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Decoy oligodeoxynucleotide targeting activator protein-1 (AP-1) attenuates intestinal inflammation in murine experimental colitis

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Various therapies are used for inflammatory bowel diseases (IBD), though none seem to be extremely effective. AP-1 is a major transcription factor that upregulates genes involved in immune and proinflammatory responses. We investigated decoy oligodeoxynucleotide (ODN) targeting AP-1 to prevent dextran sulfate sodium (DSS)-induced colitis in mice. Functional efficacies of synthetic decoy and scrambled ODNs were evaluated *in vitro* by a reporter gene luciferase assay and measuring flagellin-induced IL-8 expression by HCT-15 cells transfected with ODNs. Experimental colitis was induced in mice with a 2.5% DSS solution in drinking water for 7 days, and decoy or scrambled ODNs were intraperitoneally injected from days 2 to 5. Colitis was assessed by weight loss, colon length, histopathology, and detection of myeloperoxidase (MPO), IL-1β, and TNF-α in colon tissue. Therapeutic effects of AP-1 and NF-κB decoy ODNs were compared. Transfection of AP-1 decoy ODN inhibited AP-1 transcriptional activity in reporter assays and flagellin-induced IL-8 production *in vitro*. In mice, AP-1 decoy ODN, but not scrambled ODN, significantly inhibited weight loss, colon shortening, and histological inflammation induced by DSS. Further, AP-1 decoy ODN decreased MPO, IL-1β, and TNF-α in colonic tissue of mice with DSS-induced colitis. The AP-1 decoy therapeutic effect was comparable to that of NF-κB decoy ODN, which also significantly decreased intestinal inflammation. Double-strand decoy ODN targeting AP-1 effectively attenuated intestinal inflammation associated with experimental colitis in mice, indicating the potential of targeting proinflammatory transcription factors in new therapies for IBD.

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Ulcerative colitis (UC) and Crohn's disease (CD) are two major forms of inflammatory bowel diseases (IBD), which are characterized by chronic intestinal immune-mediated disorders of unknown etiology.[1–5] In sites of intestinal inflammation, granulocytes and macrophages produce high levels of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, which are directly involved in the pathogenesis of IBD.[1–3,6–9] Although several kinds of therapeutic strategies are used for IBD, none have been found to be totally effective. Conventional therapies for IBD focus on suppression and control of inflammation using 5-aminosalicylates, corticosteroids, and immune-modulating drugs, such as azathioprine and mercaptopurine.[10–14] On the other hand, recently developed novel cytokine antagonist therapies targeting TNF-α and IL-6 have been found to be quite effective in certain IBD patients.[15–18] Such molecular targeted inhibition of inflammatory processes may provide better therapeutic options for IBD and studies have been conducted to evaluate new innovative approaches.

Gene expression in a variety of biological conditions is initiated and regulated at the transcriptional level by interactions between specialized nuclear proteins, termed transcription factors, and promoter regions containing DNA elements that exhibit specific nucleotide sequences.[19,20] The transcription factor-activated protein-1 (AP-1) is a dimeric complex of basic region-leucine zipper proteins, and consists of heterodimers or homodimers of the Jun, Fos, and ATF

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families.[21–23] AP-1 is modulated by interactions with other transcriptional regulators and is further controlled by upstream kinases that link AP-1 to various signal transduction pathways.[24–26] AP-1 binds to specific DNA sequences present in a large number of genes that regulate inflammation, cellular growth, and differentiation.[21,27–30] Several agents that suppress AP-1 activation have been shown to inhibit inflammation and tumorigenesis. Recent studies have demonstrated that AP-1 is one of the key transcription factors that upregulate genes involved in immune as well as proinflammatory responses during the pathogenesis of IBD, suggesting that AP-1 may be an ideal target for the development of new therapeutic options for IBD.[31,32]

A decoy strategy that employs a synthetic double-stranded (ds) oligonucleotide (ODN) to competitively inhibit the binding of transcription factors to promoter regions of their target genes has emerged as a useful tool in a new class of antigene therapies presented in recent years.[33,34] It has been reported that a decoy ODN targeted at the transcription factor nuclear factor (NF)-κB, which plays an essential role in the regulation of a large number of genes involved in immune and inflammatory response, can exert potent immunosuppressive effects with certain inflammatory diseases.[35–37] Although the effects of AP-1 decoy ODN have been recently studied in several experimental disease models,[38–40] nothing is known regarding its therapeutic potential in gastrointestinal inflammation, including IBD.

In the present study, we investigated the anti-inflammatory effects of AP-1 decoy ODN in dextran sulfate sodium (DSS)-induced experimental murine colitis and compared its therapeutic potential to that of NF-κB decoy ODN. AP-1 decoy ODN treatment markedly inhibited colonic inflammation during DSS-induced colitis, suggesting that additional approaches with targeted inhibition of the transcription factor AP-1 including use of decoy ODN may contribute to development of a new therapeutic strategy for IBD.

MATERIALS AND METHODS

Synthesis of Double-Strand ODN

Synthetic ds decoy ODN was obtained as an HPLC-purified product from Hokkaido System Science (Sapporo, Japan). The ds decoy ODN was generated by annealing equimolar amounts of single-stranded sense and antisense phosphorothioate-modified ODN containing consensus AP-1 or NF-κB-binding sequences. Scrambled ds ODN samples were also generated as experimental controls for each decoy ODN. The phosphorothioate ODN used in this study had the following sequences (consensus sequences are underlined):

Decoy ODN for AP-1;
5′-TGTCTGACTCATGTC-3′
3′-ACAGACTGAGTACAG-5′
Scrambled ODN for AP-1;
5′-TGTCTCTCTGATGTC-3′
3′-ACAGAGAGACTACAG-5′
Decoy ODN for NF-κB;
5′-CCTTGAAGGGATTTCCCTCC-3′
3′-GGAACTTCCCTAAAGGGAGG-5′
Scrambled ODN for NF-κB;
5′-TTGCCGTACCTGACTTAGCC-3′
3′-AACGGCATGGACTGAATCGG-5′

Cell Line and Culture Condition

The human cell line HCT-15 was obtained from American Type Culture Collection (ATCC, Manassas, MD, USA). HCT-15 cells were grown in RPMI-1640 (ICN Biomedicals, Aurora, OH, USA), supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals) and penicillin-streptomycin-amphotericin B (GIBCO BRL), and maintained at 37°C in an incubator with 5% CO_2 and constant humidity.

Transfections of Decoy and Scrambled ODN and Transfection Efficiency

HCT-15 cells were cultured in 24-well plates (5×10^4 cells per well) and transfected with FITC-labeled decoy or scrambled ODN (0.25 μg per well) using Lipofectamine 2000 (Invitrogen, NY, USA), according to the manufacturer's protocol. Twenty-four hours after transfection, efficiency was assessed by fluorescence microscopy (Olympus, Tokyo, Japan) and flow cytometry. For flow cytometry, cells were harvested with trypsin-EDTA treatment, washed five times with cold PBS, and analyzed using an EPICS XL (Beckman Coulter, Tokyo, Japan), in which 10 000 events were counted for each condition and analyzed using EXPO32™ software.

Functional Efficiency of Decoy AP-1 and NF-κB ODNs

The efficiency of the decoy ODNs to mediate the inhibition of AP-1 or NF-κB-induced transcription activity was initially evaluated *in vitro* using a reporter gene luciferase assay. Reporter vectors were constructed by cloning AP-1 or NF-κB-binding consensus DNA elements into the promoter sequences, in which pNF-κB-Luc contained five copies of the NF-κB element 5′-TGGGGACTTTCCGC-3′ cloned upstream to the minimal TATA promoter (Stratagene Cloning Systems, La Jolla, CA, USA) and pAP-1-Luc contained five copies in a tandem repeat of the AP-1-binding site (TGACTAA), followed by cloning of the minimal TATA promoter into a pGL3-Basic vector (Promega, Madison, WI, USA). As an internal control for the dual luciferase assay, a pRL-TATA vector was constructed by cloning the minimal TATA promoter into a pRL-null vector (Promega) upstream of the renilla luciferase coding sequence. HCT-15 cells were cultured in 24-well plates (5×10^4 cells per well) and transfected with various concentrations of decoy or scrambled ODN, 0.1 μg of pNF-κB-Luc or pAP-1-Luc, and 0.02 μg of pRL-TATA per well using Lipofectamine 2000 transfection reagent (Invitrogen). Twenty-four hours after transfection, cells were stimulated with *salmonella* flagellin (0.1 μg/ml, Invitrogen, San Diego, USA), a ligand for toll-like receptor (TLR)-5, for 12 h. The cell lysates were then used for measurement of firefly and renilla luciferase activities with a PicaGene Dual

luciferase kit (Toyoinki, Tokyo, Japan). As a control, the effects of various concentrations of decoy or scrambled ODN on transcriptional activities in cultured cells without flagellin stimulation were also evaluated.

In addition to the reporter gene assays, the functional efficacy of AP-1 or NF-κB decoy ODN on AP-1 or NF-κB-induced endogenous proinflammatory gene expression was evaluated by their effects on flagellin-induced IL-8 expression by HCT-15 cells. Cells were grown in 24-well plates, transfected with various concentrations of decoy or scrambled ODNs, and stimulated with flagellin (0.1 μg/ml) for 24 h. Then, cell culture supernatants were collected for measurement of IL-8 using an enzyme immunoassay (EIA) (R&D Systems, Uden, Minneapolis, USA). For quantitative determination of IL-8 mRNA expression, total RNA was isolated using a guanidine thiocyanate-phenol-chloroform method (Isogen; Nippon Gene Co., Tokyo, Japan). From each sample, 1 μg of RNA was reverse transcribed into cDNA using the random primer from an affinity script QPCR cDNA synthesis kit (Stratagene, La Jolla, CA). Real-Time PCR was carried out in a total reaction volume of 30 μl using power SYBR Green PCR master mix (Applied Biosystem, Warrington, UK) and run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA). The primers used for transcript confirmation were as follows: IL-8 (sense), 5′-TGTGTGTAAACATGACTTCCAAGCT-3′; IL-8 (anti-sense), 5′-TTAGCACTCCTTGGCAAAACTG-3′, and GAPDH (sense), 5′-CCACATCGCTCAGACACCAT-3′; and GAPDH (antisense), 5′-TGACCAGGCGCCCAATA-3′. Finally, the results were expressed as the ratio of mean quantity of IL-8 to corresponding GAPDH for each sample. As a control, the effects of various concentrations of decoy or scrambled ODN on IL-8 expression in cultured cells without flagellin stimulation were also evaluated.

Effects of Decoy AP-1 and NF-κB ODNs on TNF-α-Mediated IL-8 Production *In Vitro*

Tumor necrosis factor-α, which regulates various inflammatory mediators, is considered to be one of the key molecules for the pathogenesis of IBD.[41] Because IL-8 is one of the major pro-inflammatory genes mediated by TNF-α stimulation, the effects of decoy ODNs on IL-8 production in HCT-15 cells were examined. Cells were grown in 24-well plates, transfected with decoy or scrambled ODNs (0.5 μg/ml), and stimulated with flagellin (0.1 μg/ml) for 24 h. Then, cell culture supernatants were collected for measurement of IL-8 using an EIA. As a control, the effects of decoy or scrambled ODN on IL-8 production in cultured cells without flagellin stimulation were also evaluated.

Experimental Colitis and Decoy ODN Therapy Protocol

Seven-week-old specific pathogen-free male BALB/c mice (Nihon Clea, Tokyo, Japan) were studied after receiving approval from the Ethics Committee for Animal Experimentation of Shimane University. The animals were housed under constant environmental conditions with circadian light/dark cycles. After an initial adaptation period of 1 week, a DSS solution (2.5% w/v) was administered to the experimental animals as drinking water for 7 days to produce experimental colitis. The *in vivo* study protocol is shown in Figure 3a. AP-1 or NF-κB decoy ODN (0, 5, 20, 40 μg per mouse) or scrambled ODN (0, 5, 20, 40 μg per mouse) was intraperitoneally injected once a day from days 2 to 5 during the DSS-administration period using a hemagglutinating virus of Japan (HVJ)-liposome method, as described previously.[38] The body weight (BW) of each mouse was measured daily until euthanasia. As a control, the effects of scrambled or decoy ODNs (0, 5, 20, 40 μg per mouse) on BW changes in mice without DSS treatment were also evaluated. The experimental animals were killed on day 8 after the end of DSS-administration by an overdose of diethyl ether, and then the colons were dissected out and rinsed in cold PBS. After measuring the total length of the colon, it was divided into proximal, medial, and distal portions. For this study, only the distal colon was examined, since it is considered to be most vulnerable to DSS-induced colitis. Further, a segment of the distal colon was processed for histological examinations and another was preserved for protein extraction.

Histological Examination

The colonic specimens were formalin-fixed and embedded in paraffin blocks. For the histological examinations, 3-μm paraffin sections were stained with hematoxylin and eosin. Two histopathologists blinded to the treatment groups examined each colonic section independently, as described previously.[42] During each histological examination, three different parameters were estimated, severity of inflammation (based on polymorphonuclear neutrophil infiltration; 0–3: none, slight, moderate, severe), depth of injury (0–3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0–4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost). The score for each parameter was multiplied by a factor reflecting the percentage of tissue involvement (× 1, 0–25%; × 2, 26–50%; × 3, 51–75%; × 4, 76–100%) and all values were added to a sum, in which the maximum possible score was 40.

Detection of IL-1β, TNF-α and Myeloperoxidase Activity in Colonic Tissues

IL-1β, TNF-α, and myeloperoxidase (MPO) are considered to be major inflammatory mediators involved in the pathogenesis of intestinal inflammation. In particular, IL-1β and TNF-α have been reported to be AP-1-target genes.[43,44] Therefore, expressions of IL-1β, TNFα, and MPO in colonic tissues were evaluated using an EIA and real-time PCR. Each distal colonic tissue sample was weighed, then after grinding by liquid nitrogen chilled mortar and pestles, it was completely lysed by syringing in ice cold 20 mM Tris, pH 7.6, containing 0.1% SDS, 1% Triton X-100, 1% deoxycholate,

100 μg/ml of the protease inhibitor PMSF, and a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Tissue lysates were centrifuged at 20 000 $\times$ *g* for 20 min at 4°C, then the supernatants were collected and the protein concentration was estimated by the Bradford method (Bio-Rad, Hercules, CA, USA). IL-1β contents and MPO activity were measured using EIA kits for IL-1β (R&D Systems, Uden, Minneapolis, USA) and MPO (Hycult Biotechnology, The Netherlands). The expression of TNF-α in colonic tissues was examined by real-time PCR, as described above, using the following primers: sense, 5′-AGACCCTCACACTCAGATCA TCTTC-3′; anti-sense, 5′-TCCTCCACTTGGTGGTTTGC-3′.

Statistical Analysis

All data are expressed as the mean ± s.e.m. The values were compared using Student's *t*-test with Stat-View 4.0 software (Abacus Concepts Inc., USA). A *P*-value of <0.05 was considered significant.

RESULTS

Transfection Efficiency and Functional *In Vitro* Effects of Decoy ODNs Toward HCT-15 Cells

To investigate the *in vitro* effects of the decoy ODNs, the transfection efficiency of the FITC-labeled ODNs was initially assessed using fluorescence microscopy. Twenty-four hours after transfection of the labeled decoy or scrambled ODN, high levels of fluorescence activity were observed in both the cytoplasm and nuclei of a large number of cultured cells (Figure 1a). Flow cytometry was then performed to quantify the proportion of transfected cells. Prior to flow cytometry, harvested cells were carefully washed with PBS to avoid non-specific fluorescence activity from labeled ODNs on the cell surface. The results of flow cytometry revealed high positive rates (above 90%) of fluorescence activity in the cultured cells transfected with the labeled decoy or scrambled ODN (Figure 1b).

Next, we examined the *in vitro* effects of decoy ODNs on the transcriptional activities of AP-1 and NF-κB using a reporter gene luciferase assay. Twenty-four hours after the transfection of reporter constructs that contained AP-1 or NK-κB-specific elements in their promoters along with the corresponding decoy or scrambled ODN, the cells were stimulated with flagellin. The results of the luciferase assay are shown in Figure 2a. Transfection of decoy ODNs significantly inhibited the transcriptional activities of the AP-1 and NF-κB promoter constructs in the cells in a dose-dependent manner. Further, the real-time PCR and EIA results showed significant inhibitory effects on IL-8 mRNA expression and protein production by the HCT-15 cells transfected with decoy ODNs (Figure 2b and c). In contrast, transfection of scrambled and decoy ODNs did not show any influence on luciferase activities or IL-8 expression in cultured cells without flagellin stimulation.

As TNF-α regulates various pro-inflammatory genes, the effects of decoy ODNs on TNF-α-mediated IL-8 production were also examined *in vitro*, with the results shown in Figure 2d. Treatment with decoy ODNs significantly inhibited TNF-α-mediated IL-8 production in cultured cells.

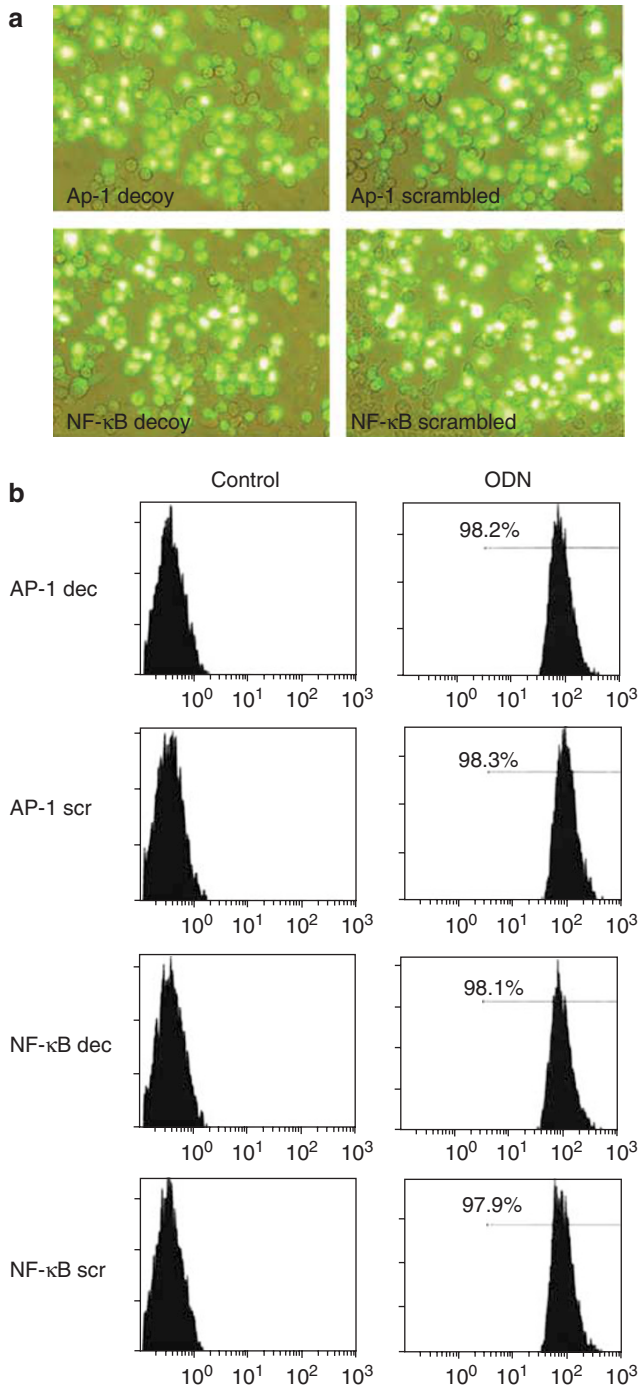

Figure 1 Transfection efficiency of decoy and scrambled ODNs in HCT-15 cells. Cells were cultured in 24-well plates and transfected with FITC-labeled decoy or scrambled ODN (0.25 μg/well). Twenty-four hours after transfection, efficiency was assessed by fluorescence microscopy (**a**) and flow cytometry (**b**). For flow cytometry, cells were harvested using trypsin-EDTA treatment, then washed five times with cold PBS and analyzed, with 10 000 events counted for each condition.

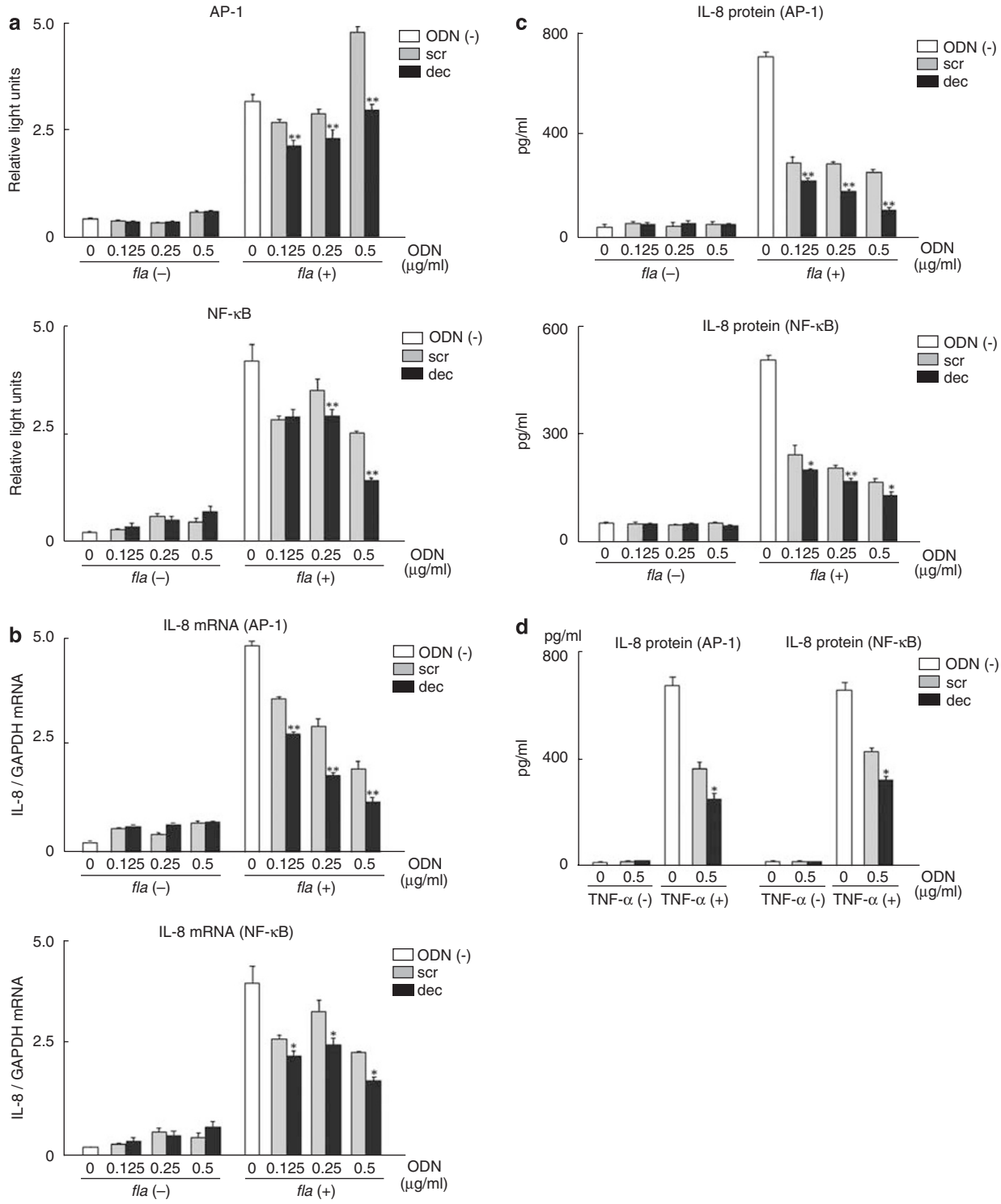

Figure 2 *In vitro* functional effects of decoy ODNs in HCT-15 cells. (**a**) The efficiency of decoy ODN-mediated inhibition of AP-1 and NF-κB-induced transcription activities was evaluated using a reporter gene luciferase assay. Cells were cultured in 24-well plates and transfected with various concentrations of decoy or scrambled ODNs, 0.1 μg of pNF-κB-Luc or pAP-1-Luc, and 0.02 μg of pRL-TATA per well. Twenty-four hours after transfection, cells were stimulated with or without flagellin (0.1 μg/ml) for 12 h, then cell lysates were used for the measurement of firefly and renilla luciferase activities. Data are expressed as relative light units obtained in dual-luciferase assays. (**b** and **c**) Functional efficacy of decoy ODNs on flagellin-induced IL-8 expression by HCT-15 cells transfected with ODNs. Cells were grown in 24-well plates, transfected with various concentrations of decoy or scrambled ODNs, and stimulated with or without flagellin (0.1 μg/ml) for 24 h. Cells and culture supernatants were used for quantitative determination of IL-8 mRNA expression and measurement of IL-8 protein content, respectively. (**d**) Effects of decoy AP-1 and NF-κB ODNs on TNF-α-mediated IL-8 production by HCT-15 cells transfected with ODNs. Cells were grown in 24-well plates, transfected with decoy or scrambled ODNs (0.5 μg/ml), and stimulated with flagellin (0.1 μg/ml) for 24 h. Cell culture supernatants were used for measurement of IL-8 using an EIA. Error bars indicate the standard error of mean values obtained from four independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ *vs* scrambled ODN.

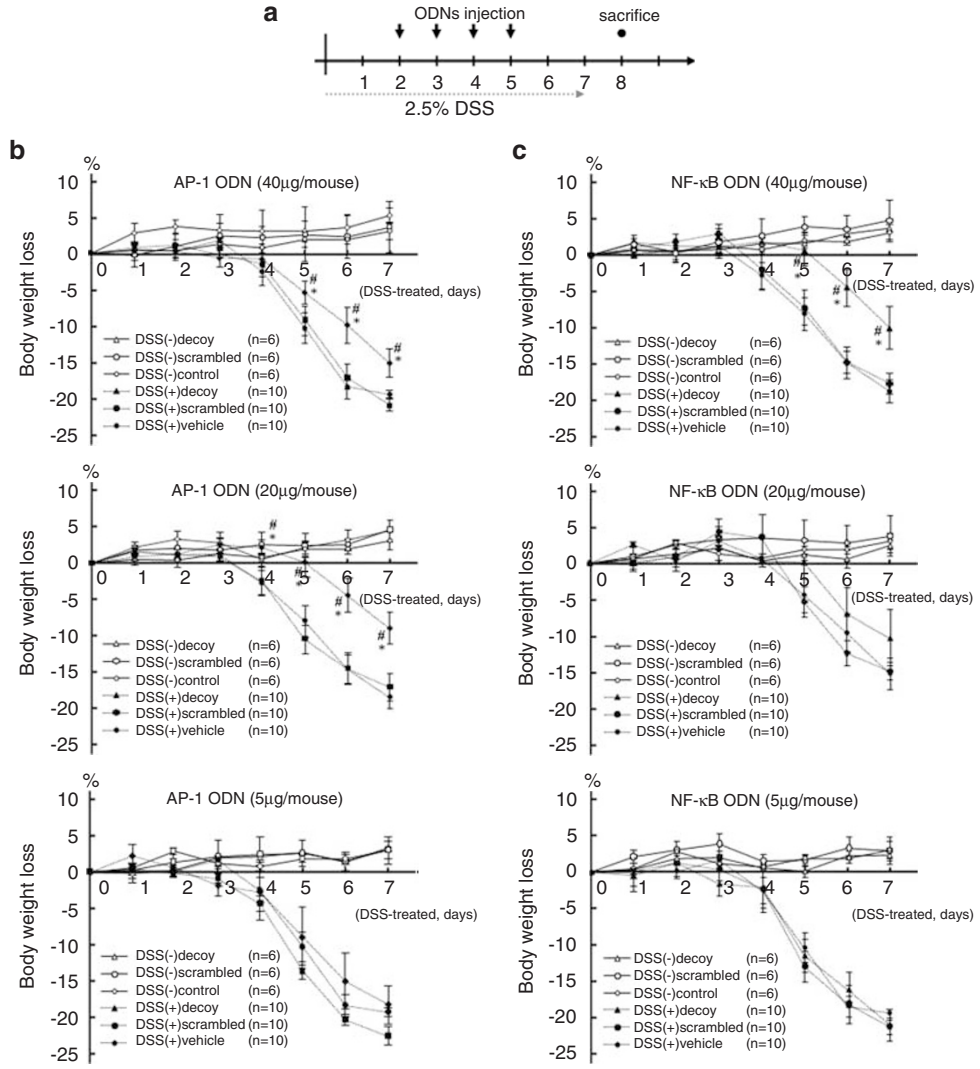

Figure 3 (**a**) Experimental colitis and decoy ODN therapy protocol. Experimental colitis models were established by administering a 2.5% DSS solution as drinking water for 7 days. Various concentrations of decoy or scrambled ODNs were intraperitoneally injected once a day from days 2 to 5 during the DSS-administration period using an HVJ-liposome method. As a control, the effects of the scrambled and decoy ODNs on BW changes in mice without DSS treatment were also evaluated. (**b** and **c**) Effects of AP-1 decoy and NF-κB decoy ODNs on BW changes in mice with or without DSS administration. Data are expressed as serial changes in percentage of BW during DSS administration. Error bars indicate the standard error of mean values obtained independently from 10 mice. $^{*}P<0.05$ *vs* DSS(+) scrambled ODN. $^{\#}P<0.05$ *vs* DSS(+) vehicle.

Effects of Decoy ODNs on BW Changes During Induction of DSS Colitis

As the decoy ODNs designed for this study had significant inhibitory effects on the transcriptional activities of AP-1 and NF-κB *in vitro*, we used them for further *in vivo* experiments. Experimental colitis models were established by administering a 2.5% DSS solution for 7 days. Each decoy, scrambled ODN, or the vehicle alone was intraperitoneally injected into mice with or without DSS administration once a day from days 2 to 5 during the experimental period (Figure 3a). In both the scrambled ODN and vehicle groups with DSS administration, BW loss of the mice commenced on day 4 and continued to day 7, whereas treatment with AP-1 decoy ODN (20, 40 μg per mouse) significantly inhibited BW loss from days 4 to 7 (Figure 3b). NF-κB decoy ODN (40 μg per mouse) also showed a significant inhibitory effect on BW loss from days 4 to 7, which was similar to the effect of AP-1 decoy ODN (Figure 3c). The BWs of mice injected with ODNs without DSS treatment were increased by about 5% on day 7, which was similar to the control mice. The doses that inhibited BW loss of DSS-treated mice most significantly were 20 μg for the AP-1 decoy ODN-injected group and 40 μg for the NF-κB decoy ODN-injected group. Colon length, histology, EIA, and real-time PCR findings for these groups are presented in Figures 4–6 and Table 1.

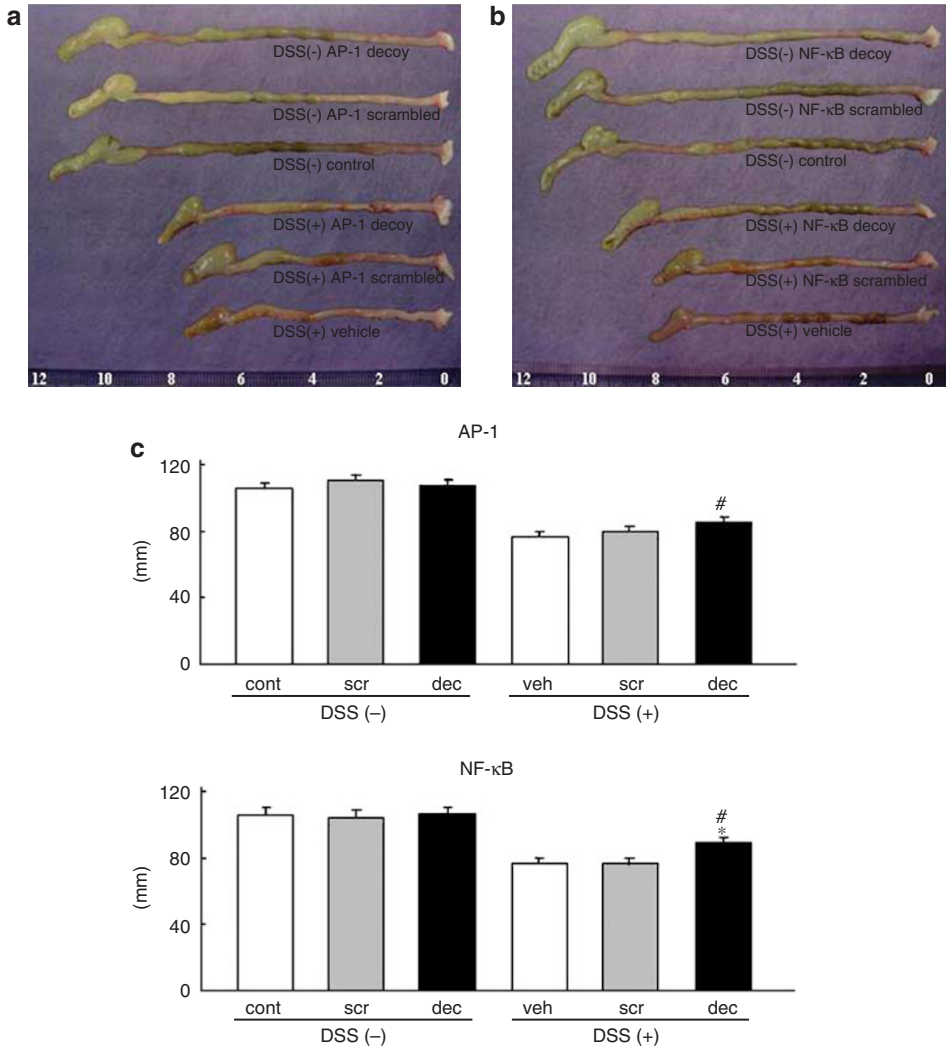

Figure 4 Effects of decoy ODNs on colon length in DSS-induced colitis. The experimental animals were euthanized on day 8, at the end of DSS administration. The total length of each colon dissected out from the mice was measured. (**a** and **b**) Representative images of colons dissected from DSS-non-treated and -treated mice following treatment with or without decoy and scrambled ODNs (AP-1: 20 μg/mouse, NF-κB: 40 μg/mouse). (**c**) Average total colon length in experimental groups treated with decoy ODN or scrambled ODN. Error bars indicate the s.e.m. values obtained independently from 10 mice. $^{*}P<0.05$ *vs* DSS(+) scrambled. $^{\#}P<0.05$ *vs* DSS(+) vehicle.

Effects of Decoy ODNs on Colon Length and Histology During Induction of DSS Colitis

The experimental animals were euthanized on day 8, at the end of DSS administration. Representative pictures of dissected colons are shown in Figure 4a and b. Treatment with AP-1 (20 μg per mouse) or NF-κB (40 μg per mouse) decoy ODNs, but not the scrambled ODNs or vehicle, significantly inhibited colon shortening induced by DSS administration (Figure 4c). Further, histological examinations showed that lamina propria infiltration by both polymorphonuclear and mononuclear cells as well as crypt epithelial damage were markedly decreased in the decoy ODN-treated mice (Figure 5a and b). All histological parameters as well as total histological score, as assessed by severity of inflammation, depth of injury, and crypt damage, in colon samples collected from decoy ODN-treated mice were significantly lower than those from scrambled ODN or vehicle-treated mice (Table 1).

Effects of Decoy ODNs on Expression of Pro-Inflammatory Cytokines and MPO Activity in Colonic Tissues

IL-1β, TNF-α, and MPO are major inflammatory mediators in the pathogenesis of IBD. As our histological examinations showed potent inhibitory effects on experimental colitis, we also evaluated the expression of pro-inflammatory cytokines and MPO activity in distal colon samples using an EIA and real-time PCR. Treatment with AP-1 or NF-κB decoy ODN significantly decreased IL-1β, TNF-α, and MPO activities in the tissue contents, which coincided well with the results of our histological examinations (Figure 6a–c).

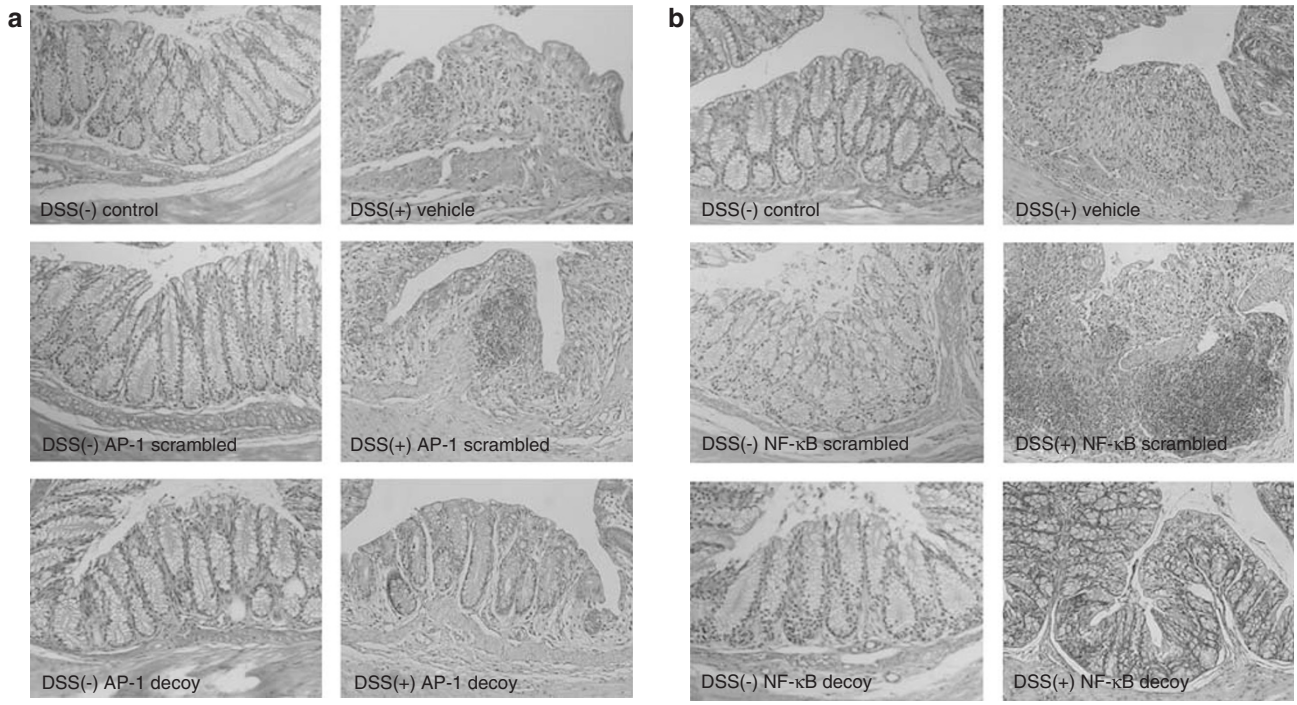

Figure 5 Effects of decoy ODNs on histological scores in mice with DSS-induced colitis. Distal colonic specimens were formalin-fixed and embedded in paraffin blocks, then 3-μm sections were stained with hematoxylin and eosin. (**a** and **b**) Representative histological images of samples from DSS-non-treated and -treated mice following treatment with or without decoy ODN or scrambled ODN (AP-1: 20 μg/mouse, NF-κB: 40 μg/mouse). (Original magnification $\times$ 100).

DISCUSSION

Studies of treatments with decoy ODNs targeting several transcriptional factors have demonstrated regulation of a variety of biological responses including inflammation and tumorigenesis in various organs.[33–40] Although AP-1 is a major immunoregulatory as well as proinflammatory transcription factor,[21,27–30,45–47] nothing is known to have been reported regarding the effect of AP-1 inhibition by a decoy ODN on intestinal inflammation. In the present study, we designed a ds decoy ODN binding to AP-1-specific nucleotide sequences and investigated its efficacy to prevent murine experimental DSS-induced colitis. Our findings demonstrated that AP-1 decoy ODN markedly inhibited DSS-induced colonic inflammation, indicating that AP-1 might be one of the potent targets for IBD therapy.

Recent advances in the elucidation of the pathogenesis of IBD have led to development of novel biological therapies that specifically inhibit molecules involved in the inflammatory cascade, including proinflammatory cytokines and their receptors, and adhesion molecules. Infliximab, a chimeric monoclonal antibody that binds with high affinity and specificity to TNF-α, has been demonstrated to be effective in both induction and maintenance therapy for patients with moderately to severely active CD, and is presently the only biological compound approved for CD therapy.[15,16,48,49] Newer therapeutic monoclonal antibodies targeting interferon (IFN)-γ,[50,51] IL-6 receptor,[18] IL-12 p40,[52] and α4-integrin[53] have also been developed, and clinical trials to evaluate the safety and efficiency of those agents for the treatment of IBD are being conducted. Although the development of therapeutic monoclonal antibodies is one of the most important advances in the care of IBD patients during the past decade, current therapies based on such biological agents are not entirely as effective as expected, as several untoward effects including immunogenicity, infusion reactions, and other serious adverse events have been reported.[13,16,17] Therefore, experimental approaches to develop new therapeutic options for IBD are encouraged.

In recent years, decoy ODNs bearing the consensus sequence of a specific transcription factor have been explored as new tools for manipulating gene expression in living cells. As a large number of transcription factors have already been identified and sequence-specific decoy ODNs are relatively easy to synthesize, the decoy approach represents a potential avenue for a wide range of clinical applications.[33,34] The multiplicity of target genes that are under the control of a single transcription factor also contributes to utility of decoy ODN strategies. Recent studies have demonstrated therapeutic applications of several decoy ODNs targeting transcription factors including NF-κB,[35–37] AP-1,[38–40] E2F,[54] Sp1,[55,56] and the signal transducer and activator of transcription (STAT) family.[57,58] There have also been a few reports suggesting an active role of AP-1 during the pathogenesis of IBD. Ishiguro *et al*[31] revealed that

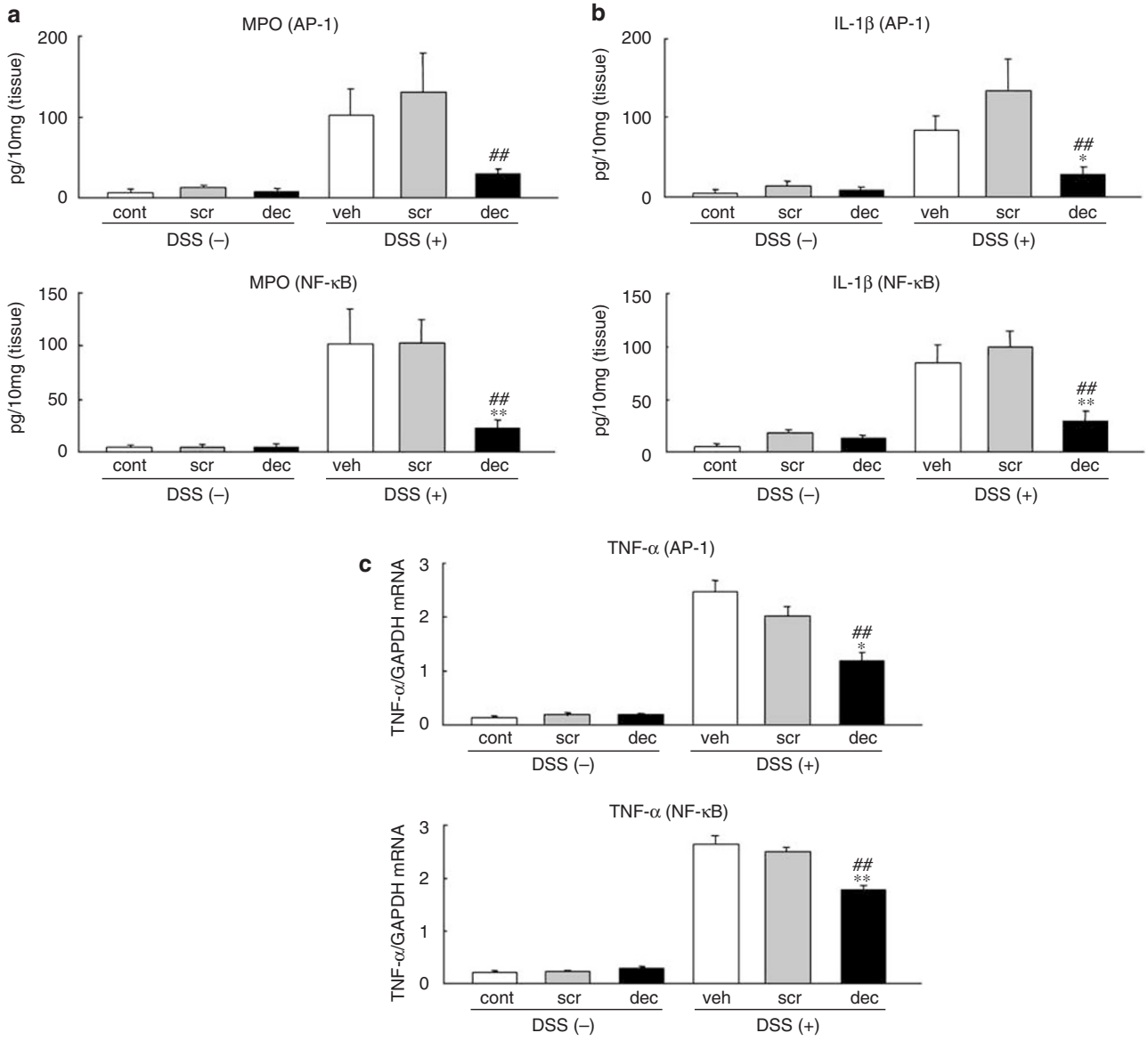

Figure 6 Effects of decoy ODNs on MPO activity (**a**), IL-1β contents (**b**), and TNF-α (**c**) in distal colonic samples collected from DSS-non-treated and -treated mice following treatment with or without decoy ODN or scrambled ODN (AP-1: 20 μg/mouse, NF-κB: 40 μg/mouse). Total protein and mRNA were extracted from each distal colonic tissue sample. MPO activity and IL-1β contents were evaluated using an EIA. Expression of TNF-α was examined by real-time PCR. Error bars indicate the s.e.m. values obtained independently from five mice. $^{*}P<0.05$, $^{**}P<0.01$, *vs* DSS(+) scrambled. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* DSS(+) vehicle.

AP-1-mediated expression of macrophage migration inhibitory factor (MIF) is involved in the glucocorticoid-resistant inflammatory process of UC. In addition, Andoh *et al*[32] recently reported that IL-22 is abundantly expressed in the colonic mucosa of IBD patients and activates the production of inflammatory cytokines, chemokines, and matrix metalloproteinases in colonic subepithelial myofibroblasts (SMEFs). They also demonstrated that IL-22-induced expression of inflammatory genes in SMEFs is mainly regulated by AP-1 signaling.

These observations suggest that control of AP-1 signaling may be a potent therapeutic target for treating IBD. In the present study, we constructed AP-1 decoy ODN and evaluated its anti-inflammatory effect on DSS-induced colitis in mice. As the therapeutic efficacy of NF-κB decoy ODN has been widely shown in a variety of studies,[33–37] the anti-inflammatory effect of NF-κB decoy ODN to prevent experimental colitis was also evaluated. Intraperitoneal treatment with AP-1 decoy ODN, but not scrambled ODN, significantly inhibited weight loss and shortening of the colon induced by DSS administration. Histological examinations clearly showed that crypt epithelial damage, as well as infiltration of lamina propria by polymorphonuclear and mononuclear cells were both markedly decreased in the AP-1 decoy

Table 1 Effect of decoy ODNs on histological inflammation

	Inflammation	Depth of injury	Crypt damage	Total
AP-1				
DSS(−) control	0.17 ± 0.17	0.17 ± 0.17	0.00 ± 0.00	0.33 ± 0.21
DSS(−) scrambled	0.33 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.21
DSS(−) decoy	0.17 ± 0.17	0.33 ± 0.21	0.00 ± 0.00	0.50 ± 0.22
DSS(+) vehicle	5.30 ± 0.90	4.20 ± 1.18	4.60 ± 1.18	14.10 ± 2.33
DSS(+) scrambled	4.60 ± 0.67	2.60 ± 0.27	4.90 ± 0.77	11.90 ± 1.17
DSS(+) decoy	2.20 ± 0.59*,#	2.10 ± 0.57	2.00 ± 0.64	6.30 ± 1.35*,#
NF-κB				
DSS(−) control	0.17 ± 0.17	0.17 ± 0.17	0.00 ± 0.00	0.33 ± 0.21
DSS(−) scrambled	0.33 ± 0.21	0.17 ± 0.17	0.00 ± 0.00	0.50 ± 0.22
DSS(−) decoy	0.17 ± 0.17	0.33 ± 0.21	0.00 ± 0.00	0.50 ± 0.22
DSS(+) vehicle	5.00 ± 1.14	3.50 ± 0.89	4.40 ± 1.07	13.40 ± 2.76
DSS(+) scrambled	4.30 ± 1.15	3.20 ± 0.84	4.60 ± 1.56	13.10 ± 3.59
DSS(+) decoy	1.40 ± 0.31*,#	1.60 ± 0.40	2.00 ± 0.45	5.10 ± 0.85*,#

AP-1 ODNs (scrambled; 20 μg per mouse, Decoy; 20 μg per mouse).
NF-κB ODNs (scrambled; 40 μg per mouse, Decoy; 40 μg per mouse).
*$P<0.05$ *vs* DSS(+) scrambled, #$P<0.05$ *vs* DSS(+) vehicle AP-1.

ODN-treated mice, which also coincided well with our findings regarding the colonic tissue contents of IL-1β, TNF-α, and MPO. Similar therapeutic effects were also observed in NF-κB decoy ODN-treated mice. Fichtner-Feigel *et al*[59] demonstrated the efficacy of NF-κB decoy ODN against murine experimental colitis induced by trinitrobenzene sulfonic acid (TNBS) and oxazolone, with prophylactic and therapeutic effects caused by apoptosis of $CD4^+$ cells, and suppressed production of inflammatory cytokines from colonic lamina propria cells was shown. Their findings support our view that decoy ODNs targeting transcription factors may become effective tools for preventing intestinal inflammation.

In the present study, we injected AP-1 decoy ODN into mice once a day from days 2 to 5 during the DSS administration period. The main purpose of our study was to evaluate whether AP-1 decoy ODN is applicable for induction IBD therapy, thus the decoy ODN was injected during the effector phase of DSS induction of colonic inflammation. We considered that this experimental protocol was useful to show the therapeutic potential of AP-1 decoy ODN to attenuate intestinal inflammation. On the other hand, AP-1 has also been recognized to play important roles in cell proliferation, migration, and differentiation in various organs.[21,27–30] Particularly, epithelial proliferation and migration are essential steps in the healing process of intestinal inflammation, which is regulated by a variety of AP-1-mediated genes. Therefore, blockade of AP-1-mediated signaling may influence mucosal regeneration during the healing of chronic intestinal inflammation including IBD, whereas the appropriate dosage period of AP-1 decoy ODN should be carefully evaluated for future clinical applications.

Host pattern recognition receptors can be activated by ds RNA or ds CpG-ODNs, and their signalling occurs in a type I IFN-dependent fashion. Anti-inflammatory response induced by such an ODN may be due to its immunostimulatory role. In the present study, we used decoy ODNs to inhibit the expression of pro-inflammatory genes whose promoters have binding sites for transcription factor AP-1. To avoid a possible immunostimulatory role of decoy ds ODNs, including corresponding scrambled ODNs, we selected sequences that were devoid of CPG motifs so that the ODNs would be less likely to produce a type-I IFN response. Moreover, we performed an *in vitro* control experiment to examine whether the anti-inflammatory responses induced by the AP-1 or NF-κB decoy ODNs were mediated through induction of type I IFN response. RT-PCR as well as an EIA were utilized for detection of IFN-β production in cells transfected with decoy ODNs, which showed that IFN-β production by cells transfected with AP-1 or NF-κB decoy ODNs was similar to that of those with scrambled ODNs. Moreover, the difference in IFN-β production remained insignificant even after stimulation of the ODN transfected cells with flagellin. The RT-PCR data were in line with the EIA results (data not shown). Therefore, it is not likely that the decoy ODNs inhibited intestinal inflammation through production of type-I IFN in the present experiments.

Recently, Suzuki *et al*[60] reported two clinical cases that utilized NF-κB decoy ODN, which showed its efficacy and safety for the prevention of restenosis after percutaneous coronary intervention. Indeed, decoy ODN strategies may have an effective therapeutic potential for a variety of diseases. However, there are several issues to be addressed before such clinical use is employed. One problem is related to how to efficiently deliver decoy ODNs in an organ- or cell-specific manner, because AP-1 and NF-κB are involved in a number of important physiological functions, whereas upregulation in certain organs or cells may be linked to pathological conditions like IBD that affect intestine and intestinal epithelia. Another is how to increase the stability of administrated decoy ODNs *in vivo*. ODN-based therapy requires high doses and frequent administrations, because ODNs are rapidly degraded *in vivo*. To resolve this issue, researchers have used end-modifications or conjugation of ODNs to increase their biological half life.[61–64] Further, viral envelopes including HVJ have been used to deliver ODNs *in vivo*, however, their safety remains unknown.[33,34,38] In the present study, injection of scrambled or decoy ODNs at 5, 20, or 40 μg per mouse did not show any influence on BW changes in mice without DSS treatment, thus those doses were considered to be safe for use in treatment of experimental colitis. Nevertheless, additional investigations addressing *in vivo* efficacy and safety should be carefully performed before ODNs can be considered for use in a clinical therapeutic strategy.

To conclude, we investigated the anti-inflammatory effect of a double-strand decoy ODN targeting AP-1 against experimental colitis in mice. The present results showed for the first time that AP-1 decoy ODN effectively attenuated intestinal inflammation, indicating the potential of targeting proinflammatory transcription factors in the development of new therapies for IBD.

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