The effect of cytoglobin gene inhibition on fibroblast keloid cells proliferation

S W A Jusman1,2*, F M Siregar3,4, M Sadikin1,2, and N S Hardiany1,2
1Department of Biochemistry & Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
2Center of Hypoxia & Oxidative Stress Studies, Jakarta, Indonesia
3Master Programme in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta, Indonesia
4Department of Biochemistry, Faculty of Medicine, Universitas Riau, Pekanbaru, Indonesia

* sri.widia@ui.ac.id

Abstract. Cytoglobin (Cygb) is a new protein from the globin family whose function is reported to be either a tumor suppressor gene or oncogene. The aim of this study was to determine the function of Cygb on fibroblast keloid cells proliferation. The relative expressions of Cygb were compared between the siRNA (+) Cygb and siRNA (-) groups using qRT PCR, and their effect on cells proliferation, which was determined using the MTS assay. The results showed that the expression level of Cygb on siRNA (+) Cygb group were decreased compared to siRNA (-) group (0.315 vs 1.056 ; p = 0.000). However at the level of cell proliferation, there were no significant differences between the two groups (1.489 vs 1.359 ; p = 0.087). Based on the results, it is concluded that Cygb has no effect on fibroblast keloid cells proliferation.

Keywords : human keloid fibroblasts, siRNA, cytoglobin, cell proliferation

1. Introduction
Keloid is an abnormal form of wound healing, the cause of which has not been fully elucidated [1]. Fibroblasts in keloids have a very high ability to proliferate, causing increased collagen production, up to 20 times higher compared to normal tissue [2,3]. This enhanced collagen synthesis will result in excessive deposition of extracellular matrix components, which then contributes to the emergence of keloids [4].

Our previous research found that expression of Cytoglobin (Cygb) in keloids was significantly enhanced when compared to normal skin [5]. Cytoglobin is a relatively new protein from the globin family which is found in the cytoplasm and also in the nucleus. Cygb is thought to have a role in regulating the cell cycle [6]. Several previous studies on the role of Cygb on cell growth have reported conflicting results [7–9]. The variability in the results reported may be due to both the cell type and the micro-environmental conditions [10]. To the best of the authors knowledge, there are currently no studies investigating the role of Cygb in keloid fibroblasts. Therefore in this study, we want to elucidate the role of Cygb on keloid fibroblast cells proliferation.

2. Experimental Method
2.1. Study design
This is an in vitro study using fibroblasts from keloid. Keloid samples were obtained from Budi Kemuliaan Hospital, Jakarta. Primary culturing was carried out to obtain keloid fibroblast cells (KFs). There were 2 groups of KFs, namely the group that received siRNA (+) Cygb and the group that received siRNA (-) as a control.

2.2. Cell culture and treatment
The primary culture procedure was performed using the protocol from previous studies [11]. Cells were grown in Low Glucose Dulbecco Modified Eagle Medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% amphotericin. All samples were incubated at a temperature of 37°C in
an atmosphere containing 5% CO₂. Fibroblast cells transfected with the Cygb siRNA (+) were given to the treatment group, while siRNA (-) were applied to the control group.

2.3. Small interfering RNA (siRNA) transfection
The transfection procedure adopted the protocol from Santacruz. Initially, about 6x10⁴ fibroblast cells were grown on a 12-well plate. After 24 hours incubation transfection was performed at a dose of 20 pmol; the treatment group used siRNA (+) cytoglobin, while the control used siRNA (-). After 6 hours, the addition of a medium (containing 2 times the normal serum and antibiotics concentration) was carried out. Fibroblast cells were then incubated for a further 18 hours. After that, the medium was replaced using a normal medium, and fibroblast cells were incubated again for 48 hours, and then harvested for variable measurements.

2.4. MTS
Cell proliferation were counted by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium or MTS assay. Cells used as control were plated in a similar number. The fibroblast were placed in 96-well plates at a density of 1x10⁵ cell/well. Following transfection the cells were incubated for 60 minutes with 120 ml medium containing 20 ml of MTS mixture (a combination of PMS and MTS with a ratio of 1: 20). The absorbance of the cells in each group was monitored at 490 nm using a microplate reader (Nanodrop Thermo Scientific Varioscan Flash).

2.5. Quantitative real-time RT-PCR
RNA isolation was performed using a Tripure reagent (Promega). Quantitative RT-PCR was performed using a LightCycler® 480 Real-Time PCR together with a SensiFAST SYBR NoROX Kit. Each reaction uses RNA (up to 50 ng). The specific primers for Cygb were Forward 5’-CAGTTCAAGCAGCTGGGAGGA-3’ and Reverse 5’-GTGGGAAGTCTGCTGCAAAT-3’ and for 18S RNA (used as a control) were Forward 5’-AAACGGCTACCACATCCAAG-3’ and Reverse 5’-CCTCCAATGGATCCTCGTTA-3’. Quantification of relative expressions was calculated using the Livak formula.

2.6. Statistical analysis
Data are presented as the mean ± SD of triplicate experiments. Statistical tests were completed using an unpaired T-Test. Statistical significance uses a 95% confidence level.

3. Result and Discussion
Figure 1 shows Cygb expression in the siRNA (+) and siRNA (-) groups. Based on these results it was found that Cygb expression in the siRNA (+) group was significantly lower than the siRNA (-) group.

**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 = 0.000; Independent T-Test)
The results of the cell proliferation is shown in Figure 2. Although cell growth in the siRNA (+) group was slightly higher than the control, the difference was not statistically significant.

Figure 2. Comparison of cell proliferation rate in KFs between siRNA (+) Cygb and control group. There was a slightly increased in MTS absorbance level in siRNA (+) Cygb compared to the control group (p = 0.087; Independent T-Test)

Correlation analysis between Cygb expression and cell proliferation are shown in Figure 3. A negative correlation between Cygb expression and cell proliferation rates can be seen, although this was not statistically significant (p>0.05). These results indicate that the suppression of the Cygb gene tend to increase the proliferation of keloid fibroblast cells.

Figure 3. A correlation of Cygb mRNA and cell proliferation rate showing a weak negative correlation (Pearson, r = -0.454, p = 0.059)

The results of previous studies regarding the relationship of Cygb expression with cell proliferation have shown variable results. Some researchers report suppressor tumor activity from Cygb. In one study almost all cancer cells and tissues exhibited decreased expression of Cygb both in vitro and in vivo, accompanied by an increase in methylation of Cygb promoter [12]. Chen et al’s research on ovarian
cancer cells also showed similar results. They reported that there was an increase in cell proliferation in the group treated with the siRNA Cygb [7].

Another fact that supports the nature of Cygb as a tumor suppressor is the location of the gene; it is located on chromosome 17q25. A number of suppressor tumor genes are located on both arms of chromosome 17. For example, TP53, which is a tumor suppressor gene that often mutates in cancer is located at 17p13.1 [13]. A recent report from John et al reported that Cygb can affect the cell cycle through its involvement in the degradation of the Skp2 protein (which is highly oncogenic) [14].

Other studies on NE1 oesophageal keratinocytes and CCD-18 cocolonic myofibroblasts reported that there was no effect of Cygb gene suppression on cell growth [8]. In contrast Singh et al reported Cygb suppression on myoblast C2C12 cells which exhibited decreased cell viability and increased cell death [9].

From these findings, it can be said that Cygb has a dual role both as a tumor suppressor and oncogenes depending on cell type, tumor stage, micro-environmental conditions and availability of oxygen [10]. The function of the tumor suppressor is mainly found in the normoxic state, whereas the role of an oncogene is observed in a state of stress [15,16].

4. Conclusion

Based on the results, although there is an indication that the suppression of the Cygb gene tends to increase the proliferation of keloid fibroblast cells, these results were not statistically significant resulting in the conclusion that Cygb has no effect on fibroblast keloid cells proliferation.

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