RESEARCH ARTICLE

Negative Correlation between Cytoglobin Expression and Intracellular ROS Levels in Human Skin Keloid Fibroblasts

Fajri Marindra Siregar[1,2], Novi Silvia Hardiany[3,4], Sri Widia Azraki Jusman[3,4,*]

[1]Master Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta, Indonesia
[2]Department of Biochemistry, Faculty of Medicine, Universitas Riau, Jl. Diponegoro No.1, Pekanbaru, Indonesia
[3]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta, Indonesia
[4]Center of Hypoxia & Oxidative Stress Studies, Jl. Salemba Raya No. 6, Jakarta, Indonesia

*Corresponding author. E-mail: sri.widia@ui.ac.id

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Abstract

BACKGROUND: In our previous study, we found higher cytoglobin (Cygb) expression in keloid than normal tissue. Cytoglobin is a new globin family protein which function is still being studied to date. The purpose of this research is to elucidate the function of Cygb in human skin keloid fibroblasts (KFs), especially its role in intracellular reactive oxygen species (ROS) levels.

METHODS: The study was conducted on human skin KFs obtained from primary culture. Inhibition of Cygb expression was achieved by using siRNA targeting Cygb. We compared the relative expression of Cygb between treatment and control group, and its effect on intracellular ROS levels. Gene expression was measured using quantitative real-time polymerase chain reaction (qRT-PCR) while the ROS level counted by dichlorodihydrofluorescein diacetate (DCFHDA) assay.

RESULTS: There was an increase in intracellular ROS levels in the small interfering RNA (siRNA) (+) Cygb group compared to control group (1.673 *vs.* 1.260; 1.773 *vs.* 1.393; 1.710 *vs.* 1.360; respectively). There is a negative correlation between Cygb expression and ROS level ($p<0.05$; r=-0.651).

CONCLUSION: There is a negative correlation between Cygb expression and intracellular ROS levels, we suggest Cygb acts as a ROS scavenger in human skin KFs.

KEYWORDS: skin keloid fibroblasts, cytoglobin, siRNA, ROS

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Introduction

Keloid, also known as keloid disorder and keloidal scar, is a benign tumor that can grow beyond the wound limit and has a high recurrence rate.(1) In the skin, keloids are typically raised, firm, red to pink, and sometimes itchy. (2) Keloid usually cause aesthetic problems so it can initiate psychological disorders in patients.(3) The exact cause of keloid to date is unknown. Presumably, there are abnormalities of wound healing process caused by an imbalance between the process of synthesis and collagen degradation.(2,4)

It has been reported that skin keloid fibroblasts (KFs) have stronger proliferating activity.(3) In keloids, there is also a state of hypoxia and an increase in reactive oxygen species (ROS) production characterized by increased hypoxia-inducible factor (HIF)-1α expression and higher intracellular ROS levels than normal tissue.(5-7) One of the regulated genes regulated by HIF-1α is cytoglobin (Cygb). (8-10)

Currently, optimal treatment of keloid remains undefined (2), because there is still limited knowledge about factors that may play a role in keloid pathophysiology, include Cygb. Cygb is a new globin family protein which function is still widely studied to date. Cygb is found not

only in the cytoplasm but also in the nucleus so it is thought to have a wider function than other globin proteins.(11) Our findings in previous studies found that Cygb gene expression was higher in keloid compared to normal tissue.(6) We suspect the high expression of Cygb is related to its ability to act as a ROS scavenger.(11) This study aims to elucidate the role of Cygb in human skin KFs.

Methods

Study Design

This research is an experimental *in vitro* research conducted on fibroblasts cell cultures taken from human skin keloid lesion. We collected keloid scar specimens from 3 patients treated at Budi Kemuliaan Hospital (Jakarta, Indonesia), who underwent a second subcutaneous operation and had keloids in their previous surgical wounds. All patients agreed to participate in this study after receiving the inform consent. A clinical and pathological investigation from all keloid cases were fully proven. Each sample was divided into 2 groups, the group treated with small interfering RNA (siRNA) (+) Cygb and untreated group (as control). All experiment was repeated 3 times.

The research was conducted in the laboratory of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia from August 2017 to May 2018. The study was reviewed and approved by Faculty of Medicine Universitas Indonesia Research Committee (No. 472/UN2.F1/ETIK/2016).

Cell Culture and Treatment

The primary skin KFs culture has been establishing, as noted previously.(12) We were grown the specimens in Dulbecco Modified Eagle Medium (DMEM) and amplified it in 10% Fetal Bovine Serum (FBS) (Gibco, New York, USA), 1% Penicillin/Streptomycin and 1% Fungizone (Gibco). All specimens were cultured with 5% CO_2 at 37°C in a humidified incubator. For siRNA (+) Cygb group, skin KFs were treated with human siRNA (+) Cygb.

siRNA Transfection

The siRNA targeting Cygb (siRNA (+) Cygb) were purchased from Santacruz. During 24 hours, skin KFs cells grown to 50-60% confluence in antibiotic-free medium for *in vitro* transfection, and then transfected with siRNA (+) Cygb using siRNA Transfection Medium sc-36868 (Santacruz) at dose 20 pmol according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis performed to validate the efficiency of silencing.

Dichlorodihydrofluorescein Diacetate (DCFHDA)

Intracellular levels of ROS were estimated by using the fluorescent dye 2',7'-Dichlorodihydrofluorescein diacetate (DCFHDA) (Invitrogen, California, USA). Cells which used as control and experimental were plated in a similar number. We were plated the skin KFs in 96-well plates at a density $1x10^4$ cell/well, then for treatment group, we typically transfected it with siRNA (+) Cygb. Three days after transfection, the cells were incubated for 30 minutes with 10 µM of DCFHDA washed in phosphate-buffered saline (PBS). The absorbance of the cells in each group was monitored at an excitation of 490 nm and emission of 520 nm using a microplate reader (Nanodrop Thermo Scientific Varioscan Flash) (Thermo Fisher Scientific, Massachusetts, USA).

qRT-PCR

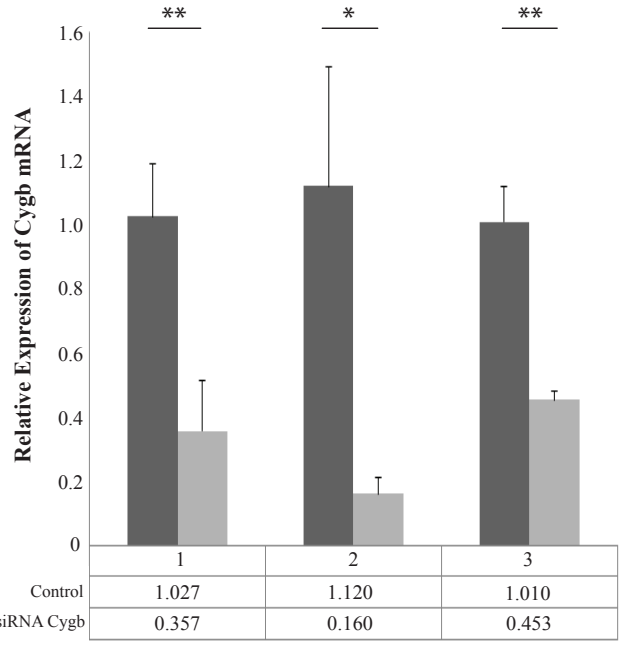
Total RNA was extracted from skin KFs using TriPure Isolation Reagent (Promega, Wisconsin, USA) according to the manufacturer's instructions. An amount of 50 ng of total RNA isolate per 10 µL of RT reaction was performed using LightCycler® 480 RT-PCR with SensiFAST SYBR NoROX Kit (Bioline, London, UK). The specific primers for Cygb were Forward 5'- CAGTTCAAGCACATGGAGGA -3'and Reverse 5'-GTGGGAAGTCACTGGCAAAT–3'; and for 18S RNA were Forward 5'-AAACGGCTACCACATCCAAG-3'and Reverse 5'- CCTCCAATGGATCCTCGTTA–3'. The 18S RNA was performed as an internal control. Then, the Livak formula was deliberated for relative quantification of the target gene. All reactions were performed in duplicates.

Statistical Analysis

Data are presented as the mean±SD of triplicate experiments. The independent T-test was employed for statistical analysis, with significant differences determined as $p<0.05$.

Results

To analyze the effect of Cygb in human skin KFs, we generated transfection of siRNA targeting Cygb. The qRT-PCR analysis indicated that the expression of Cygb was obviously decreased by siRNA (+) Cygb (Figure 1) compared to control. Furthermore, we examined the effect



	1	2	3
Control	1.027	1.120	1.010
siRNA Cygb	0.357	0.160	0.453

Figure 1. Comparison of relative expression of Cygb mRNA in KFs between siRNA (+) Cygb and control group. There was a decrease in Cygb mRNA expression in siRNA (+) Cygb compared to the control group. **p<0.05; *p<0.1; analized by independent T-test.

of Cygb inhibition on human skin KFs intracellular ROS level. As shown in Figure 2, treatment with siRNA (+) Cygb significantly increased the intracellular ROS level of human skin KFs.

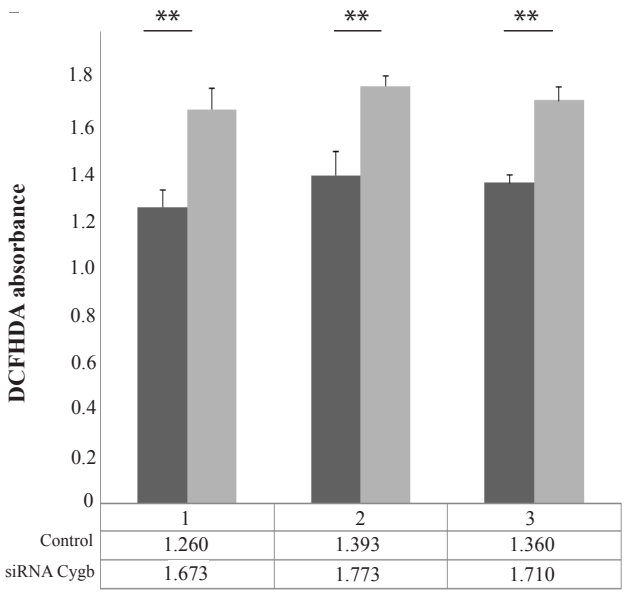
Figure 3 represents the correlation of Cygb mRNA and intracellular ROS levels in human skin KFs. The correlation between the Cygb mRNA and intracellular ROS levels is moderate negative correlation (Pearson, r=-0.651; p<0.05; n=27). This indicates that if the Cygb expression decreases then the ROS level will increase, or vice versa.

Discussion

There is a negative correlation between Cygb expression and intracellular ROS levels on human skin KFs. This observation is consistent with previous reports who showed that Cygb has a role as a ROS/reactive nitrogen species (RNS) scavenger. Similar to neuroglobin and cytochrome C, Cygb has a hexacoordinated structure and ability to detoxify radicals by reaction with their heme.(13)

Based on previous *in vitro* studies, the ability of Cygb has been proved. The Cygb had a peroxidase activity and able to create a bond with various ligands (such as oxygen, nitric oxide and other free radicals). It indicates the important role of Cygb for scavenging the free radicals (*i.e.*, reactive oxygen and nitrogen species) and modulating the cellular redox signaling than modulating the O_2 metabolism. (14,15)

In this study, we used siRNA to suppress Cygb because siRNA is one of the recent methods mediating RNA interference effect with high sensitivity and specificity.(16) Singh, *et al*., showed that administration of Cygb siRNA in myoblast cells under normoxia increased intracellular ROS levels in a small number but statistically significant compared to controls. The difference is increased when cells are cultured under hypoxia.(14) Previous study reported knockout of Cygb *in vitro* and *in vivo* will induce cells to express proinflammatory genes and increase ROS production.(17) Another investigation in NRK49F cells show that Cygb has an antioxidative stress effect. (11) McRonald, *et al.*, also received similar results where Cygb overexpression can lower ROS levels and provide protection against prooxidant-induced injuries (such as lipid peroxidation and breakdown of DNA chains due to oxidative reactions). However, the study says that there is no evidence of elevated ROS levels after suppression by RNA interference (RNAi) on cells expressing Cygb at physiological levels.(18) This may be explained because besides Cygb cells have complex antioxidant systems to detoxify ROS. Under normal conditions, antioxidant enzymes will work together to remove the various ROS produced by free radical reactions (Cu/Zn-superoxide dismutase (SOD), Mn-SOD, catalase, and selenium-glutathione peroxidase (GSH-Px)).(19)



	1	2	3
Control	1.260	1.393	1.360
siRNA Cygb	1.673	1.773	1.710

Figure 2. Comparison of intracellular ROS level in KFs between siRNA (+) Cygb and control group. There was a increased in DCFHDA level in siRNA (+) Cygb compared to the control group. **p<0.05; *p<0.1; analized by independent T-test.

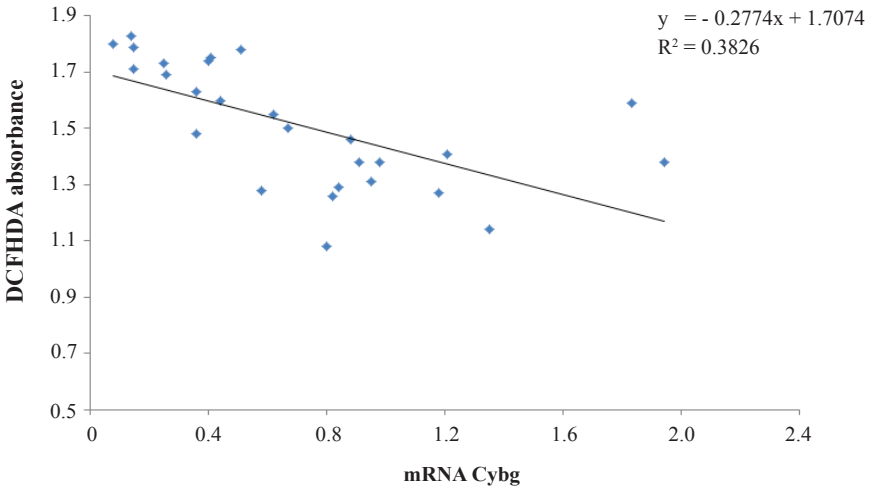


Figure 3. Correlation of Cygb mRNA and intracellular ROS level showed moderate negative correlation (Pearson, r=-0.651, $p<0.05$).

Conclusion

There is a negative correlation between Cygb expression and intracellular ROS levels, we suggest Cygb acts as a ROS scavenger in human skin KFs.

Acknowledgements

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