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Antisense IRAK-2 oligodeoxynucleotide inhibits interleukin-1-induced nuclear factor-κB activation *in vitro*

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KEY WORDS antisense oligonucleotides; interleukin-1; NF-kappa B; human embryonic kidney 293 cells; enzyme-linked immunosorbent assay

ABSTRACT

AIM: To study the inhibitory effects of antisense interleukin-1 receptor associated kinase-2 (IRAK-2) oligodeoxynucleotide (ODN) on interleukin-1 (IL-1)-stimulated nuclear factor-κB (NF-κB) activation. **METHODS:** Antisense IRAK-2 ODN was delivered by lipofectin encapsulation into human embryonic kidney 293 cells (HEK 293 cells). The levels of NF-κB were assayed by sandwich ELISA. **RESULTS:** (1) Treatment of HEK 293 cells with IL-1 enhanced NF-κB level in nuclei by 518.5 % ± 2.1 %. (2) Antisense IRAK-2 ODN inhibited IL-1-induced NF-κB activation in a concentration (1 – 8 μg)- and time (5 – 24 h)-dependent manner. A maximum inhibition was 70.7 % ± 1.0 % from $A_{control}$ 0.834 ± 0.014 to 0.244 ± 0.008 after treatment with antisense IRAK-2 ODN 4 μg for 8 h. **CONCLUSION:** Antisense IRAK-2 ODN inhibited IL-1-induced NF-κB activation.

INTRODUCTION

Interleukin-1 (IL-1) is a cytokine that has several effects in the inflammation response. Many of the proinflammatory effects of IL-1, such as up-regulation of IL-6 on human embryonic kidney 293 cells (HEK 293 cells), are regulated at transcriptional level. The transcriptional activation by IL-1 or IL-6 and other genes appears to be mediated largely by nuclear factor-κB (NF-κB)[1]. Therefore, therapeutic interventions aimed at limiting NF-κB activation could prove to be beneficial in alleviating IL-1-induced inflammation[2]. Previous studies predominantly focused on inhibiting directly the process of NF-κB activation (ie, interfering with phosphorylation and degradation of IκB, blocking translocation to the nucleus and DNA binding of NF-κB[3–5]), while few reports took notice upstream of NF-κB activation.

IL-1 receptor associated kinase-2 (IRAK-2) was identified recently as an upstream signal leading to NF-κB activation[6]. Thus inhibitory effects of IRAK-2 may block NF-κB activation and should ultimately have an anti-inflammatory effect. Since antisense oligodeoxynucleotides (ODN) present an innovative and attractive gene therapy strategy to block the transcription or translation of specific genes, the purpose of our studies was to examine the inhibitory effects of antisense IRAK-2 ODN on IL-1-stimulated NF-κB activation.

MATERIALS AND METHODS

Materials HEK 293 cell line was generously provided by Dr XU Wei (originally from ATCC, USA). DMEM and lipofectin were purchased from Gibco, BRL. IL-1β was purchased from Tian Xiang Ren Co, Beijing. Rabbit polyclonal antibody against the p65 subunit of NF-κB was obtained from Santa Cruz Biotechnology, Inc, USA. Sheep anti-rabbit IgG-HRP was purchased from Hua Mei Co, Henan.

Cell culture HEK 293 cells were cultured as adherent monolayers in DMEM supplemented with 10 % fetal bovine serum and grown to confluency in 6-well plates at 37 ℃ in a humidified atmosphere of 5 % CO_2 + 95 % air.

Synthesis of ODN and selection of sequence targets The sequences of ODN against IRAK-2 were: antisense, 5′-GTAGATGTAGCAGGCCAT-3′; sense, 5′-ATGGCCTGCTACATCTAC-3′ (1 to 18 of human IRAK-2 sequences). Sense ODN was used as control. ODN were synthesized by Sangon Co Ltd, Shanghai, and modified with phosphorothioate.

Preparation of lipofectin-encapsulated ODN

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Four μL of lipofectin 1 g·L^{-1} was diluted in 96 μL serum-free media and kept at room temperature (23 ℃) for 45 min. Indicated dose of ODN was diluted in serum-free media to 100 μL. Then lipofectin was mixed with ODN for 15 min.

Nuclear protein extraction and sandwich ELISA for detecting NF-κB Nuclear proteins in ODN-treated cells were isolated by the method described previously[7] and dissolved in buffer C. NF-κB was measured by ELISA method[8]. The capture antibody against NF-κB p65 (3 mg·L^{-1}, 100 μL/well) was coated overnight at 4 ℃ on 96-well microtiter plates (Sigma). Then the plates were blocked with 1 % BSA overnight at 4 ℃. The plates were washed. One hundred μL of dilutions of nuclear proteins in a ratio of 1∶500 were added to the plates at 37 ℃ for 2 h. The plates were washed, after which the NF-κB p65 polyclonal antibody was added in wells and incubated at 37 ℃ for 2 h. After further washing, the plates were incubated with sheep anti-rabbit IgG-HRP diluted at 1∶1000 (100 μL/well) at 37 ℃ for 2 h. The plates were washed, and OPD 2 mmol·L^{-1} with 0.006 % H_2O_2 in citric acid-phosphate buffer (100 μL/well) was added. Then the plates were covered and incubated at 37 ℃ for 1 h. The reaction was stopped by adding citric acid 2 mol·L^{-1} (50 μL/well). The absorbence (A) at 450 nm was measured with a Model 450 microplate reader (BIORAD). Background reading was determined from wells in which PBS containing buffer C without nuclear proteins was incubated. The background reading was subtracted from all results. Assays were done in triplicate wells.

Statistics Data were expressed as $\bar{x} \pm s$ and compared with t test.

RESULTS

Lipofectin-encapsulated antisense IRAK-2 ODN inhibited NF-κB activation To determine the effects of antisense IRAK-2 ODN on NF-κB activation, we delivered the lipofectin-encapsulated ODN 4 μg into HEK 293 cells under conditions of steady-state turnover of IRAK-2 and in cells undergoing rapid turnover of their IRAK-2. Thus, in the later case, we pretreated cells with IL-1β 100 kU·L^{-1} for 30 min to downregulate IRAK-2. IL-1β was then removed to allow recovery of IRAK-2 via new IRAK-2 synthesis[7]. Exposure of cells during steady-state turnover of IRAK-2 to the lipofectin-encapsulated antisense ODN was without effect on response to IL-1. If, however, cells were exposed to the lipofectin-encapsulated antisense ODN during a period of new IRAK-2 expression, the significant blockade of IL-1-stimulated NF-κB activation ($P < 0.01$ *vs* steady-state) was observed (Tab 1).

Concentration response and time course for inhibition of IL-1-induced NF-κB activation by antisense IRAK-2 ODN Antisense IRAK-2 ODN inhibited IL-1-induced NF-κB activation in a concentration- and time-dependent fashion. Concentration-response studies showed that antisense IRAK-2 ODN remarkably inhibited IL-1-induced NF-κB activation at a dose of 1 μg. The inhibitory effects were also observed when the cells were treated with the antisense ODN 2 μg, 4 μg, and 8 μg. Time-course experiments revealed that a maximum inhibition of NF-κB activation occurred 8 h after incubation of cells with the antisense ODN 4 μg. The inhibitory effects of antisense IRAK-2 ODN was seen up to 24 h. The levels of NF-κB were comparable in the control untreated cells (Tab 2).

DISCUSSION

In the present studies, we found for the first time that antisense IRAK-2 oligodeoxynucleotide blocked IL-1-

Tab 1. Effects of antisense ODN on IL-1-induced NF-κB activation. $n = 3$ wells. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* sense.

Condition	NF-κB/A_{450} per well
Steady-state turnover of IRAK-2	
Basal	0.238 ± 0.006
No IL-1, incubated for 8 h with serum-free media, IL-1 1 h	1.472 ± 0.005
No IL-1, incubated for 8 h with lipofectin antisense ODN, IL-1 1 h	1.632 ± 0.045
Rapid turnover of IRAK-2	
IL-1 30 min, incubated for 8 h with serum-free media, IL-1 1 h (control)	0.835 ± 0.013
IL-1 30 min, incubated for 8 h with lipofectin sense ODN, IL-1 1 h	0.732 ± 0.022
IL-1 30 min, incubated for 8 h with lipofectin antisense ODN, IL-1 1 h	0.261 ± 0.008^{cf}

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Tab 2. Concentration response and time course for inhibition of IL-1-stimulated NF-κB activation by antisense IRAK-2 oligodeoxynucleotide. $n = 3$ wells. $\bar{x} \pm s$. ${}^{c}P < 0.01$ *vs* control.

Antisense IRAK-2 ODN/μg	NF-κB/A_{450} per well 5 h	8 h	24 h
Control	0.820 ± 0.011	0.834 ± 0.014	0.831 ± 0.011
1	0.573 ± 0.013^c	0.377 ± 0.009^c	0.490 ± 0.006^c
2	0.325 ± 0.032^c	0.277 ± 0.047^c	0.296 ± 0.007^c
4	0.257 ± 0.002^c	0.244 ± 0.008^c	0.255 ± 0.027^c
8	0.277 ± 0.002^c	0.261 ± 0.002^c	0.269 ± 0.016^c

induced NF-κB activation.

IRAK-2 is a proximal mediator of IL-1 signaling pathway. After IL-1 binds to its receptor type I (IL-1RI), IRAK-2 is activated and phosphorylated, which triggers a signaling cascade resulting in the activation of NF-κB. Therefore, IRAK-2 may provide a therapeutic target for inhibiting IL-1-induced inflammation. Our results showed that antisense IRAK-2 ODN inhibited IL-1-stimulated NF-κB activation, so antisense IRAK-2 may potentially serve as an antiinflammatory agent. On the other hand, it was also reported that truncated mutant encoding amino acids 97 to the COOH terminal end of IRAK-2 [IRAK-2 (97 – 590)] could act as dominant negative inhibitor of IL-1-stimulated NF-κB activity[6]. But compared with the truncated mutant, antisense IRAK-2 ODN is easy to be synthesized.

In addition to size, target sequences, stability and delivering methods of antisense ODN, our experiments demonstrated that the pretreatment of the cells with IL-1 played an important role in the factors affecting biological efficacy of antisense IRAK-2 ODN. When antisense IRAK-2 ODN was added to the cells without IL-1 pretreatment, no inhibitory effects were observed. In contrast, IL-1-stimulated NF-κB activation was significantly blocked when the cells were pretreated with IL-1. It is implicated that antisense IRAK-2 ODN exerted its inhibitory effects only under conditions of new IRAK-2 syntheses.

In conclusion, antisense IRAK-2 ODN acts at a step prior to IκB phosphorylation and it is a new way to inhibit IL-1-induced NF-κB activation.

REFERENCES

1 O'Neill LAJ, Greene C. Signal transduction pathways activated by the interleukin-1 receptor family: ancient signaling machinery in mammals, insects, and plants. J Leukoc Biol 1998; 63: 650 – 7.

2 Christman JW, Lancaster LH, Blackwell TS. Nuclear factor kappa B: a pivotal role in the systemic inflammatory response syndrome and new target for therapy. Intensive Care Med 1998; 24: 1131 – 8.

3 Page S, Fischer C, Baumgartner B, Haas M, Kreusel U, Loidl G, *et al*. 4-Hydroxynonenal prevents NF-kappa B activation and tumor necrosis factor expression by inhibiting kappa B phosphorylation and subsequent proteolysis. J Biol Chem 1999; 274: 11611 – 8.

4 Yoshida S, Kato T, Sakurada S, Kurono C, Yang JP, Matsui N, *et al*. Inhibition of IL-6 and IL-8 induction from cultured rheumatoid synovial fibroblasts by treatment with aurothioglucose. Int Immunol 1999; 11: 151 – 8.

5 Ono S, Date I, Onoda K, Shiota T, Ohmoto T, Ninomiya Y, *et al*. Decoy administration of NF-kappa B into the subarachnoid space for cerebral angiopathy. Hum Gene Ther 1998; 9: 1003 – 11.

6 Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science 1997; 278: 1612 – 5.

7 Rovin BH, Dickerson JA, Tan LC, Hebert CA. Activation of nuclear factor-κB correlates with MCP-1 expression by human mesangial cells. Kidney Int 1995; 48: 1263 – 71.

8 Wang RG, Zhu XZ, Song W. Pharmacokinetics of recombinant human granulocyte macrophage colony-stimulating factor in *Macaca mulatta*. Acta Pharmacol Sin 1998; 19: 50 – 3.

白介素-1 受体相关激酶-2 的反义寡脱氧核苷酸体外抑制白介素-1 诱导的核因子-κB 活化

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关键词 反义寡核苷酸；白介素-1；核因子-κB；人胚肾 293 细胞；酶联免疫吸附测定

目的：研究白介素-1 受体相关激酶-2 的反义寡脱氧核苷酸对核因子-κB 的抑制作用. **方法**：脂质体介导反义白介素-1 受体相关激酶-2 寡脱氧核苷酸转染人胚肾 293 细胞. 夹心酶联免疫吸附测定法检测核因子-κB 的含量. **结果**：(1) 白介素-1 刺激人胚肾 293 细胞时，核因子-κB 含量明显上升(上升幅度 518.5 % ± 2.1 %). (2) 白介素-1 受体相关激酶-2 的反义寡脱氧核苷酸呈浓度(1 – 8 μg)和时间(5 – 24 h)依赖性地抑制白介素-1 的作用. 反义寡脱氧核苷酸 4 μg 与细胞孵育 8 h，约 70.7 % ± 1.0 % 的 NF-κB 活性被抑制. 吸光度由对照的 0.834 ± 0.01 下降到 0.244 ± 0.008. **结论**：白介素-1 受体相关激酶-2 的反义寡脱氧核苷酸抑制白介素-1 诱导的核因子-κB 活化.

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