A new nucleic acid–based agent inhibits cytotoxic T lymphocyte–mediated immune disorders

Chuang-Wei Wang, MS,[a,b] Wen-Hung Chung, MD, PhD,[c] Yi-Fang Cheng, PhD,[d] Nien-Wen Ying, MS,[d] Konan Peck, PhD,[d]† Yuan-Tsong Chen, MD, PhD,[d] and Shuen-Iu Hung, PhD[a,b] *Taipei and Taoyuan, Taiwan*

Background: Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and graft-versus-host disease (GVHD) are distinct immune reactions elicited by drugs or allogeneic antigens; however, they share a pathomechanism with the activation of cytotoxic T lymphocytes (CTLs). CTLs produce cytotoxic proteins, cytokines, chemokines, or immune alarmins, such as granulysin (GNLY), leading to the extensive tissue damage and systemic inflammation seen in patients with SJS/TEN or GVHD. Currently, there is no effective therapeutic agent specific for CTL-mediated immune disorders.

Objectives: By targeting $GNLY^+$ CTLs, we aimed to develop a nucleic acid–based agent consisting of an anti-CD8 aptamer with GNLY small interfering RNA (siRNA).

Methods: We performed systematic evolution of ligands using exponential enrichment to select and identify effective anti-CD8 aptamers. We developed an aptamer-siRNA chimera using a "sticky bridge" method by conjugating the aptamer with siRNA. We analyzed the inhibitory effects of the aptamer-siRNA chimera on CTL responses in patients with SJS/TEN or GVHD.

Results: We identified a novel DNA aptamer (CD8AP17s) targeting CTLs. This aptamer could be specifically internalized into human CTLs. We generated the CD8AP17s aptamer–GNLY siRNA chimera, which showed a greater than 79% inhibitory effect on the production of GNLY by drug/alloantigen-activated T cells. The CD8AP17s aptamer–GNLY siRNA chimera decreased cytotoxicity in *in vitro* models of both SJS/TEN (elicited by drug-specific antigen) and GVHD (elicited by allogeneic antigens).

Conclusions: Our results identified a new nucleic acid–based agent (CD8 aptamer–GNLY siRNA chimera) that can significantly inhibit CTL-mediated drug hypersensitivity, such as that seen in patients with SJS/TEN, as well as the alloreactivity seen in patients with GVHD. This study provides a novel therapeutic strategy for CTL-mediated immune disorders. (J Allergy Clin Immunol 2013;132:713-22.)

Key words: *Alloreactivity, aptamer, CD8, drug hypersensitivity, graft-versus-host disease, granulysin, nucleic acid–based therapeutics, Stevens-Johnson syndrome, systematic evolution of ligands by exponential enrichment, toxic epidermal necrolysis*

Stevens-Johnson syndrome (SJS) and its related disease, toxic epidermal necrolysis (TEN), are life-threatening adverse reactions most often induced by drugs.[1] Histopathologic studies of SJS and TEN identified predominantly cytotoxic T lymphocytes (CTLs), natural killer (NK)/NKT cells in the skin lesions, and marked keratinocyte apoptosis in the epidermis separation, resulting in bullae and extensive mucocutaneous shedding.[2] Recent studies on the genetic susceptibility and immune mediators of SJS/TEN proposed that the pathomechanism involves the activation of CTLs, which recognize culprit drugs/metabolites presented by HLA class I molecules on the keratinocytes.[3-8] This hypothesis is not only supported by genetic studies (eg, the strong genetic association between HLA-B*1502 and carbamazepine-SJS/TEN[3] and HLA-B*5801 and allopurinol-induced SJS/TEN[4]) but also functional analyses.[6,7] On activation, CTLs release cytotoxic proteins, cytokines, chemokines, or immune alarmins, such as granulysin (GNLY).[9,10] We have identified that GNLY is a key mediator responsible for the disseminated keratinocyte and extensive tissue damage seen in patients with SJS/TEN.[5]

Activation of CTLs and NK/NKT cells is also predominant in patients with graft-versus-host disease (GVHD), although the cause of GVHD is quite different from that of SJS/TEN, with SJS/TEN elicited by drug antigens and GVHD by allogeneic antigens.[2,11,12] Even with prophylaxis, GVHD develops in as many as 60% to 80% of recipients of stem cells from single-antigen HLA-mismatched unrelated donors, and with full HLA-matched siblings, the risk is still high (approximately 35% to 45%).[13] The CTL-mediated immune reactions of patients with GVHD are often found in the skin, gastrointestinal tract, and liver.[13-15] Upregulation of GNLY levels in sera and tissues (eg, the gut, liver, and skin) has been found in patients with GVHD.[5,16-18] GNLY might also be involved in the pathogenesis of GVHD. Targeting the GNLY-mediated cytotoxic pathway can give rise to a novel therapeutic strategy for the treatment of SJS/TEN and GVHD.

An optimal treatment standard for patients with SJS/TEN remains unavailable. Controversial results have been reported with intravenous immunoglobulin therapy[19,20] and corticosteroids.[21] Despite treatment with glucocorticoids or other immunosuppressants, SJS/TEN and GVHD still carry high morbidity and 10% to 50% mortality.[14,22,23] Currently, there is no effective

From [a]the Department and Institute of Pharmacology, School of Medicine, Infection and Immunity Research Center, National Yang-Ming University, Taipei; [b]the Molecular Medicine Program, National Yang-Ming University and Academia Sinica, Taipei; [c]the Department of Dermatology, Drug Hypersensitivity Clinical and Research Center, Chang Gung Memorial Hospitals, Keelung, Linko, Chang Gung University College of Medicine, Taoyuan; and [d]the Institute of Biomedical Science, Academia Sinica, Taipei.

†Deceased.

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Corresponding author: Shuen-Iu Hung, PhD, Institute of Pharmacology, National Yang-Ming University, 155, Linong St, Sec 2, Beitou, Taipei 11221, Taiwan. E-mail: sihung@ym.edu.tw.

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Abbreviations used
CON: Nonsilencing control
CTL: Cytotoxic T lymphocyte
GNLY: Granulysin
GVHD: Graft-versus-host disease
K_d: Dissociation constant
NF-κB: Nuclear factor κB
NK: Natural killer
RNAi: RNA interference
SEAP: Secreted embryonic alkaline phosphatase
SELEX: Systematic evolution of ligands by exponential enrichment
siRNA: Small interfering RNA
SJS: Stevens-Johnson syndrome
ssDNA: Single-stranded DNA
TCR: T-cell receptor
TEN: Toxic epidermal necrolysis

therapeutic agent specific for CTL-mediated immune disorders. One of the new therapeutic strategies is the use of mAbs. However, using mAbs in the clinic can induce unexpected immune reactions in patients. For example, anti-CD3 (muromonab) and anti-CD28 (TGN1412) antibodies have been reported to cause a life-threatening "cytokine release syndrome," resulting in multiorgan damage.[24,25] Safer and more effective therapeutics need to be developed.

Small interfering RNAs (siRNAs) have been exploited as new therapeutics for a variety of diseases.[26] The major issue in siRNA therapy is the delivery of siRNA into the target cells. Nucleic acid–based aptamers offer some important features for targeted siRNA delivery. Aptamers are artificial oligonucleotides (single-stranded DNA [ssDNA] or RNA) that can fold into a 3-dimensional structure to bind to a variety of targets, including proteins, peptides, carbohydrates, and low-molecular-weight biomolecules.[27] Aptamers with high affinity to target molecules can be screened by using the systematic evolution of ligands by exponential enrichment (SELEX) process.[28] Compared with antibodies, aptamers have shown little or no immunogenicity.[28] Here, we aimed to develop a novel anti-CD8 aptamer to deliver siRNA to $CD8^+$ CTLs.

CD8 receptor is characterized as a CTL marker and is also expressed on NKT cells. Human CD8 protein consists of 2 isoforms, termed CD8α and CD8β, expressed on the cell surface as a mixture of disulfide-linked CD8αα homodimers and CD8αβ heterodimers. CD8αβ heterodimers are predominantly found on the surfaces of T-cell receptor (TCR) αβ T cells, and CD8αβ heterodimers enhance CTL activation better than CD8αα homodimers.[29] Herein, we conjugated an anti-CD8 aptamer with GNLY siRNA to inhibit immune responses. The proposed mechanism is shown in Fig E1 in this article's Online Repository at www.jacionline.org.

METHODS

Patients and samples

This study followed the Declaration of Helsinki protocols, and approval for the use of human subjects for the study was obtained from the institutional review board. Informed consent was obtained from each participant. PBMCs were obtained from healthy donors or patients with allopurinol-induced SJS (Chang Gung Memorial Hospital Health System in Taiwan) and cultured in medium containing RPMI 1640, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids (all from Invitrogen, Carlsbad, Calif), and 10% human AB serum (BioWhittaker, Walkersville, Md). For some experiments, $CD8^+$ T cells were isolated by using the magnetic cell sorting negative selection system with the $CD8^+$ T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of $CD8^+$ T cells was greater than 95%, as determined by using flow cytometry with fluorescein isothiocyanate–labeled antibodies to CD8 (R&D Systems, Toronto, Ontario, Canada). GNLY was detected when CTLs were stimulated with 100 U/mL IL-2 and 50 ng/mL IL-15 (R&D Systems) for 48 hours. To obtain drug-specific T cells, PBMCs (2.0×10^6 cells) from patients with allopurinol-induced SJS were cultured with oxypurinol (10 μg/mL, an active metabolite of allopurinol) in culture medium for 7 to 14 days. Then the expression of GNLY in culture medium was analyzed by means of ELISA, and the expression of cell markers was analyzed by means of flow cytometry. The T cells were then subjected to a coculture containing irradiated (50 Gy), EBV-transformed, autologous, B-lymphoblastoid cell lines or autologous PBMCs and oxypurinol. After 14 days of culture, proliferated T cells were recovered and subjected to repeat culture cycles. After approximately 4 to 5 culture cycles, CTLs expressing GNLY were predominantly *in vitro* enriched. For some experiments, we used carbamazepine-specific TCR transfectants (TF-10) or C1R (human B-lymphoblastoid cells) cells expressing the full-length HLA-B*1502 or HLA-B*5801 cDNAs in this study.[6,7]

ssDNA library and SELEX procedure

The DNA oligonucleotide library composed of 40-nucleotide central random sequences flanked by 20-nucleotoide PCR priming sequences at both the 5′ and 3′ ends (ACGCTCGGATGCCACTACAG-N40-CTCATGGACGTGCTGGTGAC) was synthesized by Integrated DNA Technologies (Coralville, Iowa). The cell-based SELEX was performed in the first round of selection. The ssDNA library pool of 10^{15} molecules was incubated with intact $CD8^+$ T cells (isolated from human PBMCs) in SELEX buffer (150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and 40 mmol/L HEPES [pH 7.5]) containing 5% FBS on ice for 30 minutes. The cells were washed 3 times with SELEX buffer, and the bound ssDNA pool was eluted by heating at 95°C for 5 minutes. After that, we carried out immunoprecipitation SELEX, as previously described,[30] in the second to ninth rounds. The eluted ssDNA pool was directly incubated with CD8αβ–Fc protein complexes, which were captured by protein G beads at 37°C for 30 minutes in SELEX buffer. The beads were then washed with SELEX lysis buffer, and the CD8/aptamer complexes were eluted from the beads with citric acid. The aptamers were amplified by means of PCR with forward primers and biotin-labeled reverse primers (approximately 10-20 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 20 seconds at 72°C, followed by 3 minutes at 72°C; the PCR buffer contained 10 mmol/L Tris-HCl [pH 8.9], 1 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 20 mmol/L betaine, 1% dimethyl sulfoxide, 200 nmol/L of each primer, and 2 U of *Taq* DNA polymerase). The selected sense ssDNA pool was separated from biotin-labeled antisense reverse primer by using streptavidin-coated magnetic beads (Chemogen, Portland, Me). For each round of SELEX, the ssDNA pool was denatured at 95°C for 5 minutes and then allowed to refold to 37°C before binding. Negative selection against protein G beads alone was carried out by incubating with the ssDNA pool in the third and eighth rounds to remove the ssDNA pool bound to protein G beads. After the ninth round of SELEX, the selected ssDNA pool was PCR amplified by using unmodified primers and cloned into the TA cloning vector pGEM-T (Promega, Madison, Wis). Individual clones were determined by using DNA sequencing.

siRNAs and aptamer-stick-siRNA chimera

siRNAs and sticky sense strand RNAs were chemically synthesized by Integrated DNA Technologies. The siRNA sequences targeting *GNLY* mRNA were designed in the regions of the 9-kDa GNLY form. All sequences used for siRNA or the aptamer-siRNA chimera were shown in Table E1 in this article's Online Repository at www.jacionline.org. 2′-OMe (2′-O-Methyl group) modified bases were used at the siRNA portion of the chimera.