CpG-DNA derived from sera in systemic lupus erythematosus enhances ICAM-1 expression on endothelial cells

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Abstract

Objective—To examine the effect of transfection of oligodeoxynucleotides (ODNs) containing a CpG motif (CpG-ODN), of which the sequence was derived from circulating DNA in the sera of patients with systemic lupus erythematosus (SLE), on the expression of intercellular adhesion molecule-1 (ICAM-1) and synthesis of mRNA for proinflammatory cytokines and ICAM-1 in human umbilical vein endothelial cells (EC).

Methods—A CpG-ODN or a control analogue, GpC-ODN, was transfected into EC. ICAM-1 expression was examined by flow cytometry, and expression of mRNA in EC encoding interleukin 1 (IL1), IL6, IL8, tumour necrosis factor α (TNF α), interferon γ (IFN γ), and ICAM-1 was examined by semiquantitative reverse transcriptase-polymerase chain reaction.

Results—The CpG-ODN augmented the expression of ICAM-1 on EC determined by flow cytometry and increased mRNA levels of IL6, IL8, TNF α , IFN γ , and ICAM-1, but the GpC-ODN did not.

Conclusion—Synthesised DNA, with a sequence corresponding to that of the fragment containing the CpG motif, in sera of patients with SLE was found to enhance ICAM-1 expression on EC, suggesting the participation of circulating DNA fragments in the pathogenesis of vasculitis in SLE.

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Microbial DNA and biologically active oligodeoxynucleotides (ODNs) containing a CpG motif powerfully activate immune cells, such as macrophages and immature dendritic cells, to up regulate their expression of major histocompatibility complex class II and costimulatory molecules, transcribe cytokine mRNAs, and secrete proinflammatory cytokines.^{1,2}

Vasculitis has been believed to be one of the key pathogenic changes in systemic lupus erythematosus (SLE), in which the interaction between endothelial cells (EC) and circulating DNA fragments may be crucial.

From this point of view, an analysis of the characteristics of circulating DNA fragments may provide an important clue in the elucidation of the pathogenesis of SLE. We recently reported that DNA fragments obtained from the sera of patients with active SLE are rich in G+C and the CpG motif.³ Unmethylated CpG motifs are common in microbial DNA, but

CpG motifs in vertebrate DNA are suppressed as well as methylated, except in CpG-rich islands, where they remain unmethylated.⁴ Interestingly, DNA fragments containing the CpG motif in the sera of patients with SLE were found to be immunostimulatory, and we speculated that those DNA fragments were derived from CpG-rich islands, or from genomic DNA, where they are less intensively methylated.³

The immunostimulatory properties of CpG-ODNs on EC have not yet been studied. Therefore, we investigated whether or not a CpG-ODN, with a sequence derived from DNA fragments containing the immunostimulatory CpG motif found in a patient with SLE, is immunostimulatory to human umbilical vein EC. As an initial step in exploring the immunostimulatory properties of CpG-ODNs on EC, this study was carried out by transfecting synthesised CpG-ODNs using lipofection to enhance the effect of CpG-ODNs as was shown previously.³ We report here that synthetic CpG-ODN is immunostimulatory to EC.

Methods

PREPARATION OF ODNs

Nuclease resistant phosphorothioate modified ODNs,⁵ as described below, were synthesised using an automated DNA synthesiser (model 394; Perkin Elmer, NJ, USA):

CpG-ODN (KJ-6); 5'-TTTTCAATTGCAA GATGAAT-3'

GpC-ODN (KJ-6); 5'-TTTTCAATTGCAA GATGAAT-3', analogous to CpG-ODN (KJ-6), but with the CpG replaced by GpC.

The sequence of the synthetic 20 bp ODN was derived from DNA purified from the serum of a patient with active SLE as described previously.³

CELL PREPARATION AND TREATMENT OF EC WITH ODNs

EC were prepared in accordance with the method of Jaffe *et al.*⁶ The cells were confirmed as EC by immunofluorescence staining with rabbit antihuman von Willebrand factor antibodies (Dakopatts, Glostrup, Denmark), and there were no contaminated monocytes/macrophages. The experiments were performed by using EC at two to four passages from five different sources.

ODNs were treated with a lipofection reagent (DOTAP; Boehringer Mannheim, Mannheim, Germany) so that they were effectively incorporated into the EC.⁷ Briefly, an

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ODN solution was gently mixed with an equal volume of lipofection reagent in a polystyrene tube, and the mixture was left to stand for 15 minutes at 25°C. The ODN solution prepared thus was added to 1×10^6 EC in a 24 well, flat bottomed plate (Nunc, Roskilde, Denmark) at a final concentration of 0.02, 0.2, and 2 $\mu\text{mol/l}$, and the plates were incubated for three hours for mRNA measurement or for 48 hours for flow cytometric analysis, under 5% CO_2 at 37°C. The cell viability was over 95% as determined by the trypan blue dye exclusion method under these conditions. The incubation time and concentration of ODN were chosen based on the results of a previous study of the immunostimulatory effects of CpG-ODNs on monocytes.³

FLOW CYTOMETRIC ANALYSIS

EC incubated either with DOTAP alone or with both DOTAP and a synthetic ODN were detached from flasks using 0.001% trypsin in 0.02% EDTA. The cells (1×10^6) were analysed by staining with fluorescence isothiocyanate conjugated mouse monoclonal antibody to intercellular adhesion molecule-1 (ICAM-1; Immunotech, Marseille Cedex, France). Isotype matched control mouse IgG was used to eliminate non-specific binding.

PREPARATION OF CDNAS

Total RNA was prepared by the method of Chomczynski and Sacchi⁸ as follows. EC (1×10^6), which had been incubated for three hours at 37°C with DOTAP alone or with both DOTAP and a synthetic ODN, were immersed in Isogen-LS (Nippon Gene, Toyama, Japan) and subjected to chloroform extraction. Total RNA was precipitated with isopropanol for five minutes at -20°C, washed with 80% (v/w) ethanol, and dissolved in an appropriate amount of diethyl pyrocarbonate treated water. cDNAs were synthesised by incubating the RNA with 10 U of reverse transcriptase (RAV2; Takara, Tokyo, Japan) for 90 minutes at 42°C, in a final volume of 40 μl . cDNAs were prepared in five independent experiments using EC from five different sources.

SEMIQUANTITATIVE RT-PCR ANALYSIS FOR EXPRESSION OF IL1, IL6, IL8, TNF α , IFN γ , AND ICAM-1 MRNAS

A semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to measure the mRNA levels of each cytokine and ICAM-1 in the EC by the method described by Choi *et al.*⁹ For PCR, 4 μl aliquots of each cDNA sample, 1 U of recombinant Taq DNA polymerase (TaKaRa Taq; Takara, Tokyo, Japan), and 10 pmol of each of the primers specific for interleukin 1 (IL1), IL6, IL8, tumour necrosis factor α (TNF α), interferon γ (IFN γ), ICAM-1, or β_2 microglobulin were added in a final volume of 20 μl . Each primer pair for IL1, IL6, IL8, TNF α , IFN γ , and ICAM-1 was designated using the excellent computer facility at the European Molecular Biology Laboratory and the GenBank database for searches (Software Development,

Tokyo, Japan). The sequences of the primer pairs were as follows:

IL1 β sense primer, 5'-TTT CTG CCT GAA GAA CAG-3' and antisense primer, 5'-GCT CAT GAT TTC TGC TCT GAC A-3'; IL6 sense primer, 5'-CCT TCT CCA CAA GCG CCT TC-3' and antisense primer, 5'-GGC AAG TCT CCT CAT TGA ATC-3'; IL8 sense primer, 5'-TCT GCA CTG TGT GAA GGT GCA GTT-3' and antisense primer, 5'-AAC CCT CTG CAC CCA GTT TTC CTT-3'; TNF α sense primer, 5'-TCT CGA ACC CCG AGT GAC AA-3' and antisense primer, 5'-TAT CTC TCA GCT CCA CAC CCA-3'; IFN γ sense primer, 5'-AGT TAT ATC TTG GCT TTT CA-3' and antisense primer, 5'-ACC GAA TAA GTC AGC AGC TT-3'; ICAM-1 sense primer, 5'-ACC TAT GGC AAC GAC TC-3' and antisense primer, 5'-TGG AGT CTG CTG GGA ATT T-3'. The primers used for β_2 microglobulin were the same as those reported by Gussow *et al.*¹⁰

The cDNA amount in each sample was quantified by measuring the amount of the PCR product for β_2 microglobulin under electrophoresis, and was standardised to determine the amount of mRNAs for proinflammatory cytokines and ICAM-1.

PCR was performed using the Gene Taq Kit (Nippon Gene, Tokyo, Japan) and a thermal cycler (Cetus; Perkin Elmer, NJ, USA). Conditions for IL1, IL6, IL8 cDNA amplification were 30 cycles of 35 seconds at 95°C for the denaturation, 25 seconds at 55°C for the annealing, and one minute at 72°C for the extension, with a denaturation step for three minutes at 95°C before the first cycle and an extension step for 10 minutes at 72°C after the 30 cycles. Amplifications of TNF α and IFN γ cDNAs were performed using a similar protocol except that the annealing temperature was set at 52°C. Amplification of ICAM-1 cDNA was performed using a similar protocol except that annealing was set for two minutes at 55°C and extension at 72°C. Amplification of β_2 microglobulin cDNA was also performed using a similar protocol except that the annealing temperature was set at 60°C.

For measurement of the amplified products, PCR was performed with an antisense primer of each of the cytokines labelled with ^{32}P . The amplified products were subjected to electrophoresis on a 2.5% agarose gel. The sheet gel was dried and exposed to an imaging plate (Fuji Photo Film, Tokyo, Japan). The intensities of the photostimulated luminescence (PSL) of the radioactivity bands were measured with a Bioimaging Analyser BAS 1000 (Fuji Photo Film). The intensities of the PSL for IL1, IL6, IL8, TNF α , IFN γ , and ICAM-1 were divided by the corresponding intensities of PSL for β_2 microglobulin mRNA (mRNA for IL1, IL6, IL8, TNF α , IFN γ or ICAM-1/mRNA for β_2 microglobulin).

Statistical analysis was carried out with the Stat View J-5.0 software package for Apple Macintosh computers. A paired Student's *t* test was performed and the results are shown in fig 2.

Results

Figure 1 shows representative results for the expression of ICAM-1 on EC transfected with DOTAP alone (A), CpG-ODN (KJ-6) (B), or

Figure 1 Flow cytometric analysis for intercellular adhesion molecule-1 (ICAM-1) expression on EC transfected with a CpG-ODN (KJ-6) or GpC-ODN (KJ-6). The endothelial cells (EC; 1×10^6 cells) were incubated for 48 hours with DOTAP alone (A), DOTAP and 0.2 $\mu\text{mol/l}$ of CpG-ODN (KJ-6) (B), or DOTAP and 0.2 $\mu\text{mol/l}$ of GpC-ODN (KJ-6) (C), and the ICAM-1 expression was determined by flow cytometric analysis as described in "Methods". Fluorescence intensity and relative cell number are displayed on the horizontal axis and on the vertical axis, respectively. A representative result of five independent experiments using EC from five different sources is shown.

GpC-ODN (KJ-6) (C). A marked increase in ICAM-1 expression was seen on EC transfected with CpG-ODN (KJ-6) (21%) compared with those treated with DOTAP alone (1%) or transfected with GpC-ODN (KJ-6) (2%). A significant increase in ICAM-1 expression was not seen on EC transfected with CpG-ODN (KJ-6) in 24 hours (data not shown).

Figure 2 shows the mRNA levels for IL1, IL6, IL8, TNF α , IFN γ , and ICAM-1 in EC treated with DOTAP alone, and those transfected with GpC-ODN (KJ-6) or CpG-ODN (KJ-6).

Neither transfection with CpG-ODN (KJ-6) nor that with GpC-ODN (KJ-6) increased the mRNA levels for IL1.

The mRNA levels for IL6 and IL8 increased from 0.25 and 1.0 to 2.6 and 7.9, respectively, after transfection with CpG-ODN (KJ-6) at a concentration of 0.2 $\mu\text{mol/l}$ ($p < 0.05$ and $p < 0.01$, respectively). On the other hand, the levels for IL6 and IL8 showed no significant increase after GpC-ODN (KJ-6) transfection.

The mRNA levels for TNF α and IFN γ increased from 0.3 and 0.1 to 1.9 and 1.5 $\mu\text{mol/l}$, respectively, after transfection with CpG-ODN (KJ-6) at a concentration of 0.2 $\mu\text{mol/l}$ ($p < 0.05$ for both). On the other hand, no significant increase of the mRNA levels was seen after GpC-ODN (KJ-6) transfection.

According to the results of flow cytometric analysis, the mRNA level for ICAM-1 increased from 0.15 to 2.2 after transfection with CpG-ODN (KJ-6) at a concentration of 0.2 $\mu\text{mol/l}$ ($p < 0.01$), but the level showed no significant increase after GpC-ODN (KJ-6) transfection.

Discussion

The molecular mechanisms underlying the activation of cells of the immune system by CpG-ODN are poorly understood. However, there is evidence that CpG-ODN binds to cell surface receptors on B cells, which subsequently transduce stimulatory signals.¹¹ To the best of our knowledge, cell surface receptors on EC have not been reported.

We presumed that the stimulatory effects of CpG-ODN on EC would be less than those on monocytes/macrophages/B cells because EC may have much less ability to phagocytose ODNs than monocytes/macrophages/B cells. Consequently, we used DOTAP to treat EC with ODNs in order to enhance the effect of CpG-ODN on EC. We assume that DNA/anti-DNA immune complexes may bind through either Fc receptors or C1q receptors and are incorporated into the EC in vivo. Moreover, macrophages around EC, which have low density lipoprotein receptors and have a scavenging role something like low density lipoprotein and immune complexes, may be implicated in the interaction between DNA/anti-DNA immune complexes and EC.

Furthermore, CpG-ODN may generate reactive oxygen species before nuclear factor κB activation.¹² Activation of cells in the immune system by CpG-ODN is considered to be mediated, at least in part, by the stress kinase

Figure 2 Interleukin 1 (IL1), IL6, IL8, tumour necrosis factor α (TNF α), interferon γ (IFN γ), and intercellular adhesion molecule-1 (ICAM-1) mRNA levels in endothelial cells (EC) transfected with a CpG-ODN (KJ-6) or GpC-ODN (KJ-6). The mean and standard deviation of cytokine mRNA levels in EC transfected with CpG-ODN (KJ-6) and those transfected with GpC-ODN (KJ-6) are shown. There were no significant differences in IL1 mRNA levels between EC transfected with CpG-ODN (KJ-6) and those transfected with GpC-ODN (KJ-6). IL6, IL8, TNF α , IFN γ , and ICAM-1 mRNA levels were significantly increased in EC transfected with CpG-ODN (KJ-6) compared with those transfected with GpC-ODN (KJ-6) ($p < 0.05$ at 0.2 and 2 $\mu\text{mol/ml}$ for IL6, $p < 0.05$ at 0.02 and 2 $\mu\text{mol/ml}$ for IL8, $p < 0.05$ at 0.2 and 2 $\mu\text{mol/ml}$ for TNF α , $p < 0.05$ at 0.02, 0.2, and 2 $\mu\text{mol/ml}$ for IFN γ , $p < 0.01$ at 0.02, 0.2, and 2 $\mu\text{mol/ml}$ for ICAM-1). * $p < 0.05$, ** $p < 0.01$.

pathway, which requires endosomal translocation and maturation.¹³ A stress kinase, p38 mitogen activated protein kinase, was found to be activated by CpG-ODN and induces the synthesis of IL6, IL8, TNF α , and IFN γ and the expression of ICAM-1 and VCAM-1.^{14,15}

Proinflammatory cytokines such as IL1, IL6, TNF α , and IFN γ are known to induce the expression of ICAM-1 on EC. Among these cytokines, expressions of mRNAs for IL6, TNF α , and IFN γ were enhanced in EC transfected with CpG-ODN, but not with GpC-ODN. Two mechanisms for the expression of ICAM-1 are proposed: (a) directly through p38 phosphorylation, and (b) indirectly through stimulation by IL6, TNF α , and IFN γ ; however, p38 phosphorylation and the effect of neutralisation of IL6, TNF α , and IFN γ were not examined in this study.

Considering that EC which had been incubated for three hours with both DOTAP and a synthetic ODN expressed the mRNA for ICAM-1, and the time lag in ICAM-1 expression on EC determined at 48 hours' incubation by flow cytometric analysis, both mechanisms may be implicated in the expression of ICAM-1 on EC.

As far as we know, no studies report the production of IFN γ by EC. Therefore, we performed the experiment using an enzyme linked immunosorbent assay (ELISA) kit (ENDOGEN, Cambridge, MA, USA) for the detection of IFN γ produced by EC. The supernatants of 1×10^6 EC cultured with DOTAP, CpG-ODN, or GpC-ODN for 24 hours were

analysed for the amount of IFN γ . IFN γ 9 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ was detected in the supernatant of EC cultured with 0.02 $\mu\text{mol/ml}$ of CpG-ODN (KJ-6) and with 0.02 $\mu\text{mol/ml}$ of GpC-ODN (KJ-6), respectively (data not shown). EC cell line or other stimulation, such as lipopolysaccharide, will be required to confirm IFN γ production by EC because the amount of IFN γ produced by the EC in this study is small.

In conclusion, DNA fragments containing the CpG motif in the sera of patients with SLE may be implicated in vasculitis through activation of proinflammatory cytokines and expression of ICAM-1. However, further studies, using serum DNA without lipofection reagent, as was shown in a previous study,³ will be required to establish the participation of circulating DNA fragments in the pathogenesis of vasculitis in SLE.

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