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RESEARCH ARTICLE

THE EXPRESSION OF CYTOGLOBIN (Cygb) IN HYPOXIA FIBROSIS TISSUE WITH KELOID AS A MODEL

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ABSTRACT

Cygb is an O_2 carrier protein expressed by fibroblasts. In fibrosis, hypoxia occurs as characterized by stabilization of hypoxia-inducible factor-1α (HIF-1α), which later form the HIF-1, a transcription factor for the expression of adaptation proteins (including Cygb). The purpose of this study was to obtain information about Cygb role in fibrosis hypoxia using keloid tissue as a model. This was an observational descriptive study using keloid tissue samples, and the preputium as control. Measurement of Cygb and HIF-1α mRNA expression by real-time RT–PCR. Cygb and HIF-1α protein level (ELISA); while Cygb and HIF-1α protein expressions in the dermis layer by IHC. Data were analyzed statistically using unpaired t-test. In keloid, Cygb mRNA expression increased 8.7 times, compared to preputium, Cygb protein increased significantly (1.19 Vs 0.78 ng/mg protein and 95% Vs 63%, $P<0.05$). HIF-1α mRNA expression increased by 5.1 times, in keloid tissue, and protein HIF-1α increased significantly (0.20 Vs 0.12 ng/mg protein and 80% Vs 38%, $P<0.05$). There is a strong positive correlation between the expression of the HIF-1α and Cygb mRNA (Pearson; R = 0.899, P = 0.000).The expression of cytoglobin (Cygb) increased in hypoxia fibrosis tissue with keloid.

INTRODUCTION

Cytoglobin (Cygb) is a third member of extra-erythrocytes hemoglobin protein family, which was discovered after the discovery of myoglobin (Mb) and neuroglobin (Ngb) - the oxygen transporter proteins (Burmester*et al*. 2002; Semenza 2001; Tosqui *et al*. 2011). Cytoglobin is first identified by Kawada in 2001. It was discovered in hepatic myofibroblast; therefore, it was named‘stellate cell activated associated protein’ (STAP) (Kawada 2015). Stellate cells, which was activated into In 2002, Burmester identified Cygbafter an evaluation of gene bank and the universal Cygb was found in various tissues, unlikeother globin types such as Hb which can onlybe found in erythrocytes, Mb also can only operate in skeletal muscles and Ngb is only found in nervous tissues (Burmester 2002;Xu *et al*. 2006). The activated stellate cells experience changes into myofibroblast. In the form of myofibroblast, the cells would produce collagen, which may lead to fibrosis similar to the development of liver fibrosis (Kawada 2015; Kanitakis 2002; Cross *et al*. 2001). Fibrosis occurs as a result of activation of proliferated fibroblast as well as increased collagen production due to fibroblast growth factor (FGF) stimulation. Therefore, FGF is a marker of fibroblast capacity to produce collagen tissue (Vincent *et al*. 2008). In fibrosis, there is an increased demand for energy and O_2(Guo *et al*. 2007). On the other hand, fibroblast-like cells also synthesize Cygb and it is assumed that they may have the role in maintaining the availability of O_2 supply, which is essential in fibrosis as the process of fibrosis has increased metabolism for energy formation. The energy is necessary for proliferation or mytosis and also for hydroxylation of amino acids such as proline and lysine, which have roles in collagen synthesis. The increased oxygen demand indicates that there is an excessive activity of fibroblasts and there is a relative hypoxia condition (Cross *et al*. 2001; Guo *et al*. 2007). Hypoxia is a condition of imbalance between oxygen supply and tissue requirements. In hypoxia, a cell tries to survive by stabilizing a hypoxia inducible factor-1α (HIF-1α) protein which will be combined with HIF-1β to become HIF-1 (2). HIF-1α will activate genes needed for adaptation to fibrosis, one of which ensures the availability of O2 in cells. The Cygb protein levels increase in hypoxia, it has been proven that its synthesis is regulated by HIF-1α through binding of hypoxia response elements (HRE) in the Cygb gene promoter (Patel *et al*. 2007). Cygb can also be a tumor suppressor protein, if there is a decrease in Cygb expression regulation due to hypermetilation of the promoter. This results in decreased fibrosis in esophageal malignancies (tylosis) and squamous cell carcinoma of the head neck. in contrast, overexpression of

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Cygb can increase the sensitivity of glioma cells, which are initially resistant to radiation or chemotherapy (Xu *et al*. 2006). Based on the background above, it is necessary to know how the role of Cygb in cell/tissue hypoxia is actively synthesizing collagen through fibrogenesis. One source of collagen that is very much and still causes clinical problems is keloid. In keloids, there is proliferation of fibroblasts and excessive collagen synthesis (Cross *et al*. 2001). The slightest injury will be followed by an increase in the expression of collagen synthesis (Cross *et al*. 2001; Lei *et al*. 2011). Although keloids are indicated as benign tumors in a histapatological perspective. This keloid tissue grows rapidly resembling a malignant tumor, where the tissue grows excessively and is not coordinated (Cross *et al*. 2001;Park *et al*. 2011, Saed *et al*. 1998), even though the stimulus that causes it has stopped (Nagy 2011). So far, it is known that HIF-1α is detected in keloids that indicate hypoxia (Guo *et al*. 2007). However, there is no explanation about the Cygb expression. This study aims to obtain information about the role of Cygb in hypoxic tissue fibrosis using keloids as a model.

MATERIALS AND METHODS

Materials used in our study were: for RT-PCR technique: Cygb primer, HIF-1α, KAPA SYBR FAST one step qRT-PCR universal Kapa Bioseystems (KK4650), isolation RNA mini Kit (Geneaid Biotech. Ltd), Water-Biotechnology Gradesterilized-nuclease proteases and pyrogen Free (BUF-1180), β-mercaptoetanol; for histological technique, the materials were 10% formalin, 70%, 80% and 95% alcohol, xylol and paraffin block; Immunohistochemistry (IHC) technique,anti-Cygb primary antibody (anti-Cygb monoclonal mouse (Santa Cruz, Sc-365246) Santacruz monoclonal mouse, Anti HIF-1α primary antibody (anti-HIF-1α monoclonal mouse (Santa Cruz, Sc-53546) Santacruz monoclonal mouse, a secondary antibody novolink polymer labeled peroxidase enzyme (Leica biosystem) in the Novolink Min Polymer detection Detection-system Kit Immunohistochemistry (Novocastra (German / RE 7290-K); for and ELISA technique: Human hypoxia inducible factor-1 ELISA Kit Cusabio (CSB-E12112h), Human Cytoglobin ELISA Kit Cusabio (CSB-EL006376HU), Phosphate Saline Buffer 7.4.

Keloid tissues were obtained from keloid biopsy or excision procedure and preputium during circumcision as the control group. Keloid specimens were obtained from biopsy performed in 10 patients with keloid who visited several different hospitals. The patients with keloid participated in our study had given their written informed consent. Preputium tissues were obtained from 10 patients during mass circumcision. The Committee of Ethic, Faculty of MedicineUniversitas Indonesia–CiptoMangunkusumo Hospital approved the study.
The age types of keloid patients and preputium donors through circumcision are indeed different. Our reasons are: (1) The preputium tissue in a child's age is still proliferating according to age, so does keloid proliferation because it is pathologically considered a benign tumor. (2) We have difficulty getting normal skin and skin from dead bodies are difficult to obtain because it is related to ethics/medicolegal. (3) If we use normal skin tissue from a keloid patient, it is suspected that a new keloid will develop or the keloid will expand. (4) The preputium tissue post circumcision is wasted tissue. There is no information to date that keloids grow in the preputium tissue despite it has genetic keloids. For this reason, this study uses a preputium tissue as a normal control.

Experimental Design: We run an analytical descriptive observational studyin the Laboratory of Molecular Biology and Stress Oxidative, Department of Biochemistry and Molecular Biology, and Department of Histology, Faculty of Medicine, UniversitasIndonesia. Then keloid specimens taken following surgical removal and the preputium specimens taken from the procedure of circumcision were subjected to a study.

Isolation RNA and amplification by RT-PCR: RNA isolation from the observed tissues were carried out using RNA mini Kit (Geneaid Biotech. Ltd) and purified using DNase with the ratio of A260/A280 $\geq$1.7. We used MiniOpticon™, BioRad for synthesis of cDNA and PCR amplification; with KAPA® SYBR FAST one step qRT–PCR universal (KAPA Biosystems). Cygb and HIF–1α sequence was found in NCBI–Gene bank.The primer usedforCygb was: forward5'CAGTTCAAGCACATGGAGGA'3, reverse 5'GTGGGAAGTCACTGGCAAAT3', product 213bp. Primer forHIF–1α: forward 5'-GGAAGCGCAAGTCTTCAAAG-3',reverse5'-TGGGTAGGAGATGGAGATGC-3', product 187 bp. The primer usedfor 18S: forward5'-AGAAACGGCTACCACATCCA–3', reverse 5'-CCCTCCAATGGATCCTCGTT –3', product 258 bp. For this RT–PCR analysis purpose, RNA samples were diluted to 200 ng/uL, with the temperature melting curve (Tm) for Cygb of 84^{o} C, HIF–1α and 18S of 80^{o}C. Level of mRNA was measured in accordance to Livak method.

Level of Cygb and HIF-1α protein by ELISA: Level of Cygb and HIF–1α protein is measured using Cusabio® ELISA Kit. The specimens used were 30 mg homogenates tissues in 100 µL phosphate buffered salinepH of 7.4 in microplates coated with primary antibody, and HRP–avidin added. TMB–substrate transferred into the wells and the absorbance read using ELISA reader at 450 nm.

Histological and immunohistochemstry: Preparing histological slides and immunohistochemistry for detecting Cygb and HIF-1α. Keloid tissue was immersed in cold 0.9% NaCl and cut in 3-5 mm thickness. These specimens were then subjected to fixation, dehydration, clearing, and embedding. It was further cut into 4-5 µm thickness by using microtome. The available histological slides were ready for IHC to detect cells expression of Cygb and HIF-1α proteins. The following steps were performed: deparaffinization, dehydration, washing, a peroxidase blocking solution, washed, incubated by Cygb and HIF-1α primary antibodies (1:25 dilution) using mouse monoclonal anti-Cygb, Santa Cruz, Sc-365246) and anti HIF-1α (mouse monoclonal anti-HIF-1α, Santa Cruz, Sc-53546). The secondary antibody (novolink polymer), 3,3'-diaminobenzidine (DAB) solution and incubating them in hematoxylin solution were the added. The samples were subsequently rinsed, dehydrated and incubated in xylol. Entelan (Canada balsam) solution was added and mounted with cover glass (coverslip). The slides were ready for observation under the light microscope. The result was considered positive when there was the brown stain in the cytoplasm and nucleus (breast cancer tissues were used as positive control). The percentage cell of Cygb or HIF-1α protein expressions was calculated and subsequently compared to the number of total cells of the same high power field and multiplied by 100.

Statistical Analysis: The variables of expression of Cygb and HIF–1α mRNA (RT–PCR) and variables of Cygb and HIF–1α protein level (ELISA/IHC)were subjected to correlation analysis using SPSS ver.22.0. The data were analyzed unpaired t-test when the distribution in each group was normal and homogeneous, or Mann-Whitney test was performed when the distribution in one of the groups was abnormal. The difference was considered significant when the $p < 0.05$ with 95% confidence interval. To express the correlation between parameters, Pearson correlation test was performed.

RESULTS

Study on mRNA expression using RT-PCR we carried out the calculation of each sample in Duplo. Mean ± SD CT HIF–1α mRNA of preputium is ranged of 20.48–27.01 (24.28 ± 2.45) with ΔCt ranged of –0.28–6.26 (3.53 ± 2.45), whilst in keloid we found in ranged of 25.12–28.46 (25.43 ± 0.24) with ΔCt ranged of –2.65–28.46 (1.18 ± 0.24). The mean ± SD of ΔΔCt is 2.35 ± 0.23, thus in accordance to the Livak method of calculation, we found the expression on HIF–1α mRNA of keloid is 5.13 ± 0.82 times to preputium; (Unpaired t-test; P = 0.000) (table 1). Mean ± SD CT Cygb mRNA of preputium is ranged of 20.89–26.05 (22.78 ± 1.74) with ΔCt ranged of 1.41–6.47 (3.20 ± 1.69), whilst in keloid we found in ranged of 21.07–27.20 (24.25 ± 1.45) with ΔCt ranged of –0.13–2.29 (–0.11 ± 0.94). The mean ± SD of ΔΔCt is 2.98 ± 1.29, thus inaccordance to the Livak method of calculation, we found the expression on Cygb mRNA of keloid is 8.74 ± 3.25 times to preputium (Unpaired t-test; P = 0.000) (table 2). Expression of Cygb mRNA was found to be in accordance with the expression of HIF–1α mRNA (fig 1A). The correlation between protein level of HIF-1α with protein level of Cygb shows strong positive and significant correlation (R = 0.785; P = 0,000) (Fig 1B).

Assessment of protein level using ELISA showed that HIF–1α protein in the preputium was in ranged of 0.106–0.149 ng/mg (0.12 ± 0.01) and in keloid was in the range of 0.11–0.33 ng/mg (0.20 ± 0.07) (Unpaired t-test; P = 0.004). Cygb protein of preputium were in ranged of 0.57–1.01 ng/mg (0.78 ± 1.48) and keloid were in ranged of 0.61–2.06 ng/mg (1.19 ± 0.48) (Unpaired t-test; P = 0.048). We found elevated of Cygb protein was in accordance with HIF–1α protein (fig.1.A).
Immunohistochemistry showed both HIF–1α and Cygb protein were detected as nucleus and cytoplasma of the dermal layer cells both keloid and preputium cells (Fig 2A-D and 3A-D). To quantify the HIF–1α and Cygb expression, we calculated the percentage of total numbers of cells expressing HIF–1α and Cygb. The expression of HIF–1α and Cygb protein in demal layer of kelod tissue was significantly higher compared to those in preputium tissue (unpaired t-test; P= 0.004 and P= 0.001) (Fig 4E and 5E).

DISCUSSION

Through a study focused on keloid as a model of uncontrolled fibrosis tissue, we found that Cytoglobin has a positive correlation with on HIF–1α. In the protein level, such a correlation is significance (P = 0.018). This Study has been shown that in keloid hypoxia. There was a significantly higher HIF-1α expression starting at mRNA level, protein and cells expressing HIF-1α.
In keloid, high cell proliferation may produce a condition of hypoxia.There was a significantly higher HIF-1α expression starting at mRNA level, protein and cells expressing HIF-1α. In keloid, high cell proliferation may produce a condition of hypoxia. There was a higher HIF-1α expression. The absence of oxygen in hypoxia may lead to inhibition of prolyl hydroxylase (PHD), an enzyme that causes HIF-1α degradation. PHD activity requires some co-factors including 2-oxoglutarate, oxygen, and Fe^{2+} (Hodges *et al*. 2008). In hypoxia, there is an increase of reactive oxygen species (ROS) formation due to oxidative stress that accompanies hypoxia (Kendall 2014). The increased ROS formation can induce the synthesis of HIF-1α mRNA through signal transduction pathway. Since increased ROS formation due to hypoxia may induce HIF-1α mRNA and relative hypoxia condition causes stable HIF-1α, therefore, it can be understood that in hypoxia caused by excessive fibrosis, there is an increase of HIF-1α mRNA and protein.

Keloid is characterized by fibrosis in an uncontrolled manner. In this kind of fibrosis, progressive fibroblast proliferation is an essential issue of which unclear causal; predominated by collagen accumulation rather than its resorption (Syed *et al*. 2011;Shaw *et al*. 2009). With progressive fibroblast proliferation that displays bioenergetics of tumor cells (Dengler *et al*. 2014), the high energy requirement is a logic consequence followed by increased oxygen demand, lead to relative hypoxia (Singh *et al*. 2009;Emara *et al*. 2010). The hypoxia becomes vigorous with the increased of reactive oxygen species (ROS) released in during hypoxia (Dengler *et al*. 2014). Studies showed that hypoxia in uncontrolled fibrosis leads to increase of HIF–1α transcription (Kendall 2014;Liu *et al* 2013), which found to be stable with the increase of ROS (Ostojic *et al*. 2006). Furthers, HIF–1α controls the expression of Cygb mRNA in the existence of hypoxia–response element (HRE) in Cygb promotor gene (Chike-Obi *et al* 2009). A condition of hypoxia will be followed by Cygb mRNA transcription, and in turn by its protein synthesis (Dengler *et al*. 2014). This Cygb protein has an essential role to maintain oxygen availability in hypoxic condition induced by excessive fibroblast proliferation in keloid, which is energy and oxygen-demanding (Park *et al*. 2011, Mammen *et al*. 2006); and has not been found as an issue in the normal scar. However, this high oxygen–consumption with a low oxygen diffusion might have a great contribution to the etiopathogenesis of keloid (Shaw *et al*. 2009).

This study there was an increase also in keloid Cygb expression of mRNA and protein levels. There was a positive correlation between HIF-1α protein and Cygb mRNA. The expression of Cygb is controlled by HIF-1α based on the presence of Hypoxia-response element (HRE) in Cygb promotor gene (Ptel *et al*. 2010). That may lead to Cygb mRNA transcription, which is followed by protein synthesis (Singh *et al*. 2009). Cygb protein that had been synthesized has the essential role in O_2 supply, i.e. to overcome hypoxia condition that occurs due to excessive proliferation (Dengler *et al*. 2014; Emara *et al*. 2010). Increased O_2 needs occur due to high energy metabolism, which occurs in proliferating cells. The keloids demonstrate increased collagen and glycosaminoglycan content with whorls of thickened hyalinized collagen bundles. Keloid tissue has been shown to be more metabolically active and to use more oxygen than normal scar tissue.

Table 1. HIF–1α mRNA expression in preputium and keloid calculated using Livak method.

Samples		CT	Mean CT	CT18S	ΔACT	ΔΔCT	-ΔΔCT	$2^{-\Delta\Delta CT}$
	1	23.84 22.48	23.16	20.75	2.41			
	2	26.40 23.95	25.17	20.75	4.42			
	3	27.14 26.26	26.70	20.75	5.95			
	4	26.92 27.10	27.01	20.75	6.26			
	5	26.16 26.03	26.10	20.75	5.34	3.53	0	1
	6	22.25 20.85	21.55	20.75	0.80			
	7	24.54 24.58	24.56	20.75	3.81			
	8	26.63 26.63	26.63	20.75	5.88			
Preputium	9	20.46 20.49	20.48	20.75	-128			
	10	22.36 20.53	21.44	20.75	0.69			
		Mean±SD			3.53±2.45			
	1	25.27 25.55	25.41	24.24	1.16	-2.36	2.36	5.13
	2	25.83 25.10	25.47	24.24	1.23	-2.30	2.30	4.92
	3	28.88 25.64	25.76	24.24	1.52	-2.01	2.01	4.02
	4	25.23 25.28	25.26	24.24	1.01	-2.51	2.51	5.70
	5	25.26 25.74	25.74	24.24	1.50	-2.03	2.03	4.08
Keloid	6	25.44 25.90	25.67	24.24	1.43	-2.10	2.10	2.29
	7	25.52 24.73	25.13	24.24	0.88	-2.64	2.64	6.23
	8	28.11 28.81	28.46	24.24	4.22	0.69	0.69	5.618
	9	25.19 27.52	25.12	24.24	0.88	-2.65	2.65	6.28
	10	25.65 28.35	25.42	24.24	1.18	-2.35	2.35	5.09
				Mean±SD			2.35±0.24	5.14±0.83*

Ct:CycleThreshold in real time RT-PCR technique. Livak Method $^{\Delta C}$t (test) = ^{C}t (target,test) - ^{C}t (ref, test) $^{\Delta C}$t (calibrator) = ^{C}t (target,calibrator) - ^{C}t (ref, calibrator) $^{\Delta\Delta C}$t = $^{\Delta C}$t (test) - $^{\Delta C}$t (calibrator) $2^{-\Delta\Delta C}$t= normalizedexpression ratio Target = X gene (HIF-1α) Test = keloid tissue Calibrator = preputium tissue Ref = 18S gene unpaired t-test *p<0,05

Table 2. Expression of Cygb mRNA in preputium and keloid calculated using Livak method.

Samples		CT	Mean CT	CT18S	ΔACT	ΔΔCT	-ΔΔCT	$2^{-\Delta\Delta CT}$
	1	22.23 22.41	22.32	19.57	2.75			
	2	26.03 26.05	26.04	19.57	6.47			
	3	26.01 23.43	24.72	19.57	5.15			
	4	23.96 23.60	23.78	19.57	4.21			
	5	21.50 20.96	21.23	19.57	1.66	3.20	0	1
	6	21.19 20.89	21.04	19.57	1.47			
	7	23.69 21.94	22.81	19.57	3.24			
	8	23.3423.26	23.30	19.57	3.73			
Preputium	9	21.26 21.10	21.18	19.57	1.61			
	10	21.28 21.38	21.33	19.57	1.76			
	Mean±SD				3.20±1.70)			
	1	24.15 24.09	24.12	23.77	0.35	-2.85	2.85	7.21
	2	24.25 24.43	24.29	23.77	0.52	-2.68	2.68	6.45
	3	24.36 24.36	24.36	23.77	0.59	-2.61	2.61	6.105
	4	21.07 21.83	21.45	23.77	-2.32	-5.52	5.52	7.21
	5	23.47 26.10	24.78	23.77	1.01	-2.19	2.19	4.287
Keloid	6	23.34 23.49	23.41	23.77	-0.36	-3.56	3.56	11.784
	7	24.10 24.10	24.10	23.77	0.33	-2.87	2.87	7.311
	8	27.20 25.91	26.56	23.77	2.79	-0.41	0.41	13.361
	9	23.12 23.30	23.21	23.77	-0.56	-3.76	3.76	13.642
	10	23.14 24.14	23.64	23.77	-0.13	-3.33	3.33	10.056
				Mean(SD)			2.98±1.30	8.74±3.25*

Ct:CycleThreshold in real time RT-PCR technique. Livak Method $^{\Delta C}$t (test) = ^{C}t (target,test) - ^{C}t (ref, test) $^{\Delta C}$t (calibrator) = ^{C}t (target,calibrator) - ^{C}t (ref, calibrator) $^{\Delta\Delta C}$t = $^{\Delta C}$t (test) - $^{\Delta C}$t (calibrator) $2^{-\Delta\Delta C}$t= normalizedexpression ratio Target = X gene (Cygb) Test = keloid tissue Calibrator = preputium tissue Ref = 18S gene unpaired t-test *p<0,05

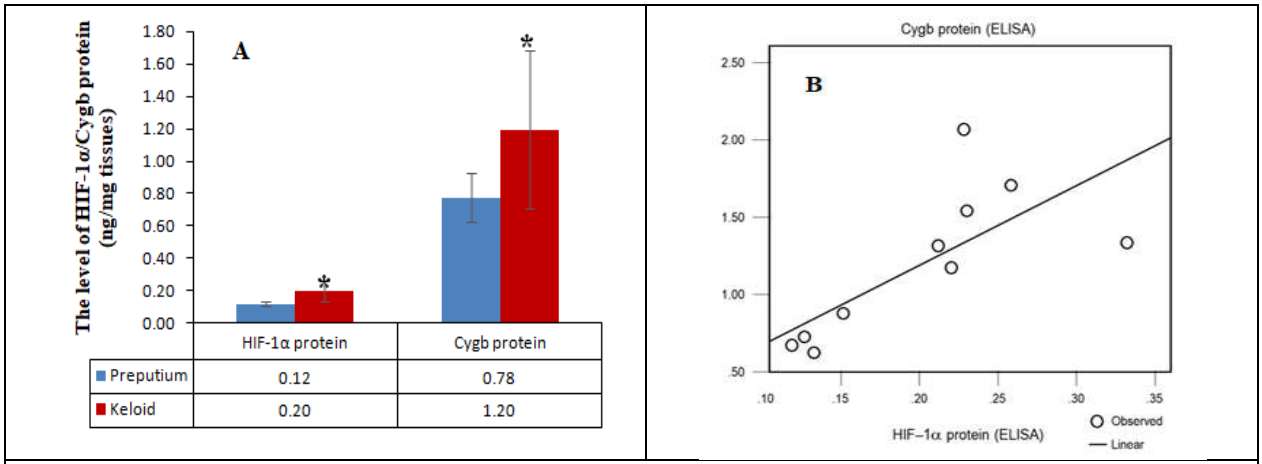
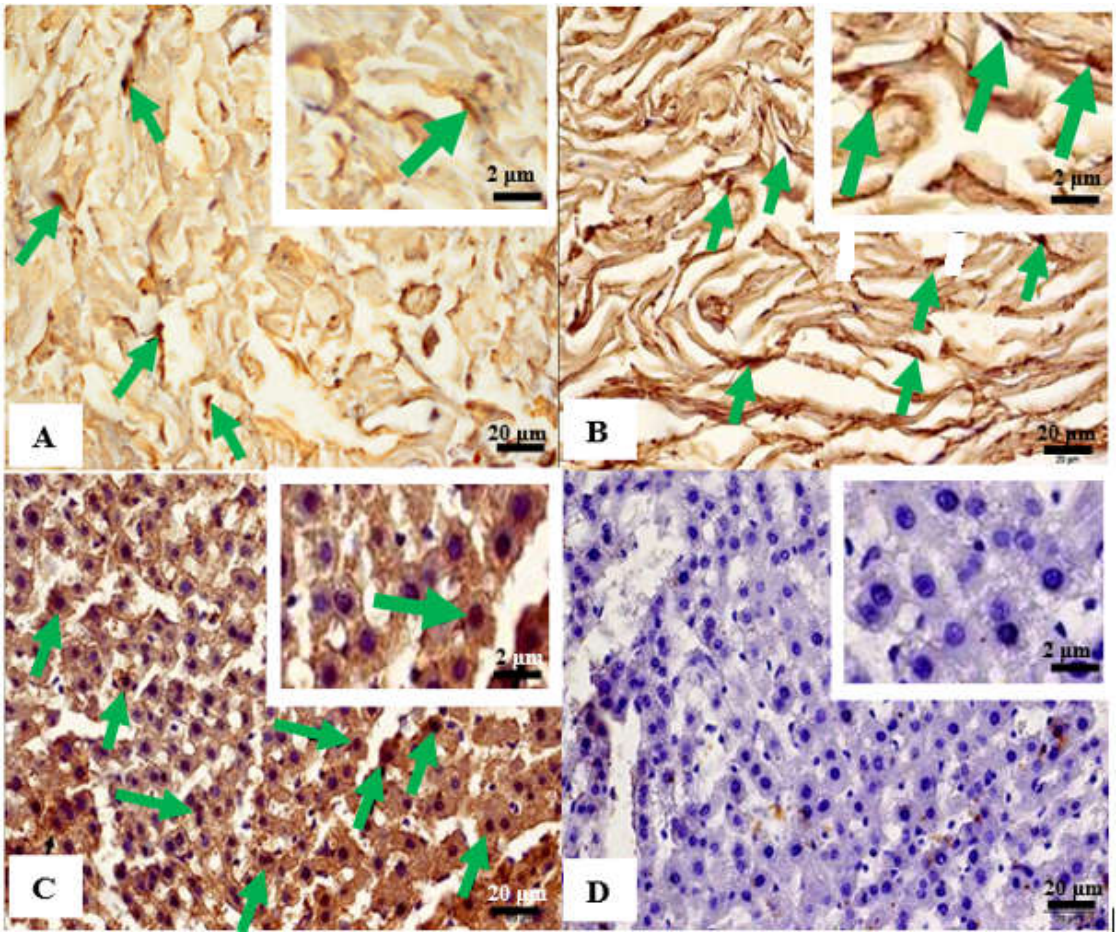


Figure1.(A) The elevated of HIF–1α protein and Cygb protein in keloid assesed using ELISA (unpaired t-test *p<0,05). (B) The correlation between protein level of HIF-1α with Cygb in keloid(Pearsoncorrelation, R=0.785; P = 0.000).



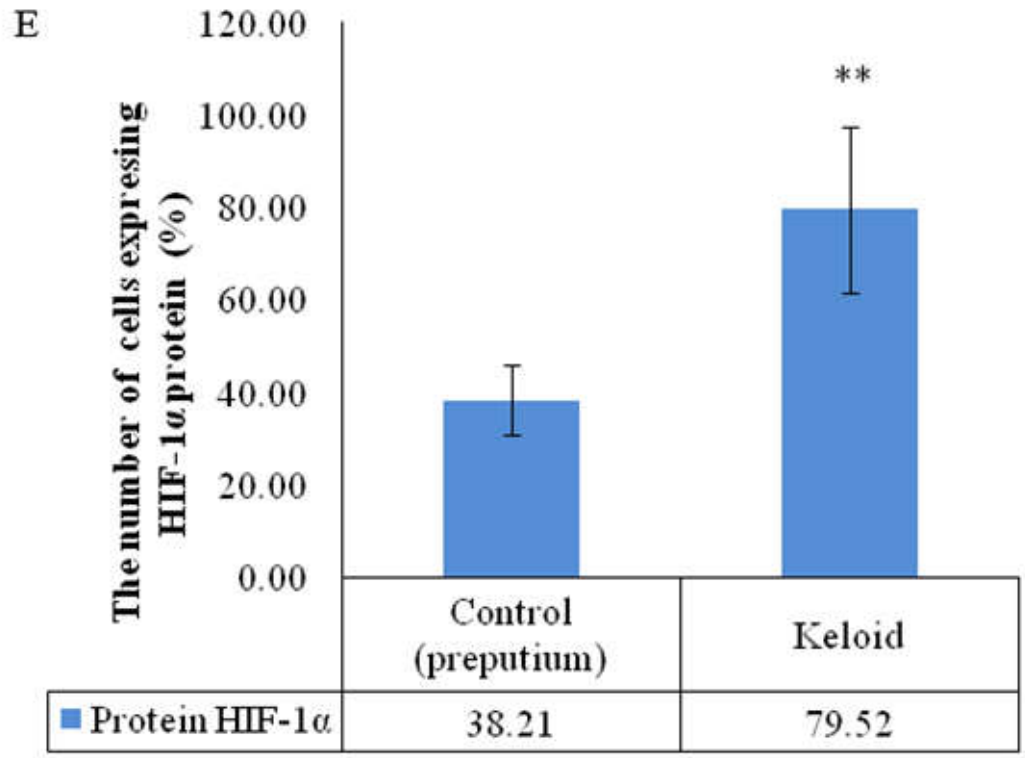
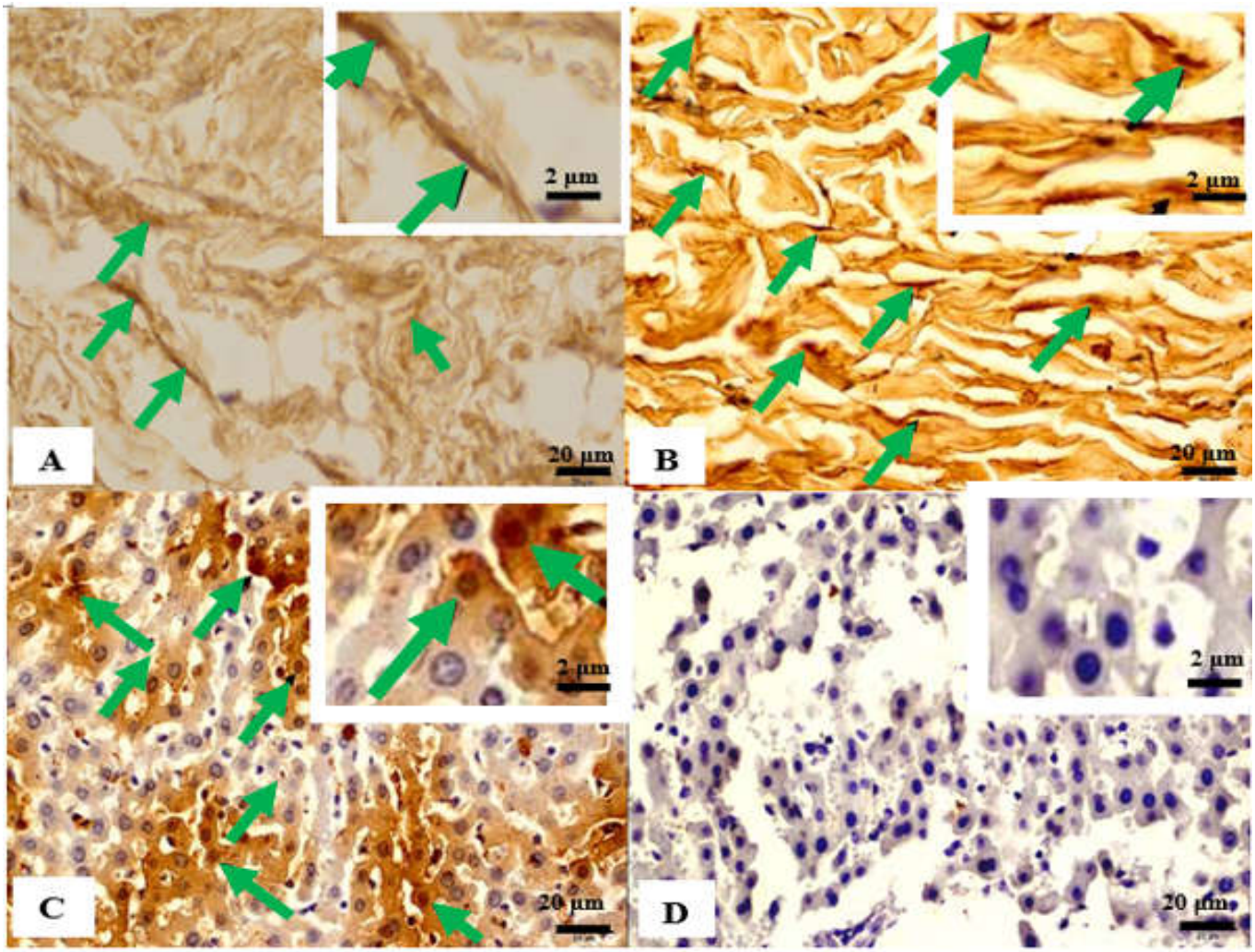


Figure 2. The expression of HIF-1α protein in the cells of dermal layer was found both in the nucleus and cytoplasm characterized by a brown staining(arrow, magnification/bar = 400 times/20 µm; inserts 1000 times/2 µm). (A)Expression of HIF-1α in demal layer cells of preputium tissue; (B) Expression of HIF-1α in demal layer cells of keloid tissue (C) Expression of HIF-1α in positive control (breast cancer tissue), (D) negative control. (E) A graph show percentage ratio of the number of cells expressing HIF-1α protein in dermal layer of keloid and preputium tissues using immunohistochemistry and it shows significant difference (unpaired t-test; **P= 0.004).



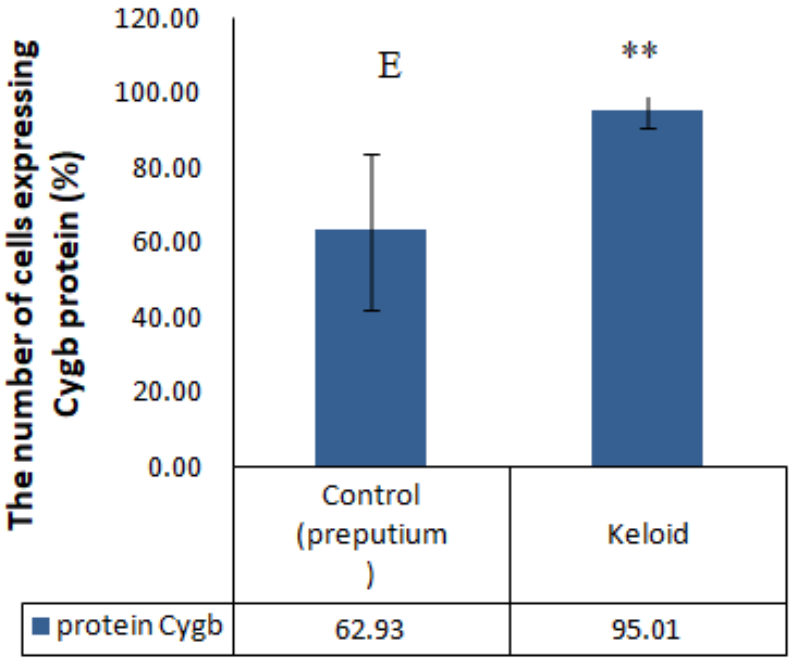


Figure 3. The expression of Cygb protein in the cells of dermal layer was found both in the nucleus and cytoplasm characterized by a brown staining(arrow, magnification/bar = 400 times/20 µm; inserts 1000 times/2 µm). (A)Expression of Cygb in demal layer cells of preputium tissue; (B) Expression of Cygb in demal layer cells of keloid tissue (C) Expression of Cygb in positive control (breast cancer tissue), (D) negative control. (E) A graph show percentage ratio of the number of cells expressing Cygb protein in dermal layer of keloid and preputium tissues using immunohistochemistry and it shows significant difference (unpaired t-test = ** *P*= 0.001).

This high oxygen-consuming potential and lowoxygen diffusion may contribute to the pathophysiology of keloid formation (Mammen *et al*. 2006). The increased of collagen expression followed by an increased for nutrients and oxygen need (Park *et al*. 2011). Energy needs mainly obtained through the process of glycolysis in the cytoplasm, which then continues with the process of oxidative phosphorylation in the mitochondria. The enzyme activity of glycolysis and oxidative phosphorylation in keloid fibroblasts showed an increased reaction. Glucose consumption, as well as the amount of lactate and ATP formed in keloid tissue, was higher than that in normal cells. Cell membrane permeability increased too, thus simplifying the entry of substrate and oxygen into the mitochondria for ATP synthesis (Guo *et al*. 2007). The increased expression of collagen can also cause by an increase in the hydroxylation of proline residues in collagen molecules in order to form a triple helix. Hydroxylation process is catalyzed by the enzyme prolyl-4-hydroxylase (P-4-HD) that the activity requires oxygen. Oxygen is required for the hydroxylation of proline and lysine to hydroxyproline and hydroxylysine, which in turnform the triple helix in the maturation of collagen, mainly in collagen type I and III (Park *et al*. 2011, Shaw *et al*. 2009). Oxygen also plays a role in triggering the differentiation of fibroblasts into myofibroblasts, to be deposed collagen appropriately. Therefore it can be said that the production of collagen is proportional to the oxygen pressure (16). Therefore, because the need for oxygen is increased, causing the cells are in a state of hypoxia, thus activating HIF-1α in keloid fibroblasts tissue (Shaw *et al*. 2009).

The expression of Cygb is also increased in overgrowth of glioblastoma cells. This mechanism explains cancer cell survival in hypoxic environments (Kendall 2014). Increased expression of Cygb also occurs in fibroblast cells, liver, heart, intestines, kidney, lungs and pancreas. The Cygb is increased as long as the fibroblast has active proliferation[5]. Cygb synthesis is obviously increased in keloid due to fibrosis. In our study, we found that there was a higher expression of Cygb mRNA (table 2), a higher Cygb protein level (figure.1.A) and the greater amount of cells expressing Cygb protein (figure.2.II) in keloid group compared to the preputium group. The high Cygb expression was obvious starting from transcription phase to protein synthesis. It has been known that fibrosis in keloid will be followed by increased needs of oxygen, which is necessary for energy metabolism through the mechanism of the respiratory chain in mitochondria (Dengler *et al*. 2014). It is assumed that one of the Cygb functions is to bind and fulfill the needs of O_2 in the cells, which is essential to assure adequate O_2 supply for mitosis that requires a lot of energy. The function of Cygb to fulfill the needs of adequate oxygen is also associated with fibroblast activity that requires oxygen for proline hydroxylation in collagen maturation, which also needs O_2 (Ostojic *et al* 2006). The function of Cygb in oxygen supply is demonstrated by the high expression of Cygb in retina, brain and peripheral tissues (Chike-Obi *et al*. 2009), i.e. those organs that cannot produce collagen. The function of Cygb in those tissues, which are vulnerable to hypoxia , it is to ensure the availability of O_2 like the function of other types of globin protein (Hb, Mgb, Ngb) (Dengler *et al*. 2014; Emara *et al*. 2010; Liu *et al*. 2013). Thus, it is now clearly understood that a positive correlation between Cygb and HIF–1α in a protein level found in ELISA to be significant rather than its expression (mRNA) is due to the fibrosis progressivity in keloid.

These findings also in accordance to studies referring Cygb might not be differentiated to myofibroblast-like phenotype leading to reduced extracellular matrix production (Dengler *et al*. 2014); and tumor suppressor gene (Syed *et al* 2011; Singh *et al* 2009), in the course of progressive fibroblast proliferation. However, in keloid as the progressive proliferation of fibroblast take place as in tumor we found increased Cygb in accordance with HIF–1α, but not downregulated.

Conclusion

Expression of Cytoglobin have the important role in hypoxic fibrosis tissue characterized by increased expressions (with keloid as a model). The expression of Cytoglobin is associated with stable HIF-1α.

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Glossary of abbreviation

Cygb - Cytoglobin
Mb - Myoglobin
Ngb - Neuroglobin
STAP - StellateCell ActivatedAssociatedProtein
FGF - FibroblastGrowth Factor
HIF-1α - hypoxia inducible factor-1α
HRE - hypoxia response elements
IHC - Immunohistochemistry
P4HD - Prolyl-4-hydroxylase

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