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Control of cytokine gene expression using small RNA interference: Blockade of interleukin-10 and interferon-gamma gene expression in pig cells

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Abstract

The ability of small RNA interference (RNAi) to reduce specific gene expression was tested using interleukin-10 (IL-10) and interferon-gamma (IFN-γ) production by cultured swine blood mononuclear cells stimulated by *Escherichia coli* lipopolysaccharide or concanavalin A. Antisense (AS) phosphorothioate oligodeoxynucleotides (ODNs) corresponding to a sequence in the region of the AUG initiation codon of swine IL-10 or IFN-γ mRNA inhibited production of IL-10 (≥93.5%) and IFN-γ (≥99%) mRNAs. Interleukin-10 and IFN-γ protein production was inhibited more than 95% by the AS ODNs. Scrambled and sense ODNs RNAi used as negative controls did not alter mRNA expression for either cytokine but slightly reduced IL-10 protein production. Cytokine-specific and control RNAi did not inhibit β_2-microglobulin mRNA expression in mitogen-stimulated blood mononuclear cells. Thus AS ODNs RNAi specifically inhibit expression of pig IL-10 and IFN-γ mRNAs by cultured, mitogen-stimulated blood mononuclear cells and may be an attractive alternative method for studying cytokine function.

Keywords: Antisense; Cytokines; IL-10; IFN-γ; Oligodeoxynucleotides; Pig; Blood monunuclear cells

1. Introduction

Functional studies of cytokines in large, outbred animals have been restricted to *in vitro* or *in vivo* methods utilizing added proteins or antibodies while methods for deleting or reducing cytokine expression have not been employed. Gene knockout (KO) methods, widely utilized in inbred laboratory animals, are not readily applied in outbred animals and are compromised when all copies of multi-copy alleles are not deleted. Also, since KO methods remove gene product throughout the ontogeny of the individual, compensation within the pleiotropic and redundant cytokine network may obscure functional aspects of individual targeted cytokines leading to false negative results (Buechler et al., 2000). The functions of individual cytokines may be additive, synergistic or antagonistic, synthesis or release of one cytokine may be controlled by others and cytokines may share receptors or parts of receptors. In this complex network it is difficult to unambiguously assign one or more biological role to each cytokine. Selectively and reversibly inhibiting the expression of one protein by

Abbreviations: Ab, antibody; AS ODN, antisense oligodeoxynucleotides; BMNC, blood mononuclear cells; c-EIA, competitive enzyme immunoassay; Con A, concanavalin A; FCS, fetal calf serum; FITC, fluoresceinated; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; KO, knockout; MuLv, murine leukemia virus; S, sense ODN; SC, scrambled ODN; PCR, polymerase chain reaction; T_m, melting temperature; 3′-UTR, 3′-untranslated region

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AS ODNs, also called RNA interference (RNAi) may enhance understanding of function (Boeve and De Ley, 1994, 2000).

Small RNA interference and their analogues directed against pre-mRNA or mature mRNA can specifically and reversibly inhibit gene expression *in vitro* and *in vivo*. Binding of ODNs to mRNA inhibits gene expression by degradation of the targeted mRNA by Rnase-H and by steric blocking of translation. Chemically modified backbones, such as phosphoramidates, 2′-*O*-alkyl RNA or peptide nucleic acids, that have reduced susceptibility to nucleases and enhanced affinity for the target RNA, are preferred. A major constraint is that ODN-mRNA hybrids must be sufficiently stable to avoid dissociation by the translational machinery (Dias et al., 1999). Phosphorothioate (PS) ODNs, in which one or more of the non-bridging oxygens in the backbone of DNA is replaced by sulfur, have markedly increased nuclease resistance (Kurreck, 2003).

While the potential utility of RNA interference methods in applications to pig health and immunology would seem to be great, recent reports deal with inhibition of porcine circovirus replication in a pig cell line (Liu et al., 2006) and control of Low Density Lipoprotein Receptor Promoter in pig ovarian granulossa cells (Natesampillai et al., 2006). In the present study, six PS-AS ODNs targeted to the translation initiation region (AUG), coding region or 3′-untranslated region (3′-UTR) of swine IL-10 and IFN-γ genes were tested for their ability to inhibit IL-10 and IFN-γ mRNA and protein production by cultured, mitogen-activated, pig blood mononuclear cells (BMNCs). Treatments were compared with untreated cells, cells treated with control sense (S) or scrambled (SC) ODNs or with the transfecting agent, DOSPER. Cytokine-specific AS ODNs were effective inhibitors of both mRNA and protein production suggesting their possible utility in studies of cytokines in pigs and likely in other species.

2. Materials and methods

2.1. Experimental design

To test the hypothesis that ODNs specifically inhibit expression of porcine IL-10 and IFN-γ mRNA and protein, pig BMNCs obtained from three weaned, specific pathogen-free Yorkshire pigs (University of Guelph, Arkel Research Station) were stimulated *in vitro* with the mitogens *Escherichia coli* lipopolysaccharide (LPS) or Con A (Sigma–Aldrich Inc., Oakville, Ont., Canada) to induce cytokine mRNA and protein. Real-time PCR (Roche Light Cycler) was used to quantify mRNA while cytokine protein was quantified using a competitive enzyme immunoassay (c-EIA). Cytokine-specific AS ODNs were designed and synthesized as were S and SC control sequences. Uptake of AS ODNs was evaluated using fluorescein-labeled nucleotides. Efficacy of the AS ODNs in down-regulating both message and protein product was confirmed by comparison with the ODN controls and with the housekeeping gene β_2-microglobulin (β_2-m).

2.2. Blood mononuclear cells

Standard ficoll-Hypaque (specific gravity 1.077, Sigma–Aldrich Inc.) density gradient methods were used to separate BMNCs from blood. For treatment with ODNs, cells were suspended in FCS-free Opti-MEM 1 medium (GIBCO BRL) and seeded into wells of tissue culture plates (GIBCO BRL) according to the total number of cells available at 3.0×10^5, 1.3×10^5 or 0.6×10^5 cells for each well of 6-, 12- or 24-well plates, respectively.

2.3. Antisense and control oligodinucleotides

Antisense ODNs for IL-10 and IFN-γ, were designed by ‘‘walking down’’ the sequences, using Primer Designer (Scientific and Educational Software, Durham, NC, USA). Sequences of the same base composition as AS ODNs but in a random, SC, or S order, were used as controls. Fluorescein isothiocynate-5′end-labeled AS ODNs were used for uptake studies. All ODNs were synthesized and phosphorothioate modified by Oligos Etc. (Eugene, Oregon, USA). The AS, SC and S are described in Table 1.

2.4. Uptake of ODNs by pig BMNCs

Fluoresceinated (FITC) ODNs were complexed with a liposome transfection reagent (DOSPER, Roche Diagnostics) according to the manufacturer’s directions. Cells were placed in serum-free medium (Opti-MEM 1, GIBCO BRL) before addition of FITC-ODNs to a final concentration of 1.5 μM/ml and incubated for 0–24 h. DOSPER was used at 3.5 or 4.5 μg/1.75 μg of ODNs. Uptake of FITC-AS ODNs was measured by fluorescence-activated cell analysis (FacScan, Beckton Dickinson, Mississauga, Ont.) at 0, 2, 4, 6, 8, 10 and 24 h after treatment. The negative control cells were treated with DOSPER only.