Expressions of EGF, FGF and VEGF In Hypoxic Fibrosis Tissue with keloid as a model
DAFTAR ISI

Pengaruh HPMC Terhadap Sifat Fisik Sedlaan Masker Peel-off Ekstrak Etanol Kulit Buah Manggis (Garcinia Mangostana L.)
Nelly Suryani, Ofa Suzanti Betha, Myra Kharisma Izzati ................................................. 101-110

Faktor Risiko Lingkungan Terhadap Angka Kesakitan Malaria di Kota Lubuk Linggau, Sumatera Selatan
Tri Bayu Purnama, Ratri Ciptaningtyas, Riaistuti Kusuma Wardani ........................................ 111-118

Expressions Of Egf, Fgf And Vegf In Hipoxic Fibrosis Tissue With Keloid As A Model
Endah Wulandari, Sri Widia A Jusman, Yefita Moenadjat, Ahmad A Jusuf, Mohamad Sadikin ......... 119-130

Pengaruh Membaca Sholawat Wahidiyah Terhadap Tingkat Kecemasan pada Pengguna Narkoba
Atik Mardiani Kholidah, Uswatun Khasanah, Ratna Pelawati .................................................. 131-144

Upaya Berhenti Merokok Mahasiswa Universitas Islam Negeri Syarif Hidayatullah Jakarta
Dela Aristi, Soekidjo Notoatmodjo ......................................................................................... 145-154

Pencegahan dan Pengendalian Infeksi yang disebabkan oleh Bakteri Multiresisten MRSA
Enike A. Suwarsono .................................................................................................................. 155-168

Aktivitas Antihyperglykemik Ekstrak Etanol Daun Angelica Keiskei Pada Tikus Galur Sprague Dawley
Faris Mohammad Hadiningrat, Yardi, Ahmad Musir .................................................................... 169-176

Gambaran Keblasaan Merokok Kuadra pada Kejadian ISPA Balita di Puskesmas Bungah Gresik
Lillis Zuhriyah, Jamaludin, Yenita Agus .............................................................................. 177-184

Penampilan Diagnostik Rapid Manual Test Metode Immunochromatography Pada Malaria
Jono Ulomo ..................................................................................................................................... 185-192

Nutrisi Pada Anak Sakit Kritis Post Operasi
Kustati Budi Lestari ................................................................................................................... 193-206

Medika Islamika Vol. 14 No. 2 Nov 2017 | iii
EXPRESSIONS OF EGF, FGF AND VEGF IN HIPPOXIC FIBROSIS
TISSUE WITH KELOID AS A MODEL

Endah Wulandari 1, Sri Widia A Jusman 1, Yelta Moenadjat 1, Ahmad A Jusuf 4, Mohamad Sadikin 1

1Department of Biochemistry, Faculty of Medicine and Health Sciences of State Islamic University Syarif Hidayatullah, Indonesia
2Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Indonesia
3Department of Surgery, Faculty of Medicine, University of Indonesia, dr. Cipto Mangunkusumo Hospital
4Department of Histology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia

Fibrosis, sebagai hasil mutasi meningkatkan proliferasi, migrasi fibroblas, dan diikuti oleh sintesis kolagen yang berlebihan. Pertumbuhan jaringan yang tidak terkontrol tidak diimbangi dengan vaskularisasi jaringan yang memadai. Pembuluh darah yang ada menjadi tidak mencukupi, sehingga fibrogenesis berlebihan diduga disebabkan oleh hipoksia yang terus menerus, seperti pembentukan tumor. Pada hipoksia, HIF-1a menjadi stabil, faktor transkripsi yang mengatur target gen berbagai proses seluler, seperti faktor pertumbuhan jaringan, eritroposis, metabolisme, angiogenesis, vaskularisasi, siklus sel, dan supresor apoptosis. Keloid adalah salah satu kasus fibrosis, karena pertumbuhan jaringan yang berlebihan yang tidak sesuai dengan tingkat keparahan trauma dan masih terus menjadi masalah klinis. Diasumsikan bahwa fibrogenesis yang berlebihan pada jaringan keloid disebabkan oleh hipoksia yang dihasilkan pada HIF-1a yang stabil, menyebabkan rangsangan lebih lanjut faktor transkripsi yang mengatur ekspresi protein faktor pertumbuhan seperti EGF, FGF, dan VEGF. Hasilnya ditunjukkan pada keloid: Ekspresi EGF lebih rendah di sel epidermis; Ekspresi FGF lebih tinggi pada sel-sel dermis; Ekspresi protein VEGF pada sel endotel lebih tinggi daripada preputium (kontrol), secara keseluruhan terdapat perbedaan yang signifikan (tidak berpasangan-t, p <0,05). Ekspresi HIF-1a berkorelasi kuat dengan sepertiga protein faktor pertumbuhan. Dalam penelitian ini menunjukkan ekspresi EGF, FGF dan VEGF yang berhubungan dengan hipoksia pada keloid fibrosis.

Kata Kunci: fibrosis, HIF-1a, EGF, FGF, VEGF

Abstract
Fibrosis, the result of mutations increased proliferation, migration of fibroblasts, followed by excessive collagen synthesis. Uncontrolled tissue growth is not matched by adequate tissue vascularization. Existing blood vessels become insufficient, so that excessive fibrogenesis allegedly caused by the continuous hypoxia, such as the formation of tumors. In hypoxia, HIF-1a becomes stable, a transcription factor that regulates gene target of various cellular processes, such as tissue growth factor, eritroposis, metabolism, angiogenesis, vascularization, cell cycle, and apoptosis suppressor. Keloid is one of the fibrosis cases, due to the excessive tissue growth which is not in accordance with the severity of trauma and it still continues to be a clinical problem. It is assumed that the excessive fibrogenesis in keloid tissue is the caused by the resulting hypoxia in stable HIF-1a, the which leads to further stimulation of transcription factors that regulate the expression of growth factor proteins such as EGF, FGF and VEGF. The results showed in the keloid; EGF expression lower in the cells of epidermis; FGF expression higher in the cells of the dermis; the expression of VEGF protein in endothelial cells is higher than the preputium (control), the overall was significantly different (unpaired-t, p <0,05). HIF-1a expression correlates strongly with a third of the growth factor protein. In these studies show the expression of EGF, FGF and VEGF that related to hypoxia in keloid fibrosis.

Key words: fibrosis, HIF-1a, EGF, FGF, VEGF

Medika Islamika Vol. 14 No. 2 Nov 2017 | 119
Expressions of EGF, FGF and VEGF In Hipoxic Fibrosis Tissue with keloid as a model

Endah Wulandari 1, Sri Widia A Jusman2, Yefta Moenadjat 3, Ahmad A Jusuf 4, Mohamad Sadikin2

1Department of Biochemistry, Faculty of Medicine and Health Sciences of State Islamic University Syarif Hidayatullah, Indonesia. 2Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Indonesia, 3Department of Surgery, Faculty of Medicine, University of Indonesia, dr. Cipto Mangunkusumo Hospital, 4Department of Histology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia.

Fibrosis, the result of mutations increased proliferation, migration of fibroblasts, followed by excessive collagen synthesis. Uncontrolled tissue growth is not matched by adequate tissue vascularization. Existing blood vessels become insufficient, so that excessive fibrogenesis allegedly caused by the continuous hypoxia, such as the formation of tumors. In hypoxia, HIF-1α becomes stable, a transcription factor that regulates gene target of various cellular processes, such as tissue growth factor, eritropoisis, metabolism, angiogenesis, vascularization, cell cycle, and apoptosis suppressor. Keloid is one of the fibrosis cases, due to the excessive tissue growth which is not in accordance with the severity of trauma and it still continues to be a clinical problem. It is assumed that the excessive fibrogenesis in keloid tissue is the caused by the resulting hypoxia in stable HIF-1α, the which leads to further stimulation of transcription factors that regulate the expression of growth factor proteins such as EGF, FGF and VEGF. The results showed in the keloid: EGF expression lower in the cells of epidermis; FGF expression higher in the cells of the dermis; the expression of VEGF protein in endothelial cells is higher than the preputium (control), the overall was significantly different (unpaired-t, \( p <0.05 \)). HIF-1α expression correlates strongly with a third of the growth factor protein. In these studies show the expression of EGF, FGF and VEGF that related to hypoxia in keloid fibrosis.

Key words: fibrosis, HIF-1α, EGF, FGF, VEGF

I. Introduction

Fibrosis is a pathological condition in which the accumulation of extracellular matrix happened as a result of mutations or damage sel (Chatziantoniou, 2005). Fibrosis is the main cause of many chronic diseases. If fibrosis is progressive fibrosis. It can cause cirrhosis such as cirrhosis of the liver. Fibrosis is a very complex process, involving fibroblast cells as primary cells, leukocytes, various cytokines, growth factors and proteinase inhibitors, reaction oxygen species (ROS), inhibition of apoptosis, and various types of kolagen (Bataller et al. 2007). The active fibroblasts will proliferate and turn into miofibroblas cells that produce collagen type I, III, and IV (Ramadori et al. 2004).

At fibrosis, activation of fibroblasts stimulated by fibroblast growth factor (FGF), which is proliferated to form a matrix extracelluler (Cross and Claesson-Welsh, 2001). The uncontrolled growth of tissue at the fibrosis is not matched by adequate tissue vascularization. Blood vessels become insufficient. For the proliferation or mitosis process, then the fibrosis increased it’s demand for energy and O\(_2\) (Vincent et al., 2008). On the fibrosis, excessive collagen synthesis takes place. Collagen maturation increases the activity prolil-4-hydroxylase and lisilhidroksilase which require high O\(_2\), for the amino acid proline hydroxylation and lisin (Smith et al. 2008). Hence the high energy requirements and O\(_2\) while the oxygen supply is not balanced, then the tissue fibrosis is generally encountered with hypoxia (Halberg et al.., 2009).

In hypoxic cells attempt to survive through the induction of protein stabilization of hypoxia inducible factor-1α (HIF-1α) in the cytosol which then will join with HIF-1β
in the nucleus becomes HIF-1 (Semensa, 2001). HIF-1 will further act as a transcription factor and activates a variety of genes that required for adaptation to hypoxia. Because of the difficulty of getting fibrosis in organ tissue, the research was conducted in keloid as a model. It is known that in keloid fibroblast proliferation and collagen synthesis are progressive (especially collagen I and III), as well as there are lot of source of collagen and it’s still cause clinical problem (Smith et al. 2008). A little wound will be followed by an increased expression of synthesis kolagen (Patel et al., 2010).

Keloid is a benign tumor with similar features to malignant tumor as it has excessive growth inconsistent with the severity of trauma. In keloid, there is imbalanced synthesis and degradation of extracellular matrix during wound healing process, which results in uncontrolled fibrogenesis (Lei et al. 2011; Park et al. 2011). In some individuals, small wounds such as parenteral injections or body piercings may cause increased expression of collagen synthesis (Patel et al. 2010; Smith et al. 2008). Until now, there is no theory explaining why there is excessive fibrogenesis in keloid tissues.

Furthermore, there is no effective therapy and treatment for keloid. Moreover, the biochemical mechanism and pathogenesis of keloid remain vague (Vincent et al. 2008). Keloid is often recurrent although it has been treated, either with pharmacological agents or surger (Sabiston, 2002). The probability of recurrent keloid following the surgery may reach 80-100% (Park dkk, 2011). Various methods of treatment modalities applied for management of keloid have not shown any effectiveness and recurrent cases are not uncommon, which are considered by most people, particularly by the patients as a form of therapeutical failure. It indicates that there is no clarity on the pathogenesis of keloid.

In keloid, there is increased needs of energy and O\(_2\) for the sake of cellular proliferation and excessive collagen synthesis; however, it is not followed by adequate O\(_2\) supply (Lei et al. 2011). Therefore, in keloid, hypoxia inducible factor-1α (HIF-1α) can be detected, which indicates that the tissue has experienced hypoxia (Vincent et al. 2008). Nagy (2011) demonstrates that if there is stable HIF-1α in hypoxia, it will subsequently further activate transcription of some target genes modulating various cellular process for adaptation including growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF).

Based on the aforementioned background, the aim of our study was to obtain information expressions of EGF, FGF and VEGF in hypoxic fibrosis tissues with keloid as a model.

II. Materials And Methods

Materials or reagents used in our study were: for histology technique, the materials were 10% formalin, 70%, 80% and 95% alcohol, xylol and paraffin block; for immunohistochemistry, the materials were EGFR monoclonal primary antibody (Bio SB USA, BSB 5473), anti-FGF antibody (rabbit polyclonal anti-FGF / Abcam USA, Ab 71928), anti-VEGF monoclonal antibody (Bio SB, BSB 6053), Immunohistochemistry Kit (Novolink Min Polymer detectionsystem, German/RE 7290-K); and the materials for ELISA technique were Human hypoxia inducible factor-1 ELISA Kit Cusabio (CSB-E12112h), PBS 7.4.

The type of this study was an analytical descriptive observational. Keloid tissues were obtained from biopsy or excision procedure and preputium tissues were obtained through circumcision as the control group. Keloid specimens were obtained from biopsy performed in 10 patients with keloid who visited several hospitals in Jakarta (Indonesia). The patients with keloid participated in our study had given their written informed consent. The study had been approved by the Medical and Health Research Ethic Committee, Faculty of Medicine, University of Indonesia. The study was conducted in Faculty of Medicine, University of Indonesia. The evaluation of HIF-1α protein level using ELISA was performed at the Laboratory of Molecular Biology, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Indonesia.
Indonesia; while the evaluation of EGF, FGF and VEGF protein expression using immunohistochemistry was performed at Department of Histology, Faculty of Medicine, University of Indonesia.

Preparing Histological Slides/Sections: keloid fibroblast tissue obtained from biopsy was immersed in cold 0.9% NaCl; then, it was cut in 3-5 mm thickness. Furthermore, a fixation was performed by transferring it into 10% formalin solution. Next, dehydration was performed by immersing the specimen in 70% alcohol incubated for 24 hours, 80% alcohol incubated for 24 hours, 95% alcohol incubated for 24 hours, 100% alcohol twice in 24 hours (in 12 hours, the 100% alcohol was removed). Afterward, clearing was performed by immersing the specimen in xylol twice in 24 hours (in 12 hours, the xylol was removed). Then, the embedding was performed, i.e. by infiltrating the specimen with liquid paraffin.

After the tissue specimen was ready, it was cut into sections with microtome in 4-5 µm thickness. The results of sections were then taken using a brush and were transferred to a water bath so that they were allowed to widen 40-46°C. At this point, the sections were trimmed and transferred onto a slide that had been smeared with eiwit (egg white and glycerin), which served as an adhesive. The slide and tissue specimen on it were set in a special shelf and transferred into incubator at 40-60°C for 24 hours or until the slide was ready for staining.

Immunohistochemistry for detecting EGF, FGF and VEGF proteins. To have a good knowledge on distribution and location of those growth factors, an observation on their expressions at different locations in the tissues through immunohistochemistry should be performed. Immunohistochemistry techniques can be used to diagnose type of cancer and may provide some assistance in predicting prognosis. It also can be used for cellular identification by having it for marking with a protein marker. Method of analysis and identification are performed using specific antigen-antibody binding within the cell. Since the reaction of the antigen-antibody binding can not be seen directly, a visualization is essential. The visualization of antigen-antibody reaction is performed by binding of antibody conjugates, e.g. by using alkaline phosphatase or horseradish peroxidase enzymes. The enzymes then catalyze reactions resulting color that can be evaluated using qualitative and quantitative analysis (Painter et al, 2010).

The available histological slides were ready for immunohistochemistry to detect cells expressing EGF, FGF and VEGF proteins. The steps were: deparaffinization by immersing the specimen in xylol for 5 minutes; dehydration by immersing the specimen in a serial of alcohol concentration of 100%, 95%, 90%, 80%, 75% for 5 minutes for each concentration and aquadest for 5 minutes. The next step was washing using PBS (pH 4) for 5 minutes. Then, a peroxidase blocking solution (0.3% H₂O₂ in methanol) was added for 15 minutes in order to inhibit peroxidase activity. The specimen was subsequently washed with PBS (2 x 5 minutes). A serum non-immune protein blocking solution was added for 15 minutes. Then, it was incubated by adding EGF, FGF, and VEGF primary antibodies for each specimen with 1:25 dilution in PBS. The specimens were washed using PBS (2 x 5 minutes). Incubation was continued by adding secondary antibody (novolink polymer) to bind biotin for 1 hour. The specimens were washed again with PBS (2 x 5 minutes). Several drops of 3,3’-diaminobenzidine (DAB) solution were added to the specimen and it was rinsed with water quickly. The specimens were then counterstained by incubating them in hematoxylin solution for 15 minutes. The specimens were rinsed again with water quickly. Next, the specimens were counterstained by incubating them in hematoxylin solution for 15 minutes. Afterward, dehydration was performed using increasing concentration of alcohol, i.e. 70%, 96% and 100% for 5 minutes each and the specimens were incubated in xylol for 2 x 2 minutes. Entelan (Canada balsam) solution was added and the specimens were
mounted with cover glass (coverslip), which was subsequently being sat in room temperature until dried. Furthermore, the slides were ready for observation under light microscope with 400x magnification. Later, quantification of cells expressing EGF, FGF and VEGF proteins was performed in 5 power fields. The result was considered positive when there was brown stain in the cytoplasm or nucleus.

The density of cells expressing protein, EGF, FGF and VEGF in keloid and preputium tissue was observed in dermal layer. Immunohistochemistry staining was performed using software Image J program; each cell that had been counted was marked (stained by the program) in order to prevent recounting. Each counting was performed by 2 different observers on the same slide and they were assisted with counting tool.

Cell quantification was performed in 5 high power field (HPF) for each slide of keloid or preputium tissue. High power field was determined as 40x magnification of dermal layer, which included: upper, lower, central, left, and right margins. Furthermore, the HPF was altered to 400x magnification to quantify the cells. The percentage of EGF, FGF and VEGF protein expressions was calculated and subsequently compared to the amount of total cells of the same power field and multiplied by 100.

ELISA technique for detecting the level of HIF-1α protein. The level of HIF-1α protein was measured using ELISA Kit Cusabio. The specimens used were 30 mg homogenates of keloid and preputium tissues in 100 µL phosphate buffered Saline (PBS) pH 7.4. The steps were as follows: the method was optimized by performing antigen titration through dilution of standard protein. The standard concentrations were 0; 0.0625; 0.125; 0.25; 0.5 (ng/mL) for quantifying protein and HIF-1α level (appendix 2 and 3). After the standard had been made, a microplate was prepared, which had been coated with primary antibody. About 100 µL of each specimen and standard were transferred to microplate well and subsequently were incubated for 2 hours at 37°C.

Following the incubation, the supernatant was discarded and about 100 µL Biotin antibody was added and then it was incubated for 1 hour at 37°C. The supernatant was discarded and the well was rinsed 3 times with Wash Buffer. About 100 µL HRP-avidin was added and it was then incubated for 1 hour at 37°C. The supernatant was discarded and the well was rinsed 5 times with Wash Buffer. About 90 µL TMB-substrate was transferred into the well. The specimens was then removed to a dark room and incubated for 15-30 minutes at 37°C. Furthermore, 50 µL stop solution was added and a color yielded, in which the absorbance could be read using ELISA reader at 450 nm wavelength.

III. Results

Results on immunohistochemistry assessment were evaluated to observe EGF, FGF and VEGF protein expression in keloid and preputium tissue. EGF was expressed in basal layer of epidermal cells; while FGF was expressed in the dermal layer and VEGF was expressed in vascular endothelial cells (shown as brown staining in nucleus and cytoplasm by using immunohistochemistry as seen in figure 1). The observation showed that EGF expression in epidermal layer was significantly lower than those in preputium tissue (figure 2A. unpaired t-test, p<0.05). Moreover, FGF expression in dermal layer was found higher in keloid compared to preputium tissue (figure 2B; unpaired t-test; p<0.05). VEGF expression of endothelial cells in keloid tissue was higher than those in preputium (figure 2C; unpaired t-test, p<0.05).

The level of HIF-1α protein is presented in figure 4.3. The level of HIF-1α is also seen significantly higher in keloid than preputium tissue (unpaired t-test; p<0.05; figure 3.B).

The correlation between HIF-1α and EGF, FGF and VEGF cytokines can be seen in table 4.3. There was a moderately positive significant correlation between HIF-1α protein and FGF as well as VEGF (R=0.540 and 0.537; both with p<0.05). There was a moderately negative significant correlation between HIF-1α protein and EGF (R= -0.529; p<0.05)
Figure 1. Expressions of growth factor proteins using immunohistochemistry technique. The expressions were found both in nucleus and cytoplasm characterized by brown staining. (A) with 1000 times magnification, the expression of EGF can be seen (arrow) in the cells of epidermal layer of preputium tissue; (B) with 1000 times magnification, EGF expression (arrow) can be seen in the cells of epidermal layer of keloid tissue; (C) with 1000 times magnification, the FGF expression (arrow) can be seen in the cells of dermal layer of preputium tissue; (D) with 1000 times magnification, FGF expression (arrow) in the cells of dermal layer of keloid tissue can be seen; (E) with 1000 times magnification, VFGF expression (arrow) in endothelial cells adjacent to preputium blood vessel can be seen; (F) with 1000 times magnification, The expression of VFGF (arrow) can be seen in endothelial cells of keloid blood vessels.
Figure 2. A graph shows a ratio of growth factors between keloid and preputium tissue using immunohistochemistry technique. (A) Ratio of EGF protein expression in the cells of epidermal layer between keloid and preputium tissue shows that they were not significantly different (*p<0.05). (B) Ratio of FGF protein expression in the cells of dermal layer between keloid and preputium tissue shows a significant difference (*p<0.05). (C) A ratio of VEGF protein expression in the cell of epidermal layer between keloid and preputium tissue shows that they were not significantly different (*p<0.05).
Table 1. Correlation between the level of HIF-1α protein and EGF, FGF as well as VEGF

<table>
<thead>
<tr>
<th>Protein</th>
<th>HIF-1α</th>
<th>R</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td></td>
<td>-0.529*</td>
<td>0.016</td>
</tr>
<tr>
<td>FGF</td>
<td></td>
<td>0.878*</td>
<td>0.014</td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td>0.537*</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Notes:
Correlation: 0-0.20 (very weak); 0.21-0.40 (weak); 0.41-0.70 (moderate); 0.71-0.90 (strong); 0.91-1 (very strong)
*Significant correlation at the level of 5%

IV. Discussion

In keloid proven hypoxia, it is shown in HIF-1α protein levels were significantly higher compared the preputium as a control (Figure 4). Hypoxic of fibrosis tissue as keloid model showed low EGF expression of epidermal, high FGF expression of dermal, and high VEGF expression of endothelial significantly (Figures 1 and 2).

The correlation between HIF-1α and EGF was a moderately negative significant correlation. It indicates that the higher the level of HIF-1α, the lesser number of cells expressing EGF. The Epidermal keloid showed thinner than preputium (control). However, the regulating effect of HIF-1α protein on EGF was weak as EGF has a role in stimulating cellular regeneration toward the keloid surface. The excessive proliferation in keloid tissue showed that HIF-1α stimulates cellular proliferation toward the skin surface;

Most cells expressing EGF are located at basal epidermal layer with upper surface of dermal layer as its border. When the dermal layer experiences increased cellular proliferation, it will be shifted to the epidermal layer stimulating epithelization and migration of epithelial cells from the wound margin that may increase cellular proliferation rate at the epidermal layer (Erlich et al, 1994).

The increased proliferation rate induces cell to start accumulation of keratin filament. The cells would progressively move to upper layer and subsequently die and shed (Velnar et al, 2009). During reepithelization, there is cellular proliferation in epidermal layer of keloid tissue, migrating keratinocytes and proliferating cells adjacent to the damaged epidermal layer. Epidermal cells move toward the wound through integrated migration after experiencing a number of changes in order to ease their movement. During migration, the cells also develop a structure of actin and stimulating the expression of proteolytic enzymes such as metalloproteinase matrix, which is useful in cellular repair and regeneration in epidermal layer (Guo and DiPietro, 2010; Shaw et al, 2009; Olczyk et al, 2014).

Hypoxic fibrosis in keloid, there is high expression of FGF. The correlation between HIF-1α and FGF showed a significant strong positive. It indicates that the higher the level of HIF-1α, the higher cellular percentage expressing FGF. The increased FGF in fibrosis has a role to stimulate fibrogenesis in developing extracellular matrix deposits (Simon et al, 2000; Shaw et al, 2009). Particularly fibroblast that produce collagen, which lead to increased collagen density (Vincent et al 2008). It is meant for wound repair (Kerbel, 2008; Olczyk et al, 2014). Fibroblasts are activated by FGF and together with PDGF and IGF-1, they proliferate and synthesize glycosaminoglycan and collagen (Olczyk et al, 2014). In excessive proliferation of keloid, there is increased number of cells expressing FGF, which may also contribute to the uncontrolled activity of keloid proliferation.

Some studies have demonstrated that during increased cellular proliferation and tumorigenesis, there is increased expression of FGF receptor (FGFR) through activation of tyrosine kinase. FGF activates JAK/STAT pathway. JAK causes phosphorylation of STATA protein in cellular membrane, which is followed by translocation of STAT protein to the nucleus and activates gene transcription associated with cellular survival with an implication on regulation of cellular proliferation. Excessive expression of FGF receptor (FGFR) through binding with tyrosine kinase is correlated to increased cellular proliferation and tumorigenesis mediated by phosphorylation of FGFR tyrosine (Olczyk et al, 2014).
Although there is a strong correlation between HIF-1α and FGF, but our study could not explain HIF-1α regulation on FGF expression. Shi et al have demonstrated that FGF may augment HIF-1α expression in breast cancer (Shi et al, 2007). In contrast, another study also has demonstrated that hypoxia may increase FGF activity in stimulating proliferation of placental endothelial cells (Wang et al, 2009). It's the same with our research.

There was a moderately significant correlation between HIF-1α and VEGF. It indicates that the higher expression of HIF-1α protein, the greater number of cells expressing VEGF. It provides evidence that during excessive cellular proliferation in keloid tissue, the VEGF is correlated with HIF-1α. Hypoxia causes a stable HIF-1α transcription factor. Following the dimer or stable HIF-1α, it will translocate to nucleus and regulate VEGF expression. Furthermore, it will bind with VEGF promoter causing increased VEGF transcription (Tabenero, 2007). In our study, through HIF-1α regulation, there was increased VEGF expression up to 4 times during excessive proliferation in keloid tissue, which is consistent with results of other studies with increased VEGF expression that may reach 6 times as in lung cancers (Wan et al, 2009).

When observing VEGF expression in keloid endothelial cells, we found a greater number of small capillary blood vessels as shown in figure 1F. In contrast, in kontrol (preputium) tissue, VEGF expression was found in endothelial cells of large-sized blood vessels (figure 1E). These findings are supported by Patel et al (2010) who demonstrated that there is hypovascularization in keloid. The lesser number of blood vessel in epidermal layer is caused by increased amount of extracellular matrix due to collagen synthesis resulting from excessive fibroblast proliferation. As a result of this excessive proliferation, hypoxia occur producing stable HIF-1α, which then regulates VEGF. Increased VEGF will cause angiogenesis and therefore, the number of capillaries in dermal layer is increasing.

VEGF belongs to angiogenic factor group, which has a function of stimulating the development of new blood vessel such as capillaries. When the amount of oxygen and nutrition needed is adequate, the angiogenesis stops and the unnecessary vascular cells will undergo apoptosis (Yolanda et al, 2014). VEGF gene is HIF-1α target gene and has a main role of regulating angiogenesis both in physiological or pathological condition, including excessive cellular proliferation (Wan et al, 2009).

During excessive cellular proliferation in keloid tissue, in addition to its role of stimulating the function of endothelial cells through angiogenesis mechanism, VEGF also has a role in stimulating proliferation of endothelial cells as survival factor, increasing vascular permeability through morphological changes of endothelial cells, altered cytoskeleton and stimulating migration and growth of the new endothelial cells. Diminished fibroblast cells expressing HIF-1α will reduce tumor growth as well as reduce vascular density. The role of VEGF in excessive cellular proliferation at the cellular level is to regulate the balance between ECM anabolism and catabolism through regulating angiogenesis (Wan et al, 2009).

Other authors suggest that VEGF will induce the development of fibrogenesis (Tabenero, 2007) by induction growth factors other, including TGF-β1 (Wan et al, 2009).

Hypoxia also induces VEGF expression and its receptor through HIF-1α (Tabenero, 2007). HIF-1α stimulates the release of VEGF and it circulates, subsequently the circulating VEGF binds to VEGF receptor on endothelial cells, Inducing tyrosine kinase pathway through angiogenesis mechanism. Shih et al suggest another role of VEGF in hypoxia that can stimulate PAI-1 expression through the extracellular signal-regulator kinase (ERK)1/2 signaling pathway. VEGF that induces PAI-1 has a role in modulating fibrosis development. The VEGF changes ECM homeostasis both through excessive degradation and accumulation (Burgess and Garrett, 2008). For the role of VEGF as mitogen, VEGF is involved in many stages of fibrogenesis response such as stimulating ECM degradation around damaged cells and then increasing proliferation and migration of cells (Wan et al, 2009). During excessive cellular proliferation in keloid, in addition to its role in angiogenic mechanism, it also more likely has a role in stimulating tissue fibrogenesis.

EGF, FGF and VEGF expressions have roles in hypoxic keloid tissue characterized by increased expressions as shown by immunohistochemistry technique. FGF and VEGF expressions are associated with stable HIF-1α in keloid tissue.

Based on results of our study, we suggest further studies on observation of EGF, FGF and VEGF expressions with HIF-1α inhibition using culture of fibroblast cell line.
V. Acknowledgements

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