Available online at www.sciencedirect.com
ScienceDirect
Toxicology in Vitro 20 (2006) 1343–1353
Toxicology in Vitro
www.elsevier.com/locate/toxinvit

Application of RNAi inducible TNFRI knockdown cells to the analysis of TNFα-induced cytotoxicity

Fumiyo Saito [a,b,*], Hirofumi Yokota [a], Yoshihisa Sudo [a], Yoshikuni Yakabe [a], Haruko Takeyama [b], Tadashi Matsunaga [b]

[a] *Chemicals Evaluation and Research Institute, Japan, 1600, Shimo-Takano, Sugito, Kitakatsushika, Saitama 345-0043, Japan*
[b] *Tokyo University of Agriculture and Technology, Department of Biotechnology, Koganei, Tokyo 184-8588, Japan*

Received 4 March 2006; accepted 16 May 2006
Available online 3 June 2006

Abstract

RNA interference (RNAi) has become a popular tool for downregulating in many species including mammalian cells. Therefore, suppression of target genes in mammalian cultured cells using RNAi may represent an ideal alternative to knockout studies for understanding the molecular mechanisms of chemical toxicity. Here, we assessed the potential of RNAi mediated gene knockdown in HeLa and HepG2 cells to cytotoxicity studies. Tumor necrosis factor receptor I (TNFRI) was chosen as a target gene because its signaling has been implicated in xenobiotic-induced toxicity. We optimized the design and performance of a vector-based RNAi experiment and then investigated viability of both HeLa and HepG2 cells exposed to TNFα. In addition, we examined gene expression profile of TNFRI knockdown HeLa cells after TNFα treatment, and then protein expression levels for several apoptosis-related genes of the cells. In both HeLa and HepG2 cells, TNFα exposure resulted in significantly reduced susceptibility of the knockdown cells to the cytotoxicity as compared with those of mock-transfected cells. Furthermore, the gene expression profiling and western blotting revealed that several genes including apoptosis and/or NF-κB pathway were downregulated in the knockdown HeLa cells. These results suggest that downregulation of the TNFRI gene in both HeLa and HepG2 cells by RNAi participates in resistance to TNFα-induced cytotoxicity. Therefore, this study raises the possibility that RNAi-based gene silencing in mammalian cells may be a valuable tool for elucidating the relationships between phenotypic changes and target gene functions in response to xenobiotic-induced cytotoxicity. Further exposure study using xenobiotics needs to be done to validate the potential utility of RNAi technology.

Keywords: TNFRI; Apoptosis; Cytotoxicity; RNA interference; Knockdown; DNA microarray

1. Introduction

RNA interference (RNAi) is one of the post-transcriptional gene silencing which is conserved in many eukaryotes, and it is initiated by introduction and cleavage of double-stranded RNA (dsRNA) that has homology in sequence to the silenced gene (Elbashir et al., 2001). This phenomenon can be observed in mammalian cells when small interfering RNAs (siRNAs) are used (Elbashir et al., 2001), and hence it has been exploited as the powerful tool for reverse genetics in the post genome era (Berns et al., 2004; Miyagishi et al., 2004). In the field of toxicological research, RNAi may offer a convenient and efficient way to enable phenotypic analysis such as cell viability to directly elucidate gene function. However, there are a few reports about it until now possibly due to technical reasons concerned with siRNA transfer (Abdelrahim et al., 2003; Stribinskis and Ramos, 2004). Synthetic siRNAs cannot induce RNAi activity over a long-term period because they do not have long half-lives once transfected into cells (Elbashir et al., 2001). Additionally, the extent of reduction of a targeted gene was depended on the transfection efficiency of the individual experiments (Abdelrahim et al., 2003). In

* Corresponding author. Address: Chemicals Evaluation and Research Institute, Japan, 1600, Shimo-Takano, Sugito, Kitakatsushika, Saitama 345-0043, Japan. Tel.: +81 480 37 2601; fax: +81 480 37 2521.
E-mail address: saito-fumiyo@ceri.jp (F. Saito).

0887-2333/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2006.05.007

order to confer significantly prolonged stability of siRNA expression and positive selection of transfected cells, a vector-based system that expresses siRNA from expression cassettes has been successfully used (Bantounas et al., 2004). However, siRNA has been frequently shown to exert multiple non-specific effects (e.g., activation of the interferon system) beyond the selective silencing of target genes (Bridge et al., 2003; Persengiev et al., 2004; Sledz et al., 2003). Therefore, particular attention should be paid to design of experimental protocols as well as interpretation of data when conducting toxicological study using RNAi technology.

In this study, we assessed the potential of RNAi inducible gene knockdown cells to be used for cytotoxicity study. As a model gene for knockdown experiments, we chose human tumor necrosis factor receptor I (TNFRI), because its ligand TNFα has been shown to involve in various models of xenobiotic-induced toxicity such as inflammation, fibrosis, and apoptotic/necrotic cell death (reviewed in Luster et al., 2001). *In vitro* studies using primary cells derived from TNFRI knockout mice reported that TNFα exaggerated cellular damage induced by xenobiotics (Knight et al., 2000; Bernardino et al., 2005). However, cost, speed and throughput considerations pose serious limitations to the study of such mammalian systems, highlighting the need for alternative strategies. In addition, the 3Rs (refining, reducing, and replacing) of animal use have become established as essential considerations (Rusche, 2003). Blocking of a target protein by a specific antibody, which is one of in vitro inhibition methods, is simple and convenient and applied into neutralization of TNFRI function (Britton et al., 1998; Lee et al., 2005). This method has some drawbacks such as instability for a long-term study. Additionally, we can not directly quantitate the level of inhibition or blockage of target protein in antibody method. Therefore, we focused on the possible use of RNAi methods in order to more easily and conveniently establish an in vitro assay based on biological mechanisms. In the first step of this investigation, we optimized siRNA transfection conditions and drug selection thereafter so that high efficiency and low cytotoxicity could be achieved. Then, TNFRI knockdown (TNFRIKD) cells, which were transfected with an RNAi expression vector and subjected to drug selection under the optimal conditions, were exposed to TNFα, and subsequent the phenotypic observation, and gene and protein expression analyses were conducted.

2. Materials and methods

2.1. Cell lines and cell culture

HeLa and HepG2 cells were obtained from the American Type Culture Collection (ATCC; VA, USA) and RIKEN BioResouce Center (Ibaragi, Japan), respectively. Cells were maintained in Dulbeco's modified Eagle's medium (DMEM; Invitrogen , CA, USA) supplemented with 10% fetal bovine serum (Hyclone; UT, USA) and 1 mM non-essential amino acids solution (Invitrogen) at 37 °C under an atmosphere of 5% carbon dioxide.

2.2. Optimization of siRNA transfection conditions

In order to optimize siRNA transfection conditions, transfections were performed with several concentrations of an expression vector which encodes a green fluorescent protein (GFP) (pZsGreen; Clontech, CA, USA) and LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's recommendations. Briefly, the day before transfection, HeLa and HepG2 cells were trypsinized, counted and seeded at 1×10^4 cells per well in 96-well tissue culture plates (Corning, NY, USA), so that they were 50–80% confluence on the day of transfection. The cells were transfected with 0–4 μg/ml of pZsGreen using 1.25–10 μl/ml of the lipofection reagent diluted in serum-fiee medium (OptiMEM; Invitrogen). Twenty-four hours post-transfection, cell viability and transfection efficiency (only for 1.25 and 2.5 μl/ml of the lipofection reagent) were measured by MTT assay using a commercially available kit (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) and morphological observation of GFP positive cells with a fluorescent microscope BX51 (Olympus, Tokyo, Japan), respectively. For cell viability assessment, 10 μl of Cell Counting Kit-8 were added to each well and incubated for 1.5 h at 37 °C. Then, absorbance at 450 nm was measured with amicroplate reader Wallac 1420 ARVO (PerkinElmer Life Sciences, MA, USA).

2.3. shRNA design and plasm id construction

A short hairpin RNA (shRNA) expression plasmid was constructed according to Miyagishi et al., 2004. Briefly, three target sites for TNFRI located downstream of the start codon were selected and oligonucleotides containing target sites were synthesized by DATE Concept Inc. (Hokkaido, Japan). These sequences are as follows: KD1-sense, 5′-caccGGAGCTTACTTGTATGATGATgtgtgctgtccGTCATTGTACAAGTAGGTTCCttttt-3′; KD 1-antisense, 5′-gcataaaaaGGAACCTACTTGTACAATGACggacagcacacAT CA-3′; KD 2-sense, 5′-caccACTATCTCAGATACTGTCTTAgtgtgctgtccTGAGGCAGTGTCTGAGGTGGTttttt-3′; KD 2-antisense, 5′-gcataaaaaACCACCTCAGACACTGCCTCAggacagcacacTAAGACAGTATCTGAGATAGT-3′; KD 3-sense, 5′-caccGAGCTAGTATCGGTATTGTTGgtgtgctgtccGAACCAGTACCGGCATTATTGttttt-3′; KD 3-antisense, 5′-gcataaaaaGAACCAGTACCGGCATTATTGggacagcacacCAACAATACCGATACTAGCTC-3′. The 21-mer targeting sequences indicated in capital letters are corresponding to human TNFRI coding nucleotides 474–494, 562–582, and 668–688, respectively. Equal amounts of sense and antisense oligonucleotides were mixed in annealing buffer (10 mM Tris–HCl, pH 7.5/1 mM EDTA/l00 mM NaCl), heated for 2 min at 99 °C, and then gradually cooled to 4 °C for 2 h. The expression plasmid that delivers an expression cassette for human U6