ERK 的激活是通过 TPK 来介导的，TPK 抑制剂 Genistein 可阻断 aFGF 诱导的 PKC 及 ERK 活化; aFGF+ Gen 组细胞 PKC 及 ERK 活性受抑制更明显，提示 CCL229 细胞中 aFGF 主要通过 TPK 途径来激活 PKC 与 ERK，从而促进细胞增生.

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