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Gene Expression Analysis in Blood Cells in Response to Unmodified and 2′-Modified siRNAs Reveals TLR-dependent and Independent Effects

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Ribonucleic nucleic acid recognition by Toll-like receptors (TLRs) induces innate immune responses. However, no comprehensive analysis of gene expression in human blood cells in response to unmodified and 2′-modified immunostimulatory RNAs has been reported. Using oligonucleotide microarrays, we show that around 400 genes were significantly ($P<0.001$) altered in peripheral blood mononuclear cells (PBMC) in response to either single-stranded (ss) or double-stranded (ds) small interfering RNAs (siRNAs). Most of the upregulated genes encode proteins involved in innate and adaptive immune responses, including proinflammatory cytokines, interferons, chemokines and chemokine receptors. Genes encoding proteins involved in lymphocyte activation (e.g. CD80, CD40, and CD69) and in regulation of the immune responses (e.g. SOCS proteins) were upregulated. Also, genes encoding for antiviral proteins (Mx1, Mx2, TRIM proteins), and interferon regulatory factors (e.g. IRF7) were upregulated. Around 90% of the genes (140 out of 160) affected by R-848, a specific ligand for TLR7 and TLR8, were also affected by ss siRNAs or ds siRNAs, indicating that the signaling pathways activated by R-848 are also activated by immunostimulatory siRNAs. In addition to immunoactivation *via* TLRs, ss siRNAs and ds siRNAs induced TLR-independent gene alterations. Surprisingly, replacement of only uridine bases with either 2′-fluoro or 2′-*O*-methyl modified counterparts abrogated all the observed bystander effects. Collectively, these microarray data offer for the first time an insight into human PMBC response to immunostimulatory RNAs such as ss siRNAs and ds siRNAs. The data should help to define strategies to either enhance or avoid the non-specific effects of siRNAs in order to develop safe therapeutics.

Keywords: siRNA; innate immunity; Toll-like receptors; chemical modifications; microarray

Introduction

RNA interference is a widely conserved mechanism in eukaryotes, in which double-stranded (ds) RNA induces a homology-dependent degradation of cognate messenger mRNA.[1,2] A key first step in this process is the cleavage of long dsRNA by Dicer enzyme into 21 nt to 25 nt ds duplexes, called small interfering RNAs (siRNAs) with two-base 3′-overhangs, which are then incorporated into the RNA-induced silencing complex (RISC). Subsequently, the antisense strand guides the programmed RISC to recognize and cleave target mRNA sequences.[3]

Gene silencing using long dsRNAs has been demonstrated in several model organisms. How-

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Abbreviations used: TLR, Toll-like receptor; PBMC, peripheral blood mononuclear cells; ss, single-stranded; ds, double-stranded; siRNA, small interfering RNA; RISC, RNA-induced silencing complex; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; PKR, dsRNA-dependent protein kinase; DOTAP, N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimrethyl-aminoniummethyl-sulfate; TRIM, tripartite motif; miRNA, microRNA.

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ever, when dsRNA larger than 30 bp is used in somatic mammalian cells, it results in the activation of the interferon pathway, which eventually leads to a global shutdown of protein synthesis and non-specific degradation of mRNAs.[4] These non-specific effects can be circumvented by the use of synthetic siRNAs, which can trigger RNAi in a sequence-specific manner without inducing the interferon pathway.[5] While the simplicity and specificity of RNAi has made the technology into a powerful tool for uncovering gene function and validation of drug targets,[6,7] recent studies have shown that chemically synthesized and *in vitro* transcribed siRNAs can activate innate immunity and that the siRNA-mediated silencing is less specific than was believed originally.[8–14] Examples include the production of interferon α (IFN-α), tumor necrosis factors alpha (TNF-α), interleukin 6 (IL6) and IL12, which are involved in inflammation and promotion of type 1 immune responses. Also, transfection of Hela S3 cells with various siRNA sequences non-specifically stimulated or repressed gene expression in a concentration-dependent manner.[15] A partial complementarity between an siRNA (sense or antisense strands) with human genes triggered off-target silencing.[11,14]

Initial studies on the activation of the interferon pathway by synthetic siRNAs indicated that the effects are sequence-independent and mediated by the dsRNA-dependent protein kinase (PKR) or Toll-like receptor (TLR) 3, a receptor known to bind viral ds RNAs.[8,13] Once activated by long ds RNA (>30 bp), PKR phosphorylates the alpha subunit of the eukaryotic translation initiation factor (eIF2α), which leads to a global inhibition of protein synthesis.[4] Also, PKR can activate interferon production. However, recently we and others have found that in immune cells neither TLR3 nor PKR is the main sensor for synthetic siRNAs.[10,16,17] We have demonstrated that the effects are sequence-dependent, and both ds siRNAs and ss siRNAs separately can activate innate immunity. Moreover, immune activation depends on the compartmentalization of ds siRNAs or ss siRNAs in endosomes. Indeed, inhibitors of endosomal acidification like bafilomycin A1, a drug that inhibits endosomal H^+-proton pumps, and chloroquine prevented activation.[10] Collectively, the available data indicate that siRNA and ss siRNAs signal through endosomal TLR7 and/or TLR8. Consistent with the role of TLR7 in this process, TLR7 knockout mice did not mount an inflammatory response to siRNAs.[17] It should be noted that in contrast to lipid-delivered siRNAs, cytoplasmic delivery of synthetic siRNAs by electroporation in human PBMC did not activate innate immunity, indicating that cytoplasmic sensors of viral ds RNAs do not recognize siRNAs.[10] Presently, it is not clear whether the lipid carriers can, to some extent, prevent siRNA recognition by the cytoplasmic sensors of RNAs. However, we believe that the siRNA molecules escape the endosomes as naked RNAs, not complexed with lipids. Consistent with our data, a recent study demonstrated that synthetic siRNAs and endogenously expressed siRNAs are not recognized by the cytoplasmic sensors of RNAs.[18] Interestingly, immune evasion of RIG-1, a major intracellular ds RNA sensor,[19] is due to the structure of the siRNA ends rather than the sequences.[20,21] Indeed, the presence of two-base 3′ overhangs impaired RIG-1 ATPase activity and subsequent activation of the downstream signaling cascades.[21]

Previous studies have shown that TLR7 and TLR8 recognized GU-rich ss RNA.[22,23] Regarding siRNAs, Judge and colleagues identified one RNA motif (5′-UGUGU-3′) and its immunostimulatory effect seems to be dependent on the GU content.[16] However, Hornung and colleagues identified a second RNA motif (5′-GUCCUU-CAA-3′) that is recognized by Toll-like receptor-7 and the induction is unrelated to GU content.[17] Although GU nucleotides may play an important role in RNA recognition by TLRs, we found recently that the uridine bases play a determinant role in ss siRNA and ds siRNA activation of the immune system.[24] When the uridine bases were replaced by either 2′-fluoro, or 2′-*O*-methyl, or 2′-deoxy-modified counterparts, immune activation was abrogated.[24]

Although immune activation is a nuisance bystander effect, immunostimulatory ds siRNAs and ss siRNAs could be effective modulators of the immune system, specifically the induction of Th-1 type response against tumor cells or virus-infected cells.[25] Given the potential medical applications of siRNAs, there is an urgent need for developing safe therapeutic siRNAs. Here, we report on the alteration of gene expression in PBMC in response to unmodified and 2′-modified ss siRNAs or ds siRNAs using oligonucleotide microarrays. Also, we compared the expression profiles to that obtained with R-848, a specific ligand for TLR7 and TLR8.[26]

Results and Discussion

Gene expression changes in PBMC after transfection with ss siRNAs

Recognition of microbial constituents by TLRs leads to a variety of signal transduction pathways that regulate the nature, magnitude and duration of the inflammatory responses.[25,27] Among ten reported human TLRs, TLR3, TLR7, TLR8, and TLR9 are involved in the recognition of microbial nucleic acids.[27] More recently, we have demonstrated that ss siRNAs (sense or antisense strands) and ds siRNA separately can activate innate immunity.[25] While immune activation by ds siRNAs and ss siRNAs in immune cells is well documented; only a few proteins have been investigated by ELISA. Therefore, the exact effects of immunostimulatory ss siRNAs and ds siRNAs on overall gene expression and cellular interactions are not known. To address