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Original Article

Selenoproteins translated by SECIS-deficient mRNA induce apoptosis in Hela cells

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Abstract: Objective: The aim of this study was to explore the biological function of selenoproteins translated by SECIS-deficient mRNAs of selenoprotein S (SelS), glutathione peroxidase 4 (Gpx4), and thioredoxin reductase 1 (TrxR1). Methods: Recombinant plasmids of normal and selenocysteine insertion sequence (SECIS)-deficient mRNAs of SelS, Gpx4, and TrxR1 were constructed and transfected into Hela cells. Total RNA was collected by TRIzol method and cDNA were obtained by mRNA reverse transcription. To ensure that target selenoprotein genes were successfully transfected into cells and highly expressed, PCR was performed for only 15 circulations. Bright bands were then observed of target genes. Results: After transfection for 24 hours, expression of green fluorescent protein was noted and the transfection efficiency was detected up to 40% by fluorescein activated cell sorter analysis. After transfection for 48 hours, cells were collected to stain for fluorescein activated cell sorter analysis. Results showed that Hela cell apoptosis could be induced by selenoproteins translated by SECIS-deficient mRNAs but not normal selenoproteins. Conclusion: Selenoproteins translated by SECIS-deficient mRNAs of SelS, Gpx4, and TrxR1 induce Hela cells apoptosis, providing the underlying mechanisms of selenium deficiency conditions or diseases.

Keywords: Selenoproteins, SECIS-deficient, apoptosis, Hela cells

Introduction

Selenium is an essential micronutrient. Much of its biological function on human health has been attributed to the presence of more than 30 selenoproteins in the human body [1]. Selenoproteins play roles in a variety of physiological processes, such as regulation of redox homeostasis [2], immune function [3], reproduction [4] and biosynthesis of thyroid hormone [5].

Unlike other metal elements that interact within proteins in the form of cofactors, selenium becomes co-translationally incorporated into the polypeptide chain as part of the amino acid selenocysteine (Sec) [6]. A remarkable feature of Sec incorporated into proteins is its shared UGA with termination codon [7]. However, the UGA codon plays the role of termination of peptide chain translation. In fact, there is competition between Sec-tRNA and the release factor for UGA decoding in the process of selenoprotein expression [8] and results are greatly influenced by dietary selenium [9]. Decoding of the UGA codon requires multiple factors, such as the selenocysteine insertion sequence (SECIS) element and other essential protein factors [6]. Secondary structures of the SECIS element are highly conserved and contain consensus sequences that are indispensable for Sec incorporation [10]. SECIS is located within the 3'-untranslated region (UTR) of mRNA in eukaryotes [11]. In the process of the selenoprotein synthesis, abnormality of any of the above factors would terminate the peptide chain without Sec incorporation.

In a low selenium environment, a different splicing form of selenoprotein induces lots of selenocysteine-deficient selenoprotein productions. Until now, the mechanisms of biologic or pathological effects caused by selenoprotein splicing form of terminating in advance or

Table 1. Primers used for amplification of selenoproteins

NCBI N0.	Gene	Primer sequences (5'-3')
NM_018445.4	SelS	For: CCGCTCGAGATGGAACGCCAAGAGG
		Rev: GCGGGCCCTTAATATACAGAAACAAACCCCATC
	trun-SelS	For: CCGCTCGAGATGGAACGCCAAGAGGAGTC
		Rev: GCGGGCCCTTAGCCTCATCCGCCAG
NM_002085.3	Gpx4	For: CCGCTCGAGATGAGCCTCGGCCGCC
		Rev: GCGGGCCCTTACCCACAAGGTAGCCAGG
	trun-Gpx4	For: CCGCTCGAGATGAGCCTCGGCCGCC
		Rev: GCGGGCCCCTAGAAATAGTGGGGCAGGTCC
NM_182743.1	trun-TrxR1	For: CCGCTCGAGATGAACGGCCCTGAAG
		Rev: GCGGGCCCTTAACCTCAGCAGCCAG

its translation product-selenocysteine-deficient peptide chain have remained unclear. The present study proposed the hypotheses that selenocysteine-deficient selenoproteins could induce cell apoptosis. Thus, five recombinant plasmids of normal and SECIS-deficient selenoprotein genes of selenoprotein S (SelS), glutathione peroxidase 4 (Gpx4), and thioredoxin reductase 1 (Trx1) were constructed and transfected into Hela cells. Cell apoptosis was then tested.

Materials and methods

Construction of normal and SECIS-deficient selenoprotein gene plasmids

For construction of pEGFP-C1-selenoprotein or pEGFP-C1-SECIS-deficient-selenoprotein recombinant plasmids, human cDNA was used for amplification. Target genes of SECIS-deficient-selenoprotein amplified by PCR only comprised the coding sequence (CDS) of the mRNA, but without the 5'untranslated region (UTR) or 3'UTR. Normal selenoproteins amplified by PCR included CDS and 3'UTR, which contain the SECIS elements without 5'UTR. Primers of the five selenoprotein genes are shown in the **Table 1**. PCR products and the pEGFP-C1 plasmids were digested by XhoI and ApaI. Fragments of double enzyme-digested products were subjected to agarose gel electrophoresis and purified by the DNA Purification Kit (BioDev-Tech, Beijing, China). Fragments of selenoprotein genes and pEGFP-C1 vectors were ligated by T4 ligase (Sigma, USA) at 4°C overnight to generate new plasmids. The plasmids were transfected into the DH5α E. coli. New plasmids were extracted by the Plasmid kit (OMEGA, USA) and were screened by XhoI and ApaI digestion and agarose gel electrophoresis. Positive plasmids were sent for sequencing (Sangong Biotech, Shanghai, China).

Cell culture and transfection with recombinant plasmids in Hela cells

Hela cells were purchased from Chinese Academy of Science Affiliated Shanghai Cell Bank. Cells were cultured in DMEM media (Hyclone, Logan, UT, USA) containing 10% fetal calf serum (Hyclone, Logan, UT, USA) at 37°C in a 100% humidified atmosphere of 5% CO_2.

Transfection of recombinant plasmids to the cells was performed with Lipofectamine™ 2000 reagent (Invitrogen, San Diego, CA, USA), according to manufacturer instructions. Briefly, 1 day before transfection, exponentially growing cells were seeded into six-well plates at a density of 1.5×10^5 cells/well in the medium without antibiotics. When they reached 70% confluence, cells were transfected with 2 μg DNA of plasmids per well using Lipofectamine 2000. Green Fluorescent Protein (GFP) expression of Hela cells was observed by a fluorescence microscope (Nikon, ELIPSE Ti, Japan) 6 hours after transfection. Transfection efficiency was evaluated with GFP by the fluorescence activated cell sorter (FACS) 24 hours after transfection.

Reverse transcription-PCR

Total RNA of the cells after transfection of recombinant plasmids for 24 hours was extracted using TRIzol (Invitrogen, USA). RNA purity and content were determined with UV/visible spectrophotometer. Synthesis of cDNA was conducted from 2 μg total RNA using the First-Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada), according to manufacturer instructions. RT-PCR was performed as follows: 1 cycle at 94°C for 3 minutes, followed by 15 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and finally 1 cycle at 72°C for 7 minutes. PCR products were subjected to agarose gel electrophoresis.

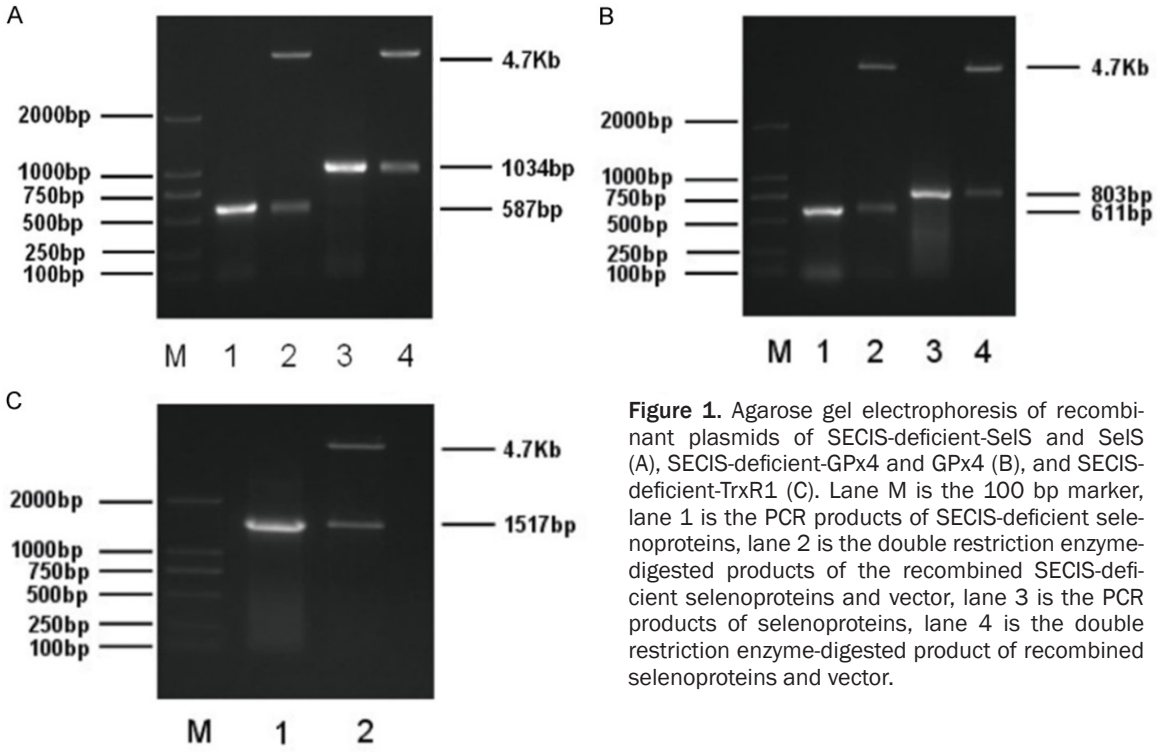

Figure 1. Agarose gel electrophoresis of recombinant plasmids of SECIS-deficient-SelS and SelS (A), SECIS-deficient-GPx4 and GPx4 (B), and SECIS-deficient-TrxR1 (C). Lane M is the 100 bp marker, lane 1 is the PCR products of SECIS-deficient selenoproteins, lane 2 is the double restriction enzyme-digested products of the recombined SECIS-deficient selenoproteins and vector, lane 3 is the PCR products of selenoproteins, lane 4 is the double restriction enzyme-digested product of recombined selenoproteins and vector.

Cell death and apoptosis assay

After transfection of recombinant plasmids for 48 hours, cell apoptosis was detected by Annexin V-PE/7-AAD apoptosis detection kit (Byotime Biotech, Jiangsu, China), according to manufacturer instructions. Briefly, cells were trypsinized and resuspended in binding buffer with 5 μL 7-AAD and 1 μL Annexin V-PE. After incubating for 15 minutes in the dark, samples were analyzed using a GuavaeasyCyt™ Flow Cytometer (EMDMillipore, Darmstadt, Germany) and percentages of 7-AAD positive and Annexin V-PE positive were valuated.

Statistical analyses

Data were analyzed using SPSS15.0 statistical software and are presented as mean ± Standard Error of the Mean (SEM). Statistical analysis of the apoptosis test was performed by the nonparametric test (Mann-Whitney U-test). *P*-values less than 0.05 are considered statistically significant and the results of *** represent P<0.001.

Results

Identification of normal and SECIS-deficient selenoprotein gene clones

Five selenoprotein genes were cloned, including SelS and SCIS-deficient-SelS (**Figure 1A**), Gpx4 and SECIS-deficient-Gpx4 (**Figure 1B**), and SECIS-deficient-TrxR1 (**Figure 1C**). Gel electrophoresis demonstrated that 5 recombinant plasmids were digested to two fragments with different lengths, respectively. Judging from the lengths, the longer products corresponded to the pEGFP-C1 vector and the shorter products corresponded to selenoprotein and SECIS-deficient-selenoprotein genes. According to sequencing results, no deletion, insertion, or mutation was found and the consistency rate was 100%. It was confirmed that the clones were successfully constructed.

Identification of transfection efficiency of normal and SECIS-deficient selenoprotein genes

After transfection with recombinant plasmids for 6 hours, cells with green fluorescence could be observed by fluorescence microscopy. Furthermore, a fluorescein activated cell sorter was used to determine GFP positive cells. Results showed that transfection efficiency was up to 40% after transfection for 24 hours (**Figure 2A**).

Total RNAs were extracted from the cells by TRIzol after transfection for 24 hours. Next, cDNA was obtained by mRNA reverse transcription and used to PCR, which was performed for 15 cycles. PCR was performed in mock, empty vector, SECIS-deficient-SelS, and SelS groups.

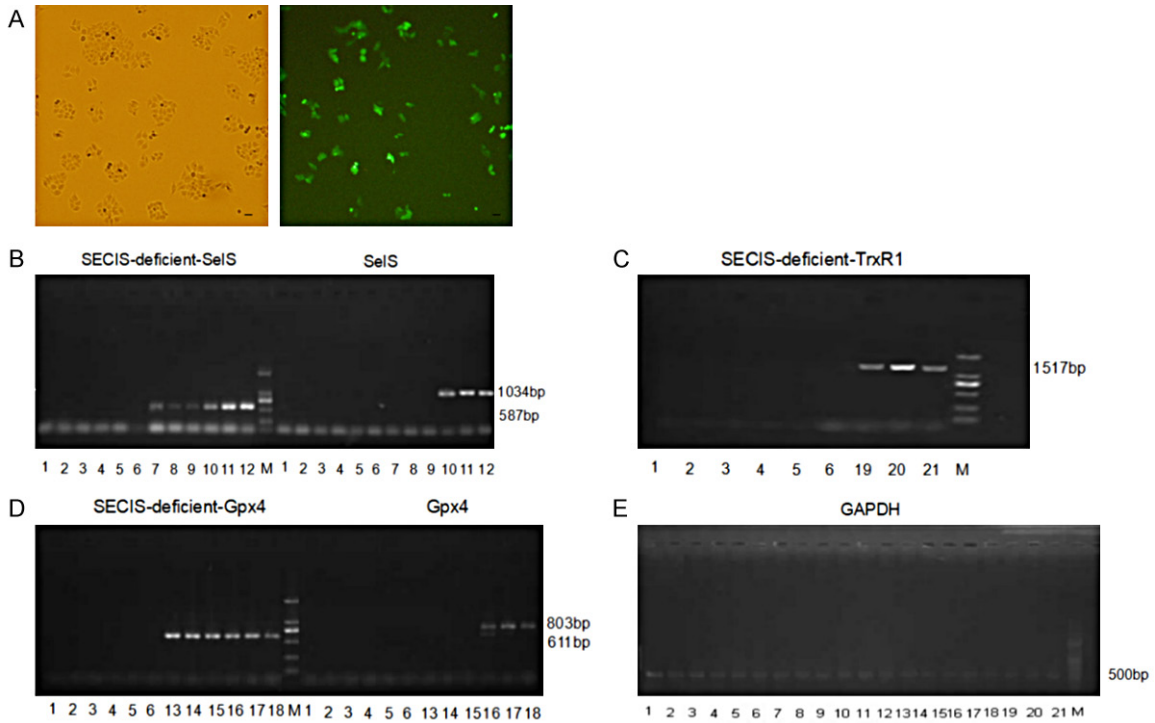

Figure 2. Identification of normal and SECIS-deficient selenoprotein genes at 24 hours after transfection. A. Transfection of recombinant plasmids in Hela cells. The left picture is under light microscope and the right one is under fluorescence microscope. The black bar indicates 10 μm. B-E. Agarose gel electrophoresis of normal and SECIS-deficient selenoprotein genes and GAPDH in Hela cells. 1-3: mock group, 4-6: empty vector group, 7-9: SECIS-deficient-SelS group, 10-12: SelS group, 13-15: SECIS-deficient-Gpx4 group, 16-18: Gpx4 group, 19 -21: TrxR1 group, each set of three parallel samples.

SECIS-deficient-SelS and SelS primers were used respectively (**Figure 2B**). As shown in the left part, SECIS-deficient-SelS gene band appeared in the SECIS-deficient-SelS group and SelS group after 15 cycles of PCR, while there was no obvious band in the mock group and empty vector group for the low copy number in these two groups. High expression of SECIS-deficient-SelS gene bands in the transfected cells indicates that the transfection was successful. Since the SelS gene fragment comprised the SECIS-deficient-SelS gene fragment, SECIS-deficient-SelS genes were also highly expressed in the SelS group. The right part indicates the results of amplifying the SelS gene fragment, manifesting the successful transfection of SelS recombinant plasmid. Similar results could be seen after transfection of GPx4 genes, SECIS-deficient-GPx4 genes (**Figure 2C**), and SECIS-deficient-TrxR1 genes (**Figure 2D**). PCR of GAPDH was also performed in all 7 groups mentioned above (**Figure 2E**).

Results demonstrated that each of the five SECIS-deficient and normal selenoprotein genes were successfully transfected into the cells and expressed at a high level. Thus, the biological effects of these target genes could be further researched.

Apoptosis induced by SECIS-deficient selenoproteins but not normal selenoproteins

After transfection of normal or SECIS-deficient selenoproteins plasmids for 48 hours, cells were collected and cell apoptosis was detected by FACS (**Figure 3A**). It was observed that, as with cells transfected with mock or empty vectors, the cells transfected with normal SelS or Gpx4 genes mainly distributed in the quadrant of living cells (lower left). Moreover, cells of these two groups distributed in the quadrants of Annexin V positive (lower right) and 7-AAD positive (upper left) were no more than the control groups. In contrast, cells transfected with SECIS-deficient-SelS, SECIS-deficient-Gpx4, or SECIS-deficient-TrxR1 genes were distributed more scattered than the control groups. Annexin V positive (lower right) and 7-AAD positive (upper left) cells in those three groups were

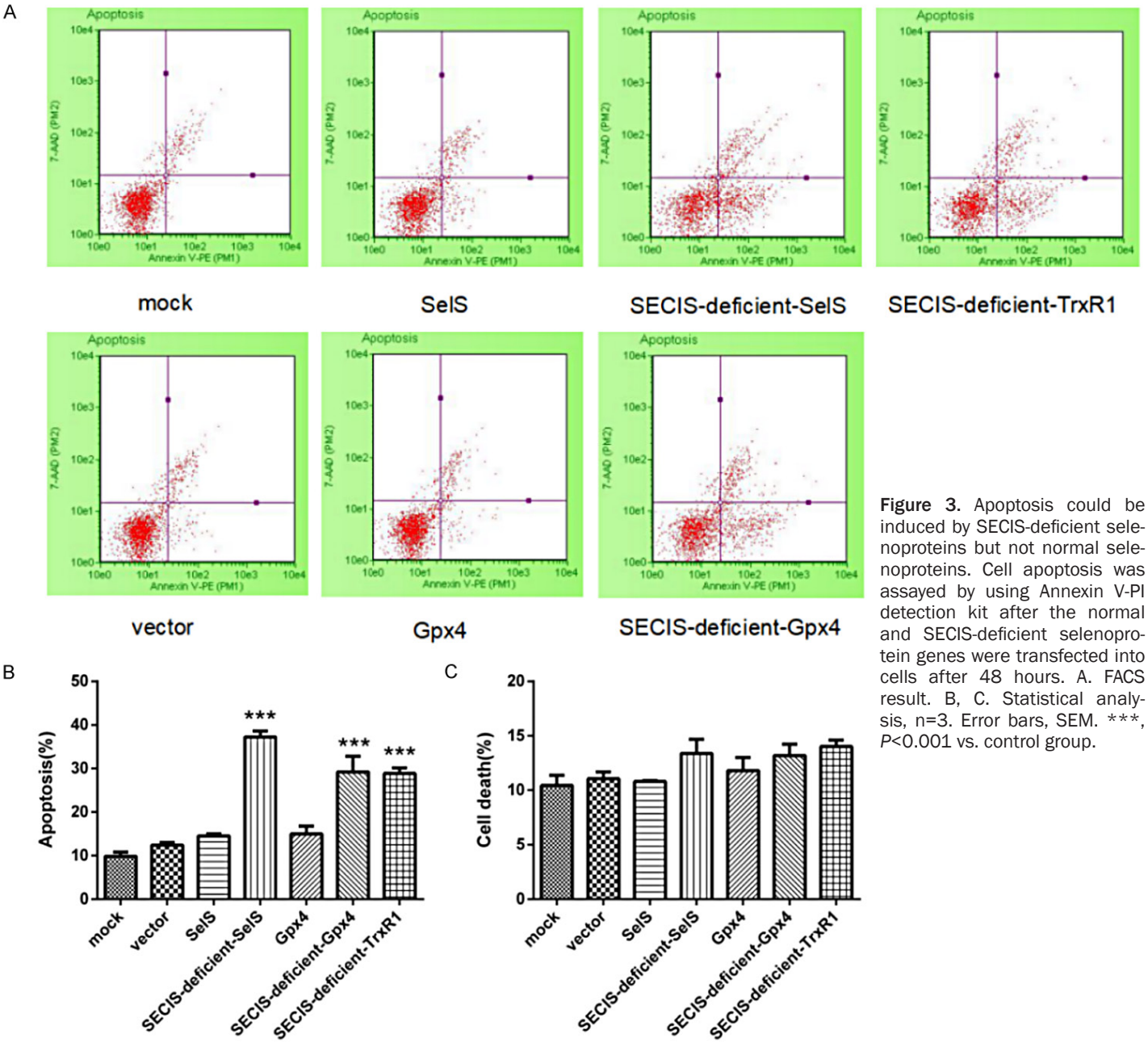

Figure 3. Apoptosis could be induced by SECIS-deficient selenoproteins but not normal selenoproteins. Cell apoptosis was assayed by using Annexin V-PI detection kit after the normal and SECIS-deficient selenoprotein genes were transfected into cells after 48 hours. A. FACS result. B, C. Statistical analysis, n=3. Error bars, SEM. ***, $P<0.001$ vs. control group.

significantly increased relative to control groups. It can be concluded from statistical analysis that the numbers of apoptotic cells in SECIS-deficient-SelS, SECIS-deficient-Gpx4, and SECIS-deficient-TrxR1 were significantly higher than those in the mock control group or empty vectors ($P<0.001$) (**Figure 3B**). However, there were no differences in the numbers of dead cells between the five experimental groups (**Figure 3C**). Hence, cell apoptosis could be induced by selenocysteine-deficient selenoproteins of SelS, Gpx4, and Trx1 but not normal selenoproteins.

Discussion

Selenium deficiency may lead to a disorder of selenoprotein synthesis and accumulation of truncated selenoproteins without active Sec in the peptides. The stability of selenoproteins has been positively correlated with selenium availability, which tunes reassignment of UGA to Sec [12]. A remarkable diversity of RNA elements conducting multiple occurrences of UGA redefine the synthesis of full length and truncated selenoprotein P isoform [13]. Nonsense-mediated mRNA decay pathways may play an important role in regulating selenoprotein mRNA levels when Se is limiting [14]. Furthermore, the mechanisms of biologic or pathological effects caused by selenoprotein splicing form of terminating in advance or its translation product-selenocysteine-deficient peptide chain remain unclear.

The present study generated the Sec-deficient selenoproteins translated by SECIS-deficient mRNAs to mimic the situation of selenium deficiency. Results showed that selenocysteine-deficient SelS, Gpx4, and TrxR1 genes could induce Hela cell apoptosis, while normal selenoproteins did not have this effect. However, the mechanisms of cell apoptosis caused by selenocysteine-deficient selenoproteins remain unclear.

It has been widely accepted that SelS, Gpx4, and TrxR1 can maintain intracellular redox levels. Sec is an essential composition of selenoprotein enzyme activity center, thus the destruction of Sec by truncating selenoproteins will destroy the activity center. As a result, intracellular ROS levels and toxic substances levels will elevate and eventually lead to cell apoptosis. To detect the effects of sec removal on the intracellular oxidative environment, research reported by Takafumi Suzuki generated a conditional knockout mouse for the tRNA-Sec gene (Trsp) [15]. This research found that deletion of Trsp in either macrophages or liver cells elevated oxidative stress and activated the transcriptional induction of cytoprotective antioxidant and detoxification enzyme genes. After double knockout of Trsp and Nrf2, macrophages and liver cells displayed reduced viability, elevated oxidative stress, and increased susceptibility to hydrogen peroxide treatment, compared with deletion of either gene alone. This study suggested that a decrease in selenoprotein activity causes Nrf2-mediated protective responses that are necessary to maintain intracellular redox homeostasis and cell viability. Results of this study support the experimental results of the present study.

The study of selenium compromised of selenoproteins has mostly focused on TrxR1. As shown in the research of Anestål et al., truncating -Sec-Gly at carboxyl terminal of TrxR1 at protein levels or forms of the enzyme compromised at the Sec residue with either cisplatin or dinitrophenyl moieties will rapidly induce cell death, while the enzymatically active full-length enzyme did not hold this property [16]. The present study generated SECIS-deficient selenoproteins at the DNA level, obtaining results that were inconsistent with previous reports.

Mechanisms of cell apoptosis induced by truncated TrxR1 have also been reported in several studies. The main function of TrxR1 is reducing Trx1, thus cell viability could be promoted. Downstream mechanisms could be summarized as inhibition of apoptosis signal-regulating kinase 1 [17], regeneration of peroxiredoxins [18], and methionine sulfoxide reductase [19]. It can be assumed that selenium deficient TrxR1 can form complexes with exogenous thioredoxin (Trx) or TrxR1 subunits. When exogenous Trx is significantly inhibited, TrxR1 activity will decline. As a downstream effect, cell apoptosis will be triggered by reduction of normal antioxidant substances and activation of apoptosis signal-regulating kinase 1. Furthermore, the link between TrxR1 and p53 [20] has been reported. TrxR1 was shown to be a prerequisite for retinoid/interferon-induced cell death but not for cell death induced by tumor necrosis factor α, etoposide, or vincristine [21]. This indi-

cates a need for a functional Trx system in the induction of apoptosis through p53. Compared to selenium-compromised TrxR1, there have been no reports concerning cell apoptosis induced by SECIS-deficient SelS or SECIS-deficient Gpx4 and the mechanisms remain unclear.

Selenium deficiency may lead to a disorder of selenoprotein synthesis and accumulation of selenocysteine-deficient selenoproteins. Cell apoptosis induced by selenocysteine-deficient selenoproteins may be through increasing intracellular redox levels. However, mechanisms under the function of each selenocysteine-deficient selenoprotein require further research.

Conclusion

Selenoproteins translated by SECIS-deficient mRNAs of SelS, Gpx4, and TrxR1 may induce Hela cell apoptosis. This may provide the underlying mechanisms of selenium deficiency conditions or diseases.

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Disclosure of conflict of interest

None.

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