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Retinoic acid receptor β is required for anti-activator protein-1 activity by retinoic acid in gastric cancer cells

WU Qiao 吴 乔, ZHANG Mingqing 张鸣青, LIU Su 刘 苏, CHEN Yuqiang 陈玉强 and SU Wenjin 苏文金

Keywords: retinoic acid receptor β · activator protein-1 · all-trans retinoic acid · gastric cancer cell

Objective To investigate the role of retinoic acid receptor β (RARβ) in mediating inhibitory effect of all-trans retinoic acid (ATRA) on activator protein-1 (AP-1) activity in gastric cancer cells.

Methods Transient transfection and chloramphenicol acetyltransferase (CAT) assay, Northern blot, gene transfection, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, and anchorage-independent growth assay were used.

Results Transient transfection of RARβ expression vector into MKN-45 cells resulted in the RARβ concentration-dependent repression of AP-1 activity induced by 12-o-tetradecanoylphorbol-13-acetate (TPA), regardless of the presence of ATRA. When the c-jun and c-fos expression vectors were cotransfected with the RARβ expression vector into MKN-45 cells, AP-1 activity was also obviously repressed. The inhibitory effect, again, was RARβ-concentration-dependent. The stable transfection of the RARβ gene into MKN-45 cells led to cell growth inhibition and colony formation inhibition by ATRA. Furthermore, Cotransfection of both RARβ/DNA binding domain (DBD) and reporter gene could not alter AP-1 activity, even in the presence of ATRA. However, when the cotransfection was substituted with the RARβ/ligand binding domain (LBD), the inhibition was significantly enhanced by ATRA.

Conclusion RARβ might be required for anti-AP-1 activity, and contribute to growth inhibition of gastric cancer cells by ATRA.

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Retinoic acids (RAs), a group of natural and synthetic vitamin A analogs, exert anticancer effect on many types of cancer cell lines, including gastric cancer cell lines. The effect of RAs is mainly mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs). The two receptors are encoded by three corresponding distinct genes, α, β and γ.[1] Retinoic acid receptor β (RARβ), an RAR subtype, plays an important role in mediating RA effect. The fact that RARβ is not expressed in lung and breast cancer cell lines implies that it might possess a tumor suppressive effect, and the lack of RARβ might facilitate tumor development.[2,3] Thus, the RARβ gene could be considered as a promising candidate of tumor suppressor gene.

We have reported that one of the anticancer mechanisms of RAs in gastric cancer cells is via the inhibition of activator protein-1 (AP-1) activity.[4] AP-1, which relates to tumor progression, consists of products of the proto-oncogenes c-jun and c-fos. Many of the AP-1 responsive genes are involved in cancer cell proliferation and transformation. Products of c-jun and c-fos form homodimers (cJun/cJun) or heterodimers (cJun/cFos), and bind to the AP-1 binding sites on their response genes, such as those of the 12-o-tetradecanoylphorbol-13-acetate (TPA) response element and collagenase promoter.[5] In the present study, the role of the RARβ in mediating inhibitory effect of all-trans retinoic acid (ATRA) on AP-1 activity was investigated in a gastric cancer cell line. The data indicated that RARβ could effectively repress AP-1 activity induced by

Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, the School of Life Sciences, Xiamen University, Xiamen 361005, China (Wu Q, Liu S and Su WJ)
PLA 175 Hospital, Zhangzhou 363000, China (Zhang MQ)
PLA 174 Hospital, Xiamen 360000, China (Chen YQ)
Correspondence to: Wu Qiao, Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, the School of Life Sciences, Xiamen University, Xiamen 361005, China (Tel: 86-592-2182542 or 86-592-2187959. Fax: 86-592-2086630. Email: xgwu@xmu.edu.cn)
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TPA in a concentration-dependent manner, or c-jun and c-fos transfection, resulting in the growth inhibition by ATRA on gastric cancer cells. In addition, the functional domain of RARβ critical to the inhibitory effect on AP-1 activity was analyzed, which was found to be located in the DNA binding domain (DBD) of RARβ. Taken together, our results demonstrated that RARβ was required for anti-AP-1 activity, and contributed to the anticancer effect of ATRA on gastric cancer cells.

METHODS

Cell lines and culture conditions

The human gastric cancer cell line MKN-45 from Japan was maintained by the Institute of Cell Biology, Shanghai, China. HeLa cells were obtained from American Type Culture Collection (ATCC). Cells were routinely maintained in RPMI-1640 medium (for MKN-45 cells) or DMEM medium (for HeLa cells), respectively, supplemented with 10% fetal calf serum (FCS), 1 mmol/L glutamine, and 100 U/ml penicillin, and further treated with 1×10^{-6} mol/L ATRA for 24 hours and/or 100 ng/ml TPA for 6 hours as required.

Reporter and plasmids

The reporter -73collagenase-chloramphenicol acetyltransferase (-73Col-CAT) contained the CAT gene linked with a collagenase promoter containing an AP-1 binding site located between residues -73 and -63. This reporter had usually been used for measuring AP-1 activity. Other expression vectors were described previously.[4,6]

RNA preparation and Northern blot

The total RNA was prepared by the guanidine hydrochloride/ultracentrifugation method. About 30 μg total RNA of each sample was electrophoretically fractionated on 1% agarose, then transferred to nylon, and probed with ^{32}p-dATP and ^{32}p-dCTP labeled probe RARβ as previously described.[3] The quantity of sample RNA could be referred to the 28S and 18S fractions of each band.

Transient transfection and CAT assay

Cells were seeded in six-well plates and were approximately 70% confluent at the time of transfection. Cells were transfected by CeLLFECTINtm (Gibco/BRI Life Technologies) according to the following procedure: 6 μl CeLLFECTINtm in 1.0 ml standard medium was added to each well containing 1.0 ml of standard medium supplemented with -73Col-CAT reporter plasmid (100 ng), β-galactosidase expression vector (400 ng, pCH110, pharmacia), and other expression vectors. CAT activity was determined using ^{3}H-acetyl-CoA as substrate. β-galactosidase activity was measured to normalize transfection efficiency.[2,3]

Stable transfection

The sense-RARβ expression vector was stably transfected into gastric cancer cells MKN-45 by CeLLFECTINtm (Gibco/BRI Life Technologies), and then screened with 400 μg/ml of G418 (Sigma). Expression of endogenous RARβ was determined by Northern blot.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay

Cells were seeded in a 96-well plate (1000 cells per well), and treated with 1×10^{-6} mol/L ATRA (Sigma). Medium supplemented with ATRA was changed once every day. Nine days later, cells were stained with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) for 3-4 hours, and their viability was examined by MTT assay.[3]

Anchorage-independent growth assay

An underlayer of 0.5% agar in medium supplemented with 10% FCS was first prepared and hardened in a 6-well plate. 1×10^{5}/ml cells, culture medium containing 10% FCS, 0.3% agar, and 1×10^{-6} mol/L ATRA (only for experimental groups), were then seeded onto the underlayer. The plate was incubated for three weeks in a CO_2 incubator. Colonies with diameter > 80 μm were counted under microscope.

RESULTS

Stable transfection of RARβ gene into MKN-45 cells

RARβ was expressed in many gastric cell lines.[4] But MKN-45 cells, a gastric cancer cell line, did not express RARβ as shown by Northern blot (and thus being used as negative control). By transfecting a relevant empty vector into MKN-45 cells, one of the resulting clones (MKN/vector) did not express RARβ, and the expression could not be induced by ATRA (being adopted as positive control). One of the clones obtained after transfection with the expression vector of sense-RARβ into MKN-45 cells (MKN/RARβ) did stably express RARβ mRNA, although its expression level was not affected by ATRA[4] (data not shown).

Effect of all-trans retinoic acid on AP-1 activity and cell growth inhibition in MKN/RARβ cells

AP-1 activity in MKN/RARβ cells was examined by CAT assay and compared with that in MKN-45 (negative control) and MKN/vector (positive control) cells. Fig. 1 shows cells treated with TPA induce a relatively strong CAT activity in MKN/RARβ, MKN/vector and MKN-45 cells. The CAT

Chinese Medical Journal 2002; 115(6): 810-814

activity was suppressed by ATRA in MKN/RARβ, but not in MKN/vector and MKN45 cells, suggesting that the RARβ gene and its expression might play a role in the inhibition of AP-1 activity.

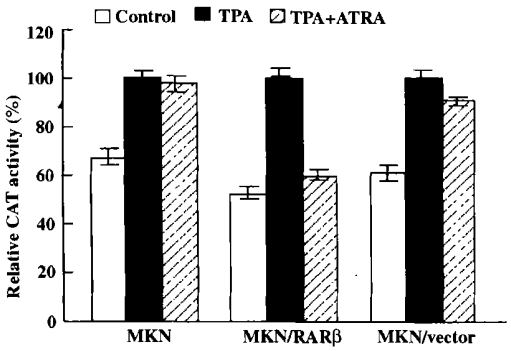

Fig. 1. -73Col-CAT reporter is transfected into relevant cells, MKN-45, MKN/vector and MKN/RARβ cells. After transfection, cells are treated with either TPA alone or with ATRA. Cells are harvested, and then CAT activity is detected by CAT assay. Data represent the mean of duplicate experiment (±SD).

Further studies on growth inhibition examined by MTT assay and anchorage-independent growth in soft agar were carried out. ATRA could effectively inhibit the growth of MKN/RARβ cells, with the inhibitory rate of 37.2%.[4] However, its parental cells MKN-45 and positive control MKN/vector cells did not show any significant response to ATRA treatment, with the inhibitory rates being 3.89% and 5.31%, respectively. The growth of MKN/RARβ cells in soft agar was clearly affected by ATRA, with more than 90% inhibition under ATRA treatment. However, the growth of MKN-45 cells and MKN/vector cells showed a much lower response to ATRA treatment, with less than 20% inhibition. These results were in accordance with those of AP-1 activity inhibited by ATRA as shown in Fig. 1, implying that RARβ is an important mediator for ATRA with regard to the anti-AP-1 activity and growth inhibition of gastric cancer cells.

Effect of exogenous RARβ on anti-AP-1 activity

To further clarify the role of RARβ in mediating inhibition of AP-1 activity, various concentrations of RARβ expression vectors were transiently transfected into MKN-45 cells that did not express RARβ. CAT assay showed that TPA induced high CAT activity without transfection of the RARβ expression vector, and ATRA treatment resulted in weak reduction of CAT activity. However, when the RARβ expression vector was transiently transfected into cells, a relatively strong inhibition on CAT activity induced by TPA was observed after ATRA treatment (Fig. 2). The inhibition of CAT activity by RARβ was concentration-dependent. More than 40% inhibition of CAT activity was seen when transfected with 100 ng RARβ.

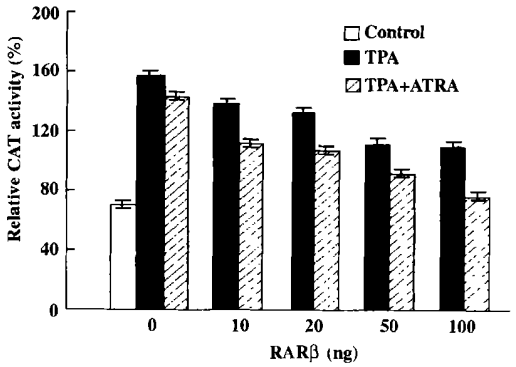

Fig. 2. Effect of exogenous RARβ on CAT activity induced by TPA. Various concentrations of RARβ expression vector is co-transfected with -73Col-CAT reporter into MKN-45 cells. After transfection, cells are treated with either TPA alone or with ATRA. Cells are harvested, and then CAT activity is detected by CAT assay. CAT activity in two independent experiments is shown (±SD).

Effect of RARβ on AP-1 activity originated from exogenous c-jun/c-fos

Our previous observation showed that in MKN-45 cells the expression level of c-jun was very low, and c-fos was not expressed, as revealed by Western blot.[4] Since the products of the c-jun and c-fos genes are components of the AP-1 protein, both c-jun and c-fos expression vectors were transiently transfected into MKN-45 cells. As expected, the CAT activity was induced obviously by c-jun and c-fos transfection in the absence of TPA. However, the inhibition of CAT activity was only observed when the RARβ expression vector was cotransfected with c-jun and c-fos expression vectors, and the strength of inhibition was positively correlated with RARβ concentration (Fig. 3). This was in accordence with the above-mentioned results observed in MKN-45 cells transfected only with RARβ.

DNA binding domain of RARβ is required for anti-AP-1 activity

Two expression vectors of RARβ mutant genes were used for the localization of the functional domain of RARβ in relation to its anti-AP-1 activity. These mutants are characterized by their special deletions, one with its DNA-binding domain (DBD) deleted (RARβ/DBD$^-$), and the other with its ligand-binding domain (LBD) deleted (RARβ/LBD$^-$). HeLa cells do not express RARs and RXRs, and therefore are usually used for the measurement of AP-1 activity.[6] As shown in Fig. 4, when HeLa cells were transfected with the RARβ/DBD$^-$ expression vector, no significant alteration of CAT activity induced by TPA could be seen, even in the presence of ATRA, suggesting that the deletion of DBD may

cause RARβ to be ineffective in mediating the inhibition of CAT activity. DBD, thus, was required for RARβ in mediating the inhibition of AP-1 activity. However, inhibition of CAT activity induced by TPA was detected when transfected with the RARβ/LBD⁻ expression vectors in the absence or presence of ATRA. This observation was similar to that of the parental RARβ receptor, as shown in Figs. 2 and 3, indicating that deletion of LBD might not be responsible for the inhibition of AP-1 activity. Taken together, these results suggested that the DNA binding domain rather than the ligand binding domain was necessary for RARβ to inhibit AP-1 activity.

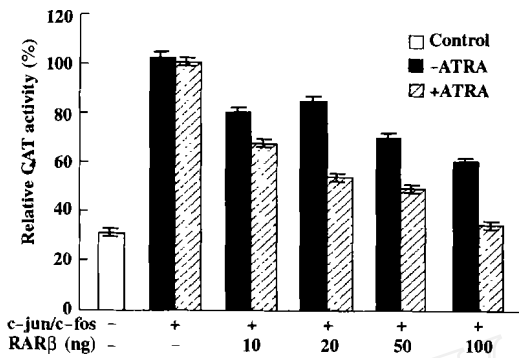

Fig. 3. Effect of RARβ on AP-1 activity originated from exogenous c-jun and c-fos. Various concentrations of RARβ expression vector is cotransfected with both -73Col-CAT reporter and c-jun/c-fos expression vectors into MKN-45 cells. After transfection, cells are treated with ATRA. Cells are harvested, and then CAT activity is detected by CAT assay. The mean of CAT activity in two independent experiments is shown (×SD).

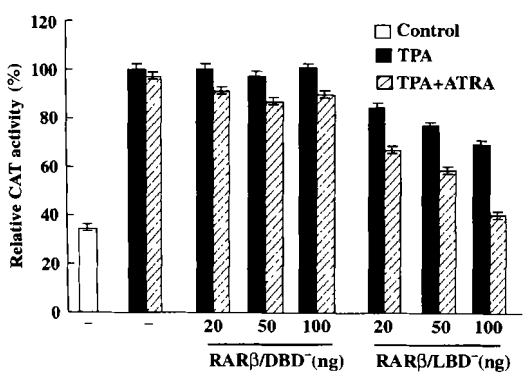

Fig. 4. Effect of two RARβ mutants, DNA binding domain deleted (RARβ/DBD⁻) and ligand binding domain deleted (RARβ/LBD⁻), on CAT activities. Data represents the mean of duplicate experiment (×SD).

DISCUSSION

Retinoic acid exerts its anticancer effect through various ways, including the induction of apoptosis and inhibition of cell growth and AP-1 activity. RA activities are mediated by RARs and RXRs. Accumulated data have indicated that RARβ plays a critical role in regulating proliferation, differentiation and apoptosis in cancer cells. In human lung cancer cell lines and primary human cancer tissue, abnormal expression of the RARβ gene rather than that of other RAR subtypes has been frequently detected.[7] The loss of RARβ expression is an early event in carcinogenesis, and is involved in the process of cancer development.[8] However, the mechanisms of RARβ for anti-AP-1 activity is still largely unknown.

Gastric cancer is one of the major lethal malignant diseases in China. We have found that ATRA could inhibit the growth of gastric cancer cells in vitro by modulating the cell cycle,[9] and suppress the metastasis of gastric cancer cells from spleen to liver in vivo.[10] We have demonstrated in this study that the inhibition of gastric cancer cell growth by ATRA relies on its repression of AP-1 activity via expressed RARβ. Gastric cancer cells MKN-45 which do not express RARβ could not be inhibited by ATRA. However, the transfection of the RARβ gene into MKN-45 cells might lead to growth inhibition in both cell proliferation and colony formation in soft agar by ATRA, suggesting that RARβ plays an important anticancer effects in gastric cancer cells.

The inhibitory function of ATRA on AP-1 activity is one of the mechanisms by which ATRA exhibits its hindrance on gastric cancer cell growth.[4] Transient transfection of the RARβ expression vector into MKN-45 cells results in a significant and RARβ concentration-dependent repression of AP-1 activity induced by TPA, regardless the presence of ATRA. AP-1 is composed of products of the oncogenes c-jun and c-fos, and the resultant AP-1 activity is closely related to tumor development.[5] When the c-jun and c-fos expression vectors were cotransfected with the RARβ expression vector into MKN-45 cells, AP-1 activity was also obviously repressed, and the inhibitory effect was RARβ-concentration-dependent. Taken together, these data strongly support that RARβ might be required for the inhibition of AP-1 activity in gastric cancer cells.

RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily that function as ligand-activated transcriptional factors.[1-3] The DNA-binding domain targets the receptor onto the response element (RE) of its response genes. Once bound to RE, the receptor may respond to its signal, e.g. ATRA, through the ligand-binding domain. The LBD functions as a modulator unit through which the transcriptional activities of the receptor could be regulated by its lidand.[11] Cotransfection of both RARβ/DBD⁻ and the reporter gene could not obviously alter the CAT activity induced by TPA, and this CAT activity was only weakly inhibited even in the presence of ATRA. In other words, the

deletion of DBD⁻ caused RARβ to be ineffective in mediating the inhibition of CAT activity, and DBD⁻ was therefore required for RARβ in mediating the inhibition of AP-1 activity by ATRA. Once the cotransfection was substituted with the RARβ/LBD⁻ and reporter gene, not only did an inhibition of TPA-induced CAT activity occur, but also the inhibition was significantly enhanced by ATRA. It meant that there might be no essential difference between RARβ/LBD⁻ and intact RARβ with regard to their inhibitory effect, and thus, LBD⁻ might not be necessary for the inhibition mediated by RARβ. These results suggest that the mechanism for AP-1 activity inhibition mediated by the retinoic acid recepter is different from the direct interaction between the RAR and the retinoic acid response element (RARE),[12] and relies on protein-protein interaction.

Certain intracellular common coactivators are involved in the molecular basis of repression of AP-1 transcriptional activity mediated by nuclear receptors.[13] The cAMP responsive element binding protein (CREB-binding protein) can be expressed by CBP whose complex has been found to be a common coactivator. The N-terminal of CBP interacts with LBD of RAR in a ligand-dependent fashion, forming a ternary complex of RAR-CBP, resulting in ligand-dependent transcriptional activation by RAR. CBP is also the coactivator required for transcriptional activation by AP-1 and CREB, but the amount of CBP is limited in cells. Therefore, the process of ligand-dependent RAR transcriptional activation is determined by its competition in CBP binding against other cellular factors, such as AP-1 and CREB. Thus, clarification of such mechanism should be of great importance.

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Chinese abstracts of original English papers

原文中文摘要

EB 病毒诱导人鼻咽上皮细胞逃避老化期的生物学研究

Epstein-Barr virus induces human nasopharyngeal epithelial cells to escape from the replicative senescence

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湖南医科大学肿瘤研究所 长沙 410078　杨　静　唐发清　顾焕华　邓锡云　翁新宪　唐　敏　曹　亚

目的　我们利用与鼻咽癌密切相关的 EB 病毒和 TPA 的协同作用，观察原代人胚鼻咽上皮细胞逃避老化期后其生物学特性的变化。

方法　应用相差显微镜观察人鼻咽上皮细胞在 EB 病毒和 TPA 感染后形态上的变化，同时通过检测群体倍增检测细胞体外培养寿命的变化。我们应用老化相关半乳糖苷酶(SA-β-Gal)染色检测 SA-β-Gal 活性的表达情况，并利用免疫组化测定 $p16^{INK4a}$ 蛋白的表达。此外，我们用免疫荧光染色法检测 EB 病毒 LMP1 是否在 EB 病毒和 TPA 感染的细胞中有所表达。

结果　EB 病毒感染的人胚鼻咽上皮细胞在原代培养后期 SA-β-Gal 活性表达降低(4.8%)，形态上发生了改变，出现转化灶样集落，其体外培养寿命延长，并且不表达抑瘤基因产物 $p16^{INK4a}$。

结论　EB 病毒和 TPA 的协同作用能促使部分原代人胚鼻咽上皮细胞逃避老化期，进入永生化早期阶段。这些研究资料为进一步阐明上皮细胞永生化的分子机制、建立人鼻咽上皮细胞永生化模型提供实验依据。

关键词　EB 病毒　鼻咽上皮细胞　老化期　永生化

视黄酸通过视黄酸 β 受体调节胃癌细胞的抗激活蛋白-1 活性

Retinoic acid receptor β is required for anti-activator protein-1 activity by retinoic acid in gastric cancer cells

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厦门大学 生命科学学院 细胞生物学与肿瘤细胞工程教育部重点实验室 厦门 361005　吴　乔　刘　苏　苏文金

中国人民解放军第 175 医院 漳州 363000　张鸣青

中国人民解放军第 174 医院 厦门 360000　陈玉强

目的　研究视黄酸 β 受体(RARβ)介导全反式视黄酸(ATRA)抑制激活蛋白-1(AP-1)活性的作用。

方法　瞬时转染和 CAT 测定，RNA 印迹，基因转染，MTT 测定，停泊非依赖性生长测定。

结果　瞬时转染 RARβ 表达载体到 MKN-45 细胞中，不论 ATRA 存在与否，TPA 诱导的 AP-1 活性受到明显抑制，并与 RARβ 浓度正相关。当 c-jun 和 c-fos 表达载体与 RARβ 表达载体一起转染到 MKN-45 细胞中，由 c-jun 和 c-fos 引起的 AP-1 活性被抑制，而且与 RARβ 浓度呈正比。当 RARβ 在 MKN-45 细胞中稳定表达时，ATRA 可以抑制 MKN-45 细胞生长及其在软琼脂中的生长。当 RARβ 的 DNA 结合区(RARβ/DBD^-)剔除后，ATRA 不能抑制 TPA 诱导的 AP-1 活性。但是，当 RARβ 的配体结合区(RARβ/LBD^-)被剔除时，ATRA 可以

显著抑制 TPA 诱导的 AP-1 活性。

结论 RARβ 对 AP-1 活性的抑制是必需的,并且与 ATRA 抑制胃癌细胞生长密切相关。

关键词 视黄酸β受体 激活蛋白-1 全反式视黄酸 胃癌细胞

产志贺样毒素大肠埃希菌的分子生物学鉴定

Identification of Shiga-like toxin Escherichia coli isolated from children with diarrhea by polymerase chain reaction

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山西省儿童医院分子微生物室 太原 030013 朱庆义 李连青

中国军事医学科学院微生物流行病研究所 北京 100071 郭兆彪 杨瑞馥

目的 探讨产志贺样毒素大肠埃希菌(SLTEC)在小儿腹泻中的病原学地位。

方法 根据肠出血性大肠埃希菌(EHEC)O157:H7 SLT1、SLT2、eaeA 基因片段设计了3对引物,采用 PCR 法检测大肠埃希菌中 SLT1、SLT2、eaeA 毒力基因。

结果 29株标准致泻大肠埃希菌(EHEC、EPEC、ETEC)和10株其它肠道病原菌中,只有1株 EHEC 检出 SLT1、SLT2、eaeA 毒力基因,其它均为阴性。从1032例腹泻患儿粪便中分离的474株未定型大肠埃希菌中,检出 SLT1 20株(4.2%),SLT2 7株(1.5%);74株产志贺样毒素侵袭性大肠埃希菌(ESIEC)中检出 SLT1 15株(20.3%),SLT2 5株(6.8%)。

结论 SLTEC 是太原地区引起小儿腹泻的一种重要的致泻病原菌。

关键词 产志贺样毒素大肠埃希菌 腹泻 聚合酶链反应

精神分裂症患者海马 parvalbumin-免疫反应神经元密度选择性减低

A selective reduction in the relative density of parvalbumin-immunoreactive neurons in the hippocampus in schizophrenia patients

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南京医科大学附属脑科医院精神科 210029 张志珺 孙 静

英国谢菲尔德大学生物医学系 Gavin P Reynolds

目的 以钙结合蛋白-parvalbumin 和 calretinin 为标记,确定海马本体 γ-氨基丁酸能神经元亚群,并定量分析精神分裂症海马 γ-氨基丁酸能中间神经元相对密度的改变及其在发病机理中的作用。

方法 用免疫组化法结合抗 parvalbumin 和抗 calretinin 抗体,测定这些钙结合蛋白免疫反应阳性细胞在精神分裂症和相匹配的正常对照(每组15例)海马齿状回和 CA1 - CA4 区的分布、胞体大小、相对密度及海马亚区面积。

结果 与正常对照相比,精神分裂症患者 calretinin-免疫反应中间神经元相对密度无显著性差异;而 parvalbumin-免疫反应中间神经元密度于海马各亚区均严重缺失,以男性患者为著,且与抗精神病药物治疗、年龄、疾病持续时间无显著相关。

结论 精神分裂症患者海马区含 parvalbumin 的 γ-氨基丁酸能抑制性中间神经元亚群选择性丢失,并可能