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Investigation of liposome-conjugated Nuclear Factor-κB Decoy Oligodeoxynucleotide in a Mouse Model of Dextran Sodium Sulphate-induced Colitis

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ABSTRACT

BACKGROUNDS/AIMS: Double strand nuclear factor-κB decoy oligodeoxynucleotide (NF-κB-Decoy) had been investigated as a potential therapeutic approach in ulcerative colitis. Here we confirm that NF-κB-Decoy inhibits inflammatory cytokines production *in vitro*, and report initial studies of the use of liposome-conjugated NF-κB-Decoy to treat dextran sodium sulphate (DSS)-induced colitis. **METHODS:** (1) Peripheral blood samples or cultured Colo 205, colon cancer cells were pretreated with NF-κB-Decoy and stimulated with lipopolysaccharide. (2) Eight-weeks-old female BALB/C mice were fed 4% DSS in drinking water for 7 days. Intra-colonic administration of liposome-conjugated NF-κB-Decoy started at the same time as DSS treatment, and continued for 14 days. **RESULTS:** (1) In the peripheral blood, NF-κB-Decoy significantly inhibited interleukin (IL)-6 production (Tukey test, $p<0.05$). In the cultured cells, NF-κB-Decoy significantly reduced IL-8 production (t-test, $p<0.01$). (2) During administration of DSS, mortality rate between NF-κB-Decoy treated and control mice (72.4% and 57.6% respectively) was not significantly different, and body weight loss and histopathological dameges were similar in both groups. But, after discontinuation of DSS, NF-κB-Decoy-treated mice regained body weight significantly earlier than did control mice. **CONCLUSIONS:** NF-κB-Decoy actually inhibited IL-6 and IL-8 production *in vitro*. Although liposome-conjugated NF-κB-Decoy could not show amelioration in DSS mice model, NF-κB-Decoy treated mice showed earlier regain of body weight.

Key words : ulcerative colitis, colon cancer cell line, interleukin-6, interleukin-8

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INTRODUCTION

Nuclear factor-κB (NF-κB) is a key transcriptional regulator of inflammation and immune response. Activation of NF-κB promotes the expression of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and IL-8 in the inflamed colonic mucosa characteristic of ulcerative colitis[1)-5)]. Double-stranded NF-κB decoy oligodeoxynucleotide (NF-κB-Decoy) has been investigated as a potential strategy to regulate NF-κB activa-tion[6)-8)]. NF-κB-Decoy attaches to the promoter site to which NF-κB binds and consequently attenuates the transcriptional activity of NF-κB. Antisense oligonucleotide to NF-κB was earlier proposed as a strategy to attenuate the effects of NF-κB[9)-11)], but has not yet reached clinical application. Theoretical considerations suggest that NF-κB-Decoy might be more stable and work more quickly than antisense to NF-κB.

Intra-colonic administration of bare NF-κB-Decoy has been reported to result in amelioration of symptoms in models of colitis induced by trinitrobenzene sulphonic acid (TNBS), oxazolone and dextran sodium sulphate (DSS)[12),13)]. De Vry et al reported that NF-κB-Decoy facilitated healing of TNBS colitis and inhibited expression of the mRNAs for inflammatory cytokines[12)]. However, to work well, NF-κB-Decoy must be distributed intracellularly[14)]. De Rosa et al reported that intracellular uptake of NF-κB-Decoy was enhanced by administration in microspheres[15)]. Therefore, we investigated the efficacy of liposomal-conjugated NF-κB-Decoy in a mouse model of DSS-induced colitis. In addition, to explore potential mechanisms, we investigated the effect of NF-κB-Decoy on inflammatory cytokines production by peripheral blood and cultured epithelial cells.

METHODS

Chemicals

NF-κB-Decoy was provided by Anges MG Inc. (Osaka, Japan) by courtesy of Professor Ryuichi Morishita. The sequence of the NF-κB-Decoy used in this study is 5'-CCTTGAAGGGATTTCCCTCC-3' and 3'-GGAACTTCCCTAAAGGGAGG-5'. The underlined sequences are identical to the binding site of NF-κB on the promoters of its target genes. FuGENE6 (Roche Diagnostics, Tokyo, Japan) was used as transfection reagent in *in vitro* experiments. Liposome COATSOME: EL-01-C (NOF Co, Tokyo, Japan) was used as carrier for intracolonic administration. DSS (ICN Biochemicals Inc, Tokyo, Japan) which molecular weights were 36000-50000 kDa, was prepared. To stimulate peripheral blood and cultures cells, lipopolysaccharide (LPS) from *E. Coli* O5:B55 (Sigma Chemical Co, St Louis, MO, USA). Each cytokines were measured using ELISA kit (R&D Systems, Minneapolis, MN, USA).

Peripheral blood

Peripheral blood samples with heparin were collected from healthy volunteers and RPMI 1640 containing 10% fetal bovine serum were prepared as culture medium. In the wells of 24 well culture plates, 0.1 ml of blood sample was suspended in the 0.9 ml of culture medium. Blood suspension was treated various concentration of NF-κB Decoy and/or 3μl of FuGENE6 2 hours prior to LPS stimulation. After adding 1 ng of LPS per well (final

concentration of 1 μg/ml), 4 hours incubation at 37°C under 5% CO_2, was done. Then the supernatant was collected by centrifugation at 3000 rpm at 4°C for 5 min and was stored -80°C until assay. TNF-α, IL-6, IL-1β, and IL-8 were measured by using an ELISA kit (R&D Systems, Minneapolis, MN, USA).

Cultured cells

The Colo 205 colon cancer cell line was provided by the Cell Source Center of Geriatric Medicine, Tohoku University School of medicine. Cells were cultured in RPMI 1640 (Invitorogen, Tokyo, Japan) containing 1mM L-glutamine, 10% FBS, and 12.5 μg/ml gentamicin. Cells ($3x10^5$ cells/well) were prepared in each well of 6-well culture plate and incubated for 24 hours at 37°C under 5% CO_2. Then the culture medium was changed to RPMI 1640 containing 10% FBS but without gentamicin. NF-κB-Decoy was added to the wells at a final concentration of 1 μg/ml and incubation was continued for 2 hours. Then LPS solution was added to the wells at a final concentration of 1 μg/ml. After 4 hours incubation at 37°C under 5% CO_2, supernatant was collected by centrifugation at 3000 rpm at 4°C for 5 min and was stored-80°C until assay. IL-8, TNF-α, IL-1β, and IL-6 were measured in the supernatant. Cell viability was verified by Trypan Blue staining.

Animal model

These animal experiments were approved by Laboratory Animal Center, Yamagata University Faculty of Medicine. BALB/C mice (female, 8 weeks) (CLEA Japan Inc., Tokyo, Japan) were used. To induce colitis, mice were allowed free access to 4% DSS solution in drinking water for 7 days (from Day 1 to Day 7). For treatment, NF-κB-Decoy was dissolved in liposome solution at a final concentration of 2 mg/ml with distilled water. Preparation of liposome-conjugated NF-κB-Decoy were followed the technique recommended by NOF Co. Saline was used as control. For 14 days (from D 1 to D 14), 0.4 ml of each solution (800 μg/body of NF-κB) was administrated intracolonically 3 cm into the colon by a thin and soft polyurethane tube. Body weight and mortality rate was evaluated. In a separate experiment, mice were sacrificed at Day 10, and histopathological changes[16] and serum cytokines levels in both groups were examined.

Statistics

Tukey test was applied to analyze examinations of peripheral blood. Student's t test was used to analyze body weight and IL-8 concentrations in culture cells. Mortality rate were tested by χ^2 or Fischer's exact calculation. IL-6 in DSS induced colitis and histological index were evaluated by Mann-Whitney's U test. Difference with p values less than 0.05 were considered as significant.

RESULTS

Peripheral blood

On stimulation with LPS, peripheral blood produced TNF-α, IL-1β, and IL-6 but not IL-8. NF-κB-Decoy did not inhibit production of TNF-α (Figure 1a). But when NF-κB-Decoy was treated with FuGENE6, TNF-α production was significantly inhibited. In contrast, NF-κB-Decoy significantly inhibited production of IL-6 and stronger inhibition were observed using FuGENE6 (Figure 2a). In another experiments, it was confirmed that FuGENE6 itself did not influence on the TNF-

α (Figure 1b) and IL-6 (Figure 2b) production. IL-1β had been measurable, but NF-κB-Decoy did not significantly inhibit production of IL-1 β even with FuGENE6 treatment.

Culture cells

Cultured Colo 205 cells produced IL-8 on stimulation with LPS. NF-κB-Decoy signifi-

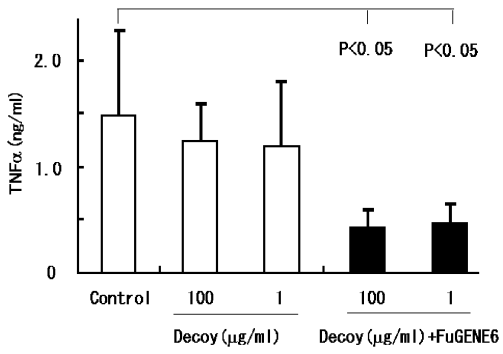

Fig. 1a. Effect of NF-κB-Decoy on the production of TNF-α in the peripheral blood
Blood samples were pretreated with NF-κB-Decoy (with or without FuGENE6) at final concentration of 100 and 1μg/ml for 2 hours. After 4 hours incubation with LPS, TNF-α concentrations of supernatant were measured (mean ± SD). NF-κB-Decoy significantly inhibited production of TNF-α in the cases with FuGENE6 (Tukey-test, $p<0.05$). "Decoy" indicated NF-κB-Decoy treatment (n=12) and "Control" indicated control (n=12).

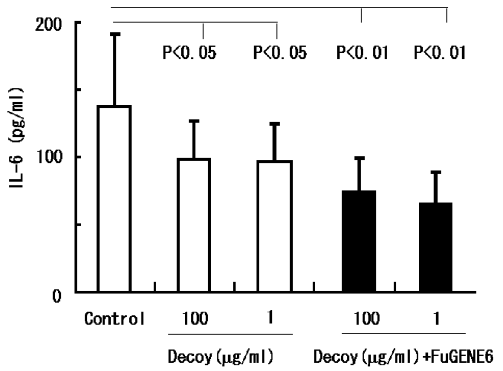

Fig. 2a. Effect of NF-κB-Decoy on the production of IL-6 in the peripheral blood
Blood samples were pretreated with NF-κB-Decoy (with or without FuGENE6) at final concentration of 100 and 1μg/ml for 2 hours. After 4 hours incubation with LPS, IL-6 concentrations of supernatant were measured (mean ± SD). NF-κB-Decoy significantly inhibited production of IL-6 (Tukey-test, $p<0.05$). "Decoy" indicated NF-κB-Decoy treatment (n=12) and "Control" indicated control (n=12).

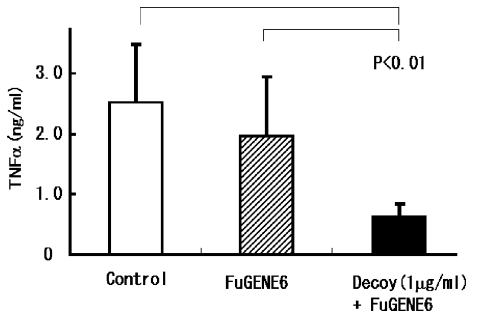

Fig. 1b. Influence of FuGENE6 on the production of TNF-α in the peripheral blood
Blood samples were pretreated with or without FuGENE6. FuGENE6 itself did not suppress TNF-α production of blood cells but NF-κB-Decoy significantly inhibited production of TNF-α (mean ± SD) (Tukey-test, $p<0.01$). "Decoy" indicated NF-κB-Decoy treatment (n=12) and "Control" indicated control (n=12).

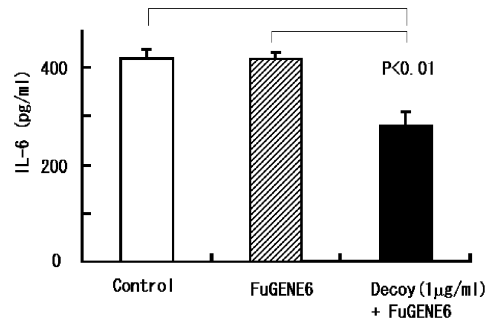

Fig. 2b. Influence of FuGENE6 on the production of IL-6 in the peripheral blood
Blood samples were pretreated with or without FuGENE6. FuGENE6 itself did not suppress TNF-α production of blood cells (mean ± SD) (Tukey-test, $p<0.05$). "Decoy" indicated NF-κB-Decoy treatment (n=6) and "Control" indicated control (n=6).

cantly inhibited production of IL-8 (t-test, $p<0.01$) (Figure 3). TNF-α, IL-6 and IL-1β were not measurable in this experiment.

Liposome-conjugated NF-κB in DSS induced colitis

We performed three separate sets of studies of survival and body weight. In total, 29 mice were treated with liposome-conjugated NF-κB-Decoy (treated mice) and 33 mice were assigned to the control group. During the study periods, 21 of 29 treated mice and 19 of 33 control mice died. These mortality rates were not significantly different (χ^2 test). The change in body weight of surviving mice in both groups is shown in Figure 4. The body weight of the NF-κB treated mice decrease similarly to the control mice until Day 9, but after Day 10 the treated mice showed earlier regain of body weight compared with the control mice (t-test, $p<0.05$). There was a significant difference at Day 10, 11, and 12, but on Day 13 and 14 the body weight of both groups became similar.

In a separated set of similar studies, mice were sacrificed on Day 10. Two sections of each colon, representing the proximal and distal one-thirds, were prepared and inflammation was scored histologically. Based on the histological colitis scoring method, score (mean ± SD) of the NF-κB-Decoy treated group (n=6) and control group (n=10) were 7.3 ± 2.8 and 7.5 ± 2.6, respectively (Table 2). These values are not significantly different, indicating that NF-κB-Decoy treatment did not ameliorate the histopathological changes characteristic of DSS-induced colitis. Then serum concentrations of IL-6 of mice were measured. As a number of serum samples were was small and varied widely, validity could not be evaluated.

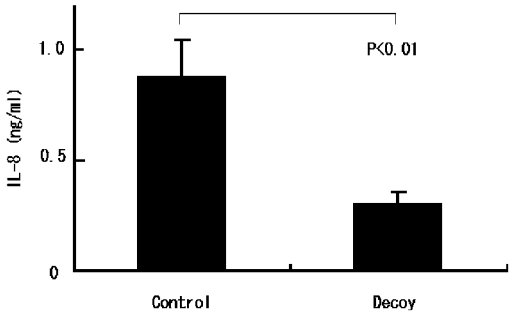

Fig. 3. Effect of NF-κB-Decoy on the production of IL-8 in cultured cells

Colo 205 cells were pretreated with NF-κB-Decoy at final concentration of 1μg/ml for 2 hours. After 4 hours incubation with LPS, IL-8 concentrations were measured in the supernatant (mean ± SD). NF-κB-Decoy significantly inhibited production of IL-8 in cultured cells (t-test, $p<0.01$). "Decoy" indicated NF-κB-Decoy treatment (n=6) and "Control" indicate control (n=6).

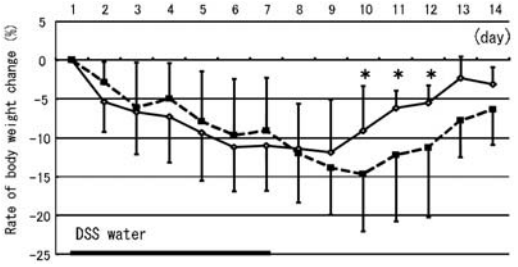

Fig. 4. Change of body weights of mice in DSS colitis model

Mice were fed 4% DSS in drinking water for 7 days, and intra-colonic administration of liposome-conjugated NF-κB decoy oligodeoxynucleotide started at the same time of beginning of DSS water-feeding for 14 days. Closed square with brake line showed NF-κB decoy oligodeoxynucleotide treated mice and open diamond with solid line showed control mice (mean ± SD). On the day 10, 11, and 12, significant higher body weight revealed in NF-κB-Decoy treated group (*; t-test, $p<0.05$).

Table 1. Mortality rate in the DSS-induced model of colitis

	dead	survive	mortality rate
NF-κB-Decoy (n=29)	21	8	(72.4%)
Control (n=33)	19	14	(57.6%)

Mice were fed 4% DSS in drinking water for 7 days, and intracolonic treatment with liposome-conjugated NF-κB-Decoy was initiated at the beginning of DSS administration and continued for 14 days. Mortality refers to the period of DSS administration. The mortality rates in the two groups were not significantly different (χ^2 test).

Table 2. Concentration of serum IL-6 and histological score after discontinuation of DSS treatment

	NF-κB-Decoy (n= 6)	Control (n=10)
Serum IL-6 (pg/ml)	127 ± 158	76 ± 42
Histological Score	7.3 ± 2.8	7.5 ± 2.6

As a separate experiment, mice were sacrificed at Day 10. There were not significant difference in serum IL-6 levels and histological colitis scores between liposome-conjugated NF-κB-Decoy and control mice group.

However, NF-κB treated mice revealed 127 ± 158 pg/ml and 76 ± 42 pg/ml control mice done so (Table 2).

DISCUSSION

At first, we confirmed that NF-κB-Decoy had certain inhibitory effects on TNF-α, IL-6, and IL-8 production in vitro. In peripheral blood experiments, we should properly use monocytes derived from inflammatory mucosa. But, as many inflammatory cells are supplied from peripheral blood, we have chosen peripheral whole blood as substitutes of inflammatory mucosal cells, in this preliminary *in vitro* study. In addition, we chose colon cancer cell line as colonic epithelial cells. As IL-6 production from peripheral blood and IL-8 production from epithelial cells were particularly inhibited, NF-κB-Decoy may be practically promising in the treatment for inflammatory bowel disease.

NF-κB-Decoy should enter inside the cells to work well. We showed that treatment of transfection reagent enhanced the inhibitory effect. In fact, intra-bronchiole administration of NF-κB-Decoy could not show efficacy in bleomycin induced pneumonia and it presumed to be problem of insufficient intracellular distribution[17]. Therefore it is an important issue that efficient delivery systems of NF-κB-Decoy should be established. Concerning NF-κB-Decoy delivery system, De Rosa et al reported that intracellular uptake of NF-κB was enhanced applying microsphere[15]. So far it had been reported that an intracolonic administration of bare NF-κB-Decoy was effective in the animal models of inflammatory bowel disease[12),13)]. Hence, based on this NF-κB-Decoy delivery system, we performed a preliminary study applying liposome-conjugated NF-κB-Decoy *in vivo*.

As NF-κB-Decoy is a double strand oligodeoxynucleotide, it might be expected to be stable. However, the half-life of NF-κB-Decoy is 26 minutes after intravenous administration

(information from Anges MG Co., Osaka, Japan). Therefore, topical (i.e. intracolonic) administration appears necessary for treatment of colitis. Analogously, in atopic dermatitis, where the target cells for NF-κB-Decoy are epidermal macrophages, topical NF-κB-Decoy ointment applied to the skin showed efficacy in an atopic mouse model[18]. Recently, to improve the stability of NF-κB-Decoy, ribbon-type of the decoy oligodeoxynucleotides had been developed and investigated[19].

In previous reports of the use of NF-κB decoy oligonucleotides in models of colitis, TNBS- and oxazolone-induced models were described in full. Precisely and sufficient improvements were reported not only in the histopathological evaluations but also mRNA expressions of inflammatory cytokines[12),13)], whereas relatively little information was provided on the DSS-induced model[12)]. Topical (intracolonic) administration should be practical for the distal type of ulcerative colitis, and the DSS-induced colitis model is generally employed as an animal model of ulcerative colitis. Consequently, we chose the DSS-induced colitis model for this study.

To establish the model, we used 5% DSS in water in initial experiments. However, all mice were lost within 14 days. The present study used 4% DSS in water, which also appears to be a severe insult as the mortality rate remained high. Under these conditions, mortality was not improved by NF-κB-Decoy-treatment. In addition, decrease of body weight, histopathological scores, and increase of IL-6 levels during DSS intake were not improved in the NF-κB-Decoy-treated mice. As a result, we could not reveal amelioration of colitis by NF-κB-Decoy. As an only benefit of NF-κB-Decoy treatment, we could observe that NF-κB-Decoy-treated mice regained body weight more quickly after discontinuation of DSS treatment. De Vry et al also mentioned that NF-κB-Decoy might contribute to the early recover in TNBS colitis model[12)].

We must discuss that liposome-conjugate NF-κB-Decoy failed to show improvements in DSS mouse model, contrary to previous reports. It may firstly rise up as an issue that DSS colitis model used in this study might be too severe. In fact, mortality rate showed more than 50%. If we attempt to make a clinical application of NF-κB-Decoy for the patients with ulcerative colitis, distal colitis type with mild inflammation may be indicated. Hence, we might have chosen mild colitis model. Secondary, liposome itself was possibly to cause deterioration of inflammation. However, we preliminary evaluated liposome solution did not exacerbate colitis in small number of animals, although data had not been shown.

Recently, Xiang et al reported that NF-κB-Decoy treatment ameliorated 5% DSS-induced murine colitis model[20)]. They used the same decoy oligodeoxynucleotides as ours, which were not liposome conjugated. It was thought that relatively small molecular weight of DSS realized the adequate colitis model. In future, using the appropriate colitis model, we must investigate whether the liposome conjugated NF-κB-Decoy is truly more effective than the bare NF-κB-Decoy.

Prior to NF-κB-Decoy, successful treatments of NF-κB p65 antisense oligonucleotides in TNBS or DSS colitis models[9)-11)]. In addition, NF-κB p65 antisense oligonucleotides actually inhibited production of pro-inflammatory cytokines in lamina propria mononuclaer cells drived from ulcerative colitis patients[21)]. In future, comparing NF-κB-Decoy and NF-κB

p65 antisense oligonucleotides, we may elucidate which is more promising to medical treatment for ulcerative colitis.

CONCLUSIONS

In conclusion, it had been confirmed that NF-κB-Decoy actually inhibited production of inflammatory cytokines such as TNF-α, IL-6 and IL-8 *in vitro*. On the other hand, liposome-conjugated NF-κB-Decoy could not show histopathological amelioration in DSS mice model, but NF-κB-Decoy treated mice showed earlier regain of body weight compared with the control mice.

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