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Intracellular processing of immunostimulatory CpG–siRNA: Toll-like receptor 9 facilitates siRNA dicing and endosomal escape

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ABSTRACT

Dicer-substrate siRNAs equipped with CpG oligodeoxyribonucleotides overcome the major hurdle in cell-specific siRNA delivery. The CpG–siRNA molecules are actively internalized by TLR9$^+$ cells, without the need for transfection reagents, leading to RNA interference both in vitro and in vivo. Here, we elucidate the molecular mechanisms of CpG–siRNA processing in target cells. We show that shortly after uptake into early endosomes (EE), CpG and siRNA parts of the conjugate are uncoupled in the presence of Dicer endonuclease. Diced siRNA molecules are translocated from endosomes to endoplasmic reticulum, where they can interact with the RNA interference machinery. We previously observed that even though TLR9 is not involved in CpG–siRNA uptake, it is indispensable for induction of gene silencing. To explain the role of TLR9 in intracellular processing of CpG–siRNA, we used primary macrophages derived from wild-type and *Tlr9*-deficient mice. Macrophages lacking TLR9 showed extended endosomal colocalization of CpG and siRNA parts of the conjugate. However, *Tlr9* ablation did not interfere with the interaction of CpG–siRNA with Dicer as shown by in situ proximity ligation assay. Using CpG–siRNA labeled with pH-sensitive dye, we finally identified that lack of TLR9 in macrophages resulted in significant retention of the siRNA in endosomes. Thus, TLR9 facilitates the critical step following CpG–siRNA uncoupling, which is cytoplasmic release of the diced siRNA. These findings suggest that the class of immunostimulatory siRNAs may benefit from activation of certain endosomal immune receptors, such as TLR9, in augmented gene silencing and therapeutic efficacy.
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1. Introduction

Since its discovery by Fire and Mellow in 1998, siRNA has proven to be a powerful tool for modulating the expression of almost any gene in various species. Several studies demonstrated the feasibility of in vivo siRNA delivery, leading to therapeutic effects not only in mice [1–5] but also in non-human-primates [6,7]. More recently, gene silencing after systemic siRNA administration was demonstrated in humans [8]. However, there are still severe hurdles to wide therapeutic application of this technology, such as cell-specific siRNA delivery and efficient release into cytoplasm [9]. There have been several innovative attempts to solve these problems using modular design of siRNA conjugated to cell-specific antibodies [1] or RNA aptamers [4,5]. Others used combinations of siRNA with lipid reagents or cholesterol to enable siRNA uptake without the need for molecule encapsulation using potentially toxic lipid reagents [9]. Nevertheless, most of the delivery methods result in endosomal rather than cytoplasmic siRNA uptake [10].

Due to rapid progress of this field, the rational design of siRNA reagents is hampered by gaps in our understanding of their intracellular processing. For many delivery strategies the mechanisms of siRNA release into cytoplasm are still unclear. In the case of modified siRNAs that undergo rapid protonation in endosomes, the suggested release mechanism is swelling and burst of endosomes called a “proton sponge effect” [10]. This process does not explain the cytoplasmic release of siRNA conjugates linked to antibodies or RNA aptamers which is usually limited due to their large size [10,11]. Timely siRNA release is critical because loaded early endosomes (EE) undergo a series of transformational events called maturation, resulting in

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significant decrease of endosomal pH and recruitment of proteases and RNAases [12]. The conversion of early into late endosomes may take as little as 20 min, and an additional 20 min is required to fuse with lysosomes leading to the degradation of the vesicle content [12]. In addition, location of various downstream components of siRNA processing machinery is not well defined. Dicer endonuclease was originally found in the cytoplasm [13], but later studies indicated that it might be associated with the endoplasmic reticulum (ER) [14]. More recent reports found that Ago2 endonuclease, a major component of the RNA-induced silencing complex (RISC), likely resides on multi-vesicular bodies (MVB), which are intermediates between early and late endosomes [15]. These findings agree well with one of two major hypotheses of RISC complex assembly, which identifies Ago2 on the surface of EE and MVBs [10]. A slight variation of this assumption is that RISC is located in P-bodies, which in turn are associated with GW-bodies located on the cytoplasmic side of MVB membranes [10,15]. In contrast, others have shown that RISC colocalizes with large subunits of ribosomes anchored to the rough ER, thus blocking translation of target mRNA [16].

We previously developed an immunostimulatory siRNA molecule by conjugating Dicer-substrate siRNA [17] to a CpG oligodeoxyribonucleotide (ODN), which acts as a targeting moiety for the intracellular toll-like receptor 9 (TLR9). CpG–siRNAs were successfully used for targeting various genes in normal and malignant TLR9-positive cells in vitro and in vivo. CpG–*Stat3* siRNA was shown to generate potent systemic antitumor immunity [18], and prevented tumor metastasis in mouse models [19,20]. Very recently, we optimized CpG–siRNA design to target human immune and cancer cells. Blocking of *STAT3* or *BCL-X_L* survival signaling inhibited in vivo growth in several xenotransplanted blood cancer models [21]. In addition, the conjugate retained its immunostimulatory properties and activated various populations of human DCs and B cells [21]. An unexpected finding of our recent studies was that both TLR9 expression and activation were required for the target gene silencing but not for the conjugate uptake. In vitro studies using CpG–siRNA in *Tlr9*$^{-/-}$ cells failed to detect RNA interference (RNAi) in target cells. Here, we delineate the critical initial steps and the role of TLR9 in CpG–siRNA processing. We believe that identifying biomarkers for target selection and molecules indispensable for the therapeutic effect will accelerate the clinical translation of the CpG–siRNA strategy for cancer therapy. Finally, we anticipate that this information will support and accelerate the rational design of other types of immunostimulatory siRNAs.

2. Materials and methods

2.1. Oligonucleotide design and synthesis

The phosphorothioated ODN and antisense strands (AS) of siRNAs were linked using 5 units of C3 carbon chain linker, $(CH_2)_3$ from Glen Research (Sterling, VA). The resulting constructs were hybridized with complementary sense strands (SS) of siRNAs to create chimeric ODN–siRNA constructs (deoxyribonucleotides are underlined). Sequences of single stranded constructs are listed below; the siRNA sequence is specific for mouse *Stat3* gene (NM_213659, bases 1898–1922). *CpG1668-mouse Stat3 siRNA(AS)*: 5′TCCATGACGTTCCTGATGCT-linker-UUAGCCCAUGUGAUCUGACACCCUGAA 3′. *Mouse Stat3 siRNA (SS)*: 5′CAGGGUGUCAGAUCACAUGGGCUAA 3′. Constructs were fluorescently labeled using fluorescein (FITC), Cy3 (Lumiprobe, Hallandale Beach, FL) or pHrodo (Life Technologies, Carlsbad, CA) at sites indicated in the text. For FRET experiments we generated the following constructs of tripartite design using complementary 2′fluoro-modified "stick" adapters (Q = 2′fluoro-U; Z = 2′fluoro-C): *CpG1668-linker^{FITC}-stick*: 5′-TCCATGACGTTCCTGATGCT-linker^{FITC}-AGGQGZGZZAZQQZZU-3′. *Mouse Stat3 siRNA(SS)-linker-stick*: 5′-CAGGGUGUCAGAUCACAUGGGCUAA-linker-AGGAAGQGGZGZAZZU-3′. *Mouse Stat3 siRNA^{Cy3}(AS)*: 5′Cy3- linker-UUAGCCCAUGUGAUCUGACACCCUGAA-3′. We have confirmed that modification of CpG–siRNA conjugates with various fluorochromes does not alter their internalization by target cells or gene silencing effect (Supplementary Fig. 9). The sequence of firefly *luciferase*-specific 25/27mer siRNA (Luc1 R 25D/27) used for the CpG(1668)-*Luc* siRNA conjugate molecule was published elsewhere [22]. The formation of siRNA duplex was confirmed by electrophoresis in 15% polyacrylamide/7.5 M urea gel.

2.2. Cells

Mouse B16 melanoma, L929 fibroblasts, and RAW 264.7 macrophage cell lines were purchased from ATCC. B16 were cultured in RPMI medium with Glutamax (Life Technologies) and 5% fetal bovine serum (FBS, CellGro, Manassas, VA); L929 and RAW 264.7 were cultured in DMEM with Glutamax and 10% FBS. Bone marrow-derived Macrophages (BMDM) were differentiated from bone marrow cells of *WT* and *Tlr9*$^{-/-}$ C57BL/6 mice as described elsewhere [23]. Differentiation status and CpG–siRNA uptake efficiency were confirmed by flow cytometry with CD11b and F4/80 antibodies.

2.3. Fluorescent confocal microscopy and fluorescence resonance energy transfer (FRET)

BMDM or RAW 264.7 cells were treated using 500 nM CpG–siRNA for 30 min in a culture medium with or without 5% FBS, respectively. The medium was then replaced and incubations were continued for indicated times. Cells were fixed with 2% paraformadehyde for 20 min, permeabilized in PBS containing 0.1% Triton X-100, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (PBST) for 10 min, and quenched in 50 mM NH_4Cl/PBST for 5 min before blocking in 1% BSA/PBST for 1 h. Samples were stained with the antibodies specific to EEA1 (H-300), Dicer (H-212, C-20) (Santa Cruz, Dallas, TX), Rab7 (D95F2 XP) (Cell Signaling, Boston, MA), Calnexin (GenScript, Piscataway, NJ), TLR9 (IMG-431) (Imgenex, San Diego, CA), FITC (Life Technologies), Ago2 (11A9) (Sigma, St. Louis, MO), and then detected with secondary Alexa 488-, 555-, 633-, and 647-coupled antibodies (Life Technologies). Slides were mounted in DAPI-containing mounting medium (Vector Labs, Burlingame, CA). Confocal imaging was carried out using C-Apochromat 40×/1.2 water-, C-Apochromat 63×/1.2 water-, or Plan-Apochromat 63×/1.4 oil-immersed objectives on cLSM510-Meta inverted confocal microscope (Zeiss, Thornwood, NY). We used LSM software v.4.2 SP1 for image acquisition, and LSM Image Browser v.4,2,0,121 for post-acquisition analysis (Zeiss).

For time-lapse confocal microscopy in living cells, cells were cultured on 35/14 mm #1.5 glass bottom tissue culture dishes (MatTek, Ashland, MA). After washing cells twice in phenol red-free media, cells were treated using CpG–siRNA for 20–30 min. Images were acquired using C-Apochromat 40×/1.2 water immersed objective, in 20–30 min time increments, in Z stacks. Microscope chamber for environmental control (37 °C, 5% CO_2) was used in all experiments with living cells. For quantification purposes, Z-stacks were collapsed post-acquisition, and signal intensity in selected regions of interest (ROI) was determined using LSM software (Zeiss). In experiments with pHrodo-labeled CpG–siRNA each ROI encompassed a single cell, and ROI settings remained constant throughout the experiment. Reverse correlation between the intensity of pHrodo succinimidyl ester emission and pH was verified in a series of 50 mM citric buffers with pH values 4.5–8.0 (Supplementary Fig. 7A). Experiment was carried out in quadruplicates, and intensity of emission was analyzed using SpectraMax fluorescence plate reader (Molecular Devices, Sunnyvale, CA) using 544 nm excitation and 590 nm emission filters. FRET was carried out in living RAW 264.7 macrophages with tripartite CpG–*Stat3* siRNA constructs labeled with FITC within the carbon linker between CpG and a "stick" adapter, and Cy3 on 5′ end of siRNA (AS). This design allowed for more control over the distance between two fluorophores for inducing the FRET effect. Cell culturing and

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