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Effect of tagitinin C isolated from kembang bulan [*Tithonia diversifolia* (Hemsley) A. Gray] leaves on VEGF and TNF-α expressions of keloid fibroblast

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ABSTRACT

Tagitinin C, an active constituent of *Tithonia diversifolia* (Hemsley) A. Gray, has been proven caninhibit the collagen deposition of keloid fibroblasts *in vitro*. However, its mechanism of action has not been widely studied. One possible mechanism involves growth factors and cytokines. Vascular endothelial growth factor (VEGF) and tumor necrosis factor alpha (TNF-α) play an important role in the collagen deposition. The study aimed to evaluate the effect of tagitinin C on VEGF and TNF-α expression in keloid fibroblasts culture. An experimental laboratory study using fibroblast cell lines at passages III and IV was performed. Treatments were divided into two groups i.e. the treatment groups after incubation with tagitinin Cat various concentration of 1, 0.5, 0.25, and 0.125µg/mL for 72 h, and the control group using culture media without tagitinin C. Following after incubation, the VEGF and TNF-α levels of keloid fibroblast culture supernatant were measured by ELISA. Kruskal-Wallis test continued using Mann-Whitney test or one way Anova continued by independent t test were applied to evaluate the differences between groups. A p value of less than 0.05 was considered statistically significant. The VEGF levels significantly decreases in concentration-dependent manner after treatment of the tagitinin C at various concentrations ($p<0.05$). However, no significantly difference in TNF-α levels was observed ($p> 0.05$). In conclusion, tagitinin C decreases the VEGF expression of keloid fibroblasts. However, it has no effect on the TNF-α expression.

ABSTRAK

Tagitinin C, suatu senyawa aktif dari *Tithonia diversifolia* (Hemsley) A. Gray, telah terbukti dapat menghambat deposisi kolagen fibroblas keloid secara *in vitro*. Namun mekanismenya belum banyak dikaji. Salah satu kemungkinan mekanisme aksinya adalah melalui keterlibatan factor pertumbuhan dan sitokin. *Vascular endothelial growth factor* (VEGF) dan *tumor necrosis factor* (TNF-α) memegang peranan penting dalam deposisi kolagen. Penelitian ini bertujuan mengkaji efek tagitinin C terhadap ekspresi VEGF dan TNF-α pada kultur fibroblas keloid. Ini merupakan penelitian eksperimental laboratorium menggunakan sel fibroblas keloid pada sub kultur ke III dan IV. Perlakuan dibagi dua kelompok yaitu kelompok perlakuan setelah perlakuan dengan tagitinin C dengan konsentrasi 1; 0,5; 0,25 dan 0;125µg/mL selama 72 jam, dan kelompok control hanya menggunakan sel fibroblast keloid tanpa tagitinin C. Setelah inkubasi, kadar VEGF dan TNF-α dalam supernantan kultur fibroblast keloid ditetapkan dengan ELISA. Uji Kruskal-Wallis dilanjutkan dengan uji Mann-Whitney atau Anova satu jalan dilanjutkan dengan uji t independent digunakan untuk mengkaji perbedaan antar kelompok. Nilai p lebih kecil dari 0,05 dianggap berbeda nyata. Kadar VEGF turun tergantung konsentrasi setelah perlakuan tagitinin C pada berbagai konsentrasi ($p<0.05$). Namun demikian, perbedaan tidak nyata pada kadar TNF-α dapat diamati ($p>0,05$). Dapat disimpulkan, tagitinine C menurunkan ekspresi VEGF fibroblas keloid, namun tidak mempunyai efek pada ekspresi TNF-α.

Keywords:
keloid fibroblast;
cell proliferation;
tagitinin C;
VEGF;
TNF-α;

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INTRODUCTION

Keloids are harmless fibroprolife ration dermal tumors that growin the scar and exceed the wound limit. Keloid is characterized by excessive accumulation of extracellular matrix components such as collagen, fibronectin, elastin, proteoglycans and growth factors.[1] Keloid incident has a correlation with the wound healing process. The abnormal process of wound healing from the formation and degradation of matrix proteins, the expression of cytokines, growth factors and apoptosis pathways greatly affect the potential for hypertrophy scars to keloid.[2]

Vascular endothelial growth factor (VEGF) belongs to the group of growth factors that participates in the angiogenic response by increasing microvascular permeability, inducing endothelial cell (EC) proliferation, migration, survival and secretion of matrix metalloproteinases (MMPs).[3] It also plays an important role in the formation of keloids by changing the extracellular matrix and increasing EC proliferation.[4]

Deregulated cytokine secretion is implicated in several disease states ranging from chronic inflammation. When macrophages are exposed to inflammatory stimuli, they secrete cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, IL-8, and IL-12. All of these molecules, in concert, may inducein creased vascular permeability and recruitment of inflammatory cells. For example, excessive production of IL-1β and TNF triggers an acute generalized inflammatory response.[5] Large amounts of TNF-α involve nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and stimulate MMPs in wound healing and angiogenesis to tumor metastasis by inhibiting caspase 8 in the process of apoptosis in keloid fibroblasts.[6]

Patients prone to healing problems should receive preventive treatment. These preventions usually include topical medical application, cryotherapy, the use of silicone gel sheets, the injection of steroids, radio therapy and an early surgical procedure for wound closure.[7] Recent reports have introduced the use of extracts from natural sources such as plant extracts in an attempt to correct these problems. One alternative therapy that could be applied is kembang bulan [*Tithonia diversifolia* (Hemsley) A. Gray]. The potency of kembang bulan as an anticancer agent has been investigated. Ethyl acetate extract of kembang bulan has cytotoxic effects and induce apoptosis in human hepatoma HepG2 cells.[8] Chloroform extract from the *T. diversifolia* plant has also been reported to inhibit the proliferation of other cancer cells (WiDr cell line).[9] Ethanolic extract of *T. diversifolia* as an antifibrosis agent was proven with its capability to inhibit the proliferation of keloid fibroblasts and collagen accumulation.[10] In addition, ethanolic extract of *T. diversifolia* inhibits fibroblast migration activity, decreases the transforming growth factor-beta1 (TGF-β1), and inhibits vascular endothelial growth factor (VEGF) expression of keloid fibroblasts.[11]

Tagitinin C, is an active substance isolated from *T. diversifolia*. It is a sesquiterpene lactone (SLS) class that shows cytotoxicity against cancer cells. Tagitinin C also inhibits collagen accumulation on keloid fibroblasts *in vitro* after 72 and 120 h of incubation.[12] Tagitinin C also inhibits the fibroblast migration activity and decrease TGF-β1 levels of keloid fibroblasts.[13] This study was conducted to evaluate the effect of tagitinin C on the VEGF and TNF-α expressions of keloid fibroblast culture.

MATERIALS AND METHODS

MATERIALS

This was an experimental laboratory study conducted at the Laboratory of Health Technology, Department of Dermatology and Venerology, Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital, Yogyakarta. The protocol of the study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital (ref. KE/FK/426/EC). Tagitinin C was isolated from the leaves of *T. diversifolia* as conducted based on previous studies.[10] The chemical structure of tagitinin C is presented in FIGURE 1.[14]

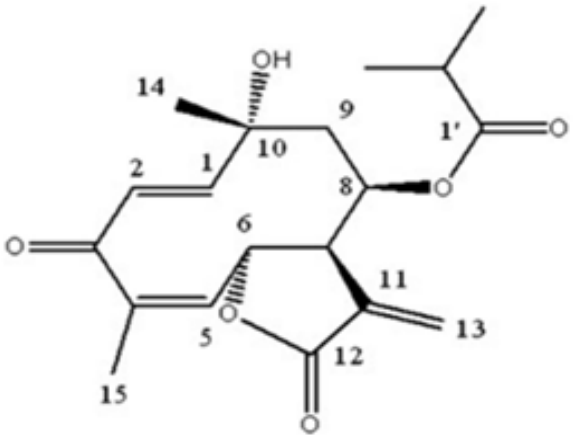

FIGURE 1. chemical structure of tagitinin C

Keloid fibroblasts and normal fibroblasts were obtained from the Laboratory of Health Technology, Department of Dermatology and Venereology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital. Keloid fibroblast and normal fibroblast cell lines were maintained *in vitro* in Dulbecco's modified eagle medium (DMEM). The primary culture was harvested after all petri fibroblasts were examined by inverted microscopy. The next step was subculture processing. The whole subculture process was repeated with sterile PBS for washing, DMEM for medium, and 2 mL 0.25% trypsin for trypsinization until mature subculture at passage III-IV.

Determination of the inhibition concentration 50% (IC_{50}) of tagitinin C

The IC_{50} of tagitinin C on keloid fibroblast was determined using MTT assay. The IC_{50}would be determined tagitinin C concentration on the VEGF and TNF-α expressions study. Keloid fibroblast cells were distributed in 96-wells microplates at 1 x 10^4 cells per well in 200 µL of complete DMEM medium. The cell cultures were then incubated in 5% CO_2 incubator at 37°C for 24 h. Followed after incubation, the medium was removed and replaced with new complete DMEM medium containing various concentrations of tagitinin C i.e. 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 µg/mL in triplicate. The DMEM medium just containing keloid fibroblast was used as negative control. After wards, the cell cultures containing tagitinin C were incubated again for 3 days. After the incubation, the medium was removed and the cells were resuspended in DMEM medium, 10 µL of 5 mg/mL MTT [3-9, 4, 5-dimethylthiazole-2-yl-2,5-diphenyltetrazolium bromide] and then further incubated for 4 h. The reaction was stopped by adding 100 µL of 10% sodium dodecyl sulfate (SDS) in 0.01N HCl. The 96-well microplate was then

shaken gently for 5 min, covered with aluminum foil and incubated at room temperature overnight. Absorbance of the 96-well microplate was measured in an ELISA plate reader at λ_{max} 595 nm. The IC_{50} values were determined by probit analysis.

Measurement of VEGF and TNF-α levels

The keloid fibroblast culture supernatant from the IC_{50} experimental result. e. was stored for further measurement of VEGF and TNF-α levels using ELISA reader. The VEGF and TNF-α levels were measured according to the protocol issued by Koma Biotech Inc. as a manufacturer of measurement kits for human VEGF (Catalog number : K0331132) and TNF-α (Catalog number K0331131).

Statistical analysis

Data of VEGF and TNF-α levels were presented as mean ± standard deviation (SD). The VEGF levels between groups were analyzed by using Kruskal-Wallis test continued using Mann-Whitney test, where as the TNF-α levels between groups were analyzed by using one way analysis of variance (ANOVA) continued by independent t test. A pvalue of less than 0.05 was considered statistically significant.

RESULTS

VEGF levels

The IC_{50} of tagitinin C against keloid fibroblast growth obtained in this study was 0.384µg/mL. Therefore, the measurement of VEGF and TNF-α levels on the keloid fibroblast culture supernatant was performed at the tagitinin C concentrations of 0.125, 0.25, 0.5 and 1 µg/mL. The results of the measurement of VEGF levels are presented in FIGURE 2 and TABLE 1. The VEGF levels significantly decreases after treatment of the tagitinin C at various concentrations ($p<0.05$). It demonstrated that the tagitinin C inhibits the VEGF expression of the keloid fibroblast.

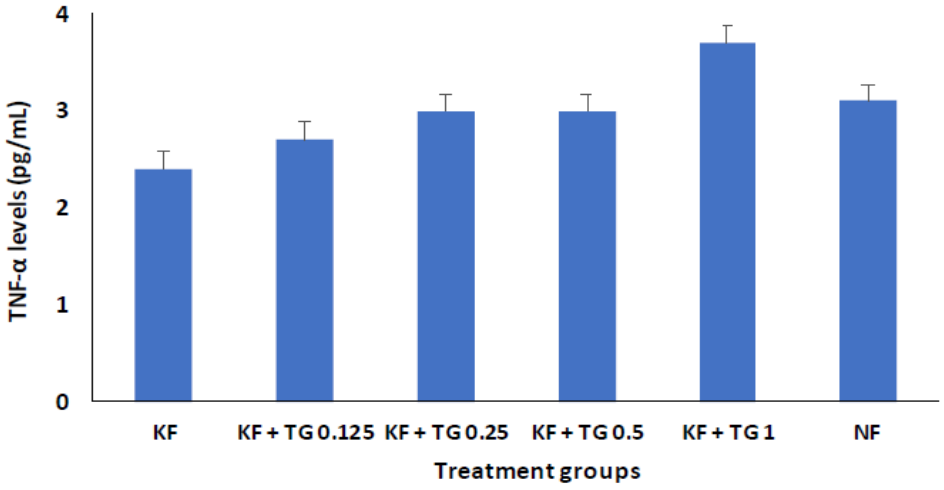

FIGURE 2. The VEGF levels on keloid fibroblast culture supernatant after tagitinin C treatment.

TABLE 1. Mean VEGF and TNF-α expression supernatant by treatment group

Variable	KF	KF+TG0.125	KF+TG0.25	KF±TG0.5	KF±TG1	NF	p
VEGF(ρg/mL)	222.9±3.6	142.2±2.0	117.3±2.7	108.9±2.5	75.7±3.6	20.2±0.6	<0.05
TNF-α (ρg/mL)	2.4±0.5	2.7±0.9	3.0±1.7	3.1±1.8	3.7±1.4	3.1±1.1	<0.05

KF: keloid fibroblast; KF+TG1:keloid fibroblast + tagitinin 1 µg/mL; KF+TG0.5: keloid fibroblast + tagitinin 0.5 µg/mL; KF+TG0.25: keloid fibroblast +tagitinin 0.25 µg/mL; KF+TG0.125: keloid fibroblast + tagitinin 0.125 µg/mL; NF: normal fibroblast.

TNF-α levels

The results of the measurement of TNF-α levels are presented in FIGURE 3 and TABLE 1. The TNF-α levels after tagitinin C treatment were not significantly different compared to that fibroblast keloid and normal fibroblast (p>0.05). It demonstrated that the tagitinin C did not affect the TNF-α expression of the keloid fibroblast.

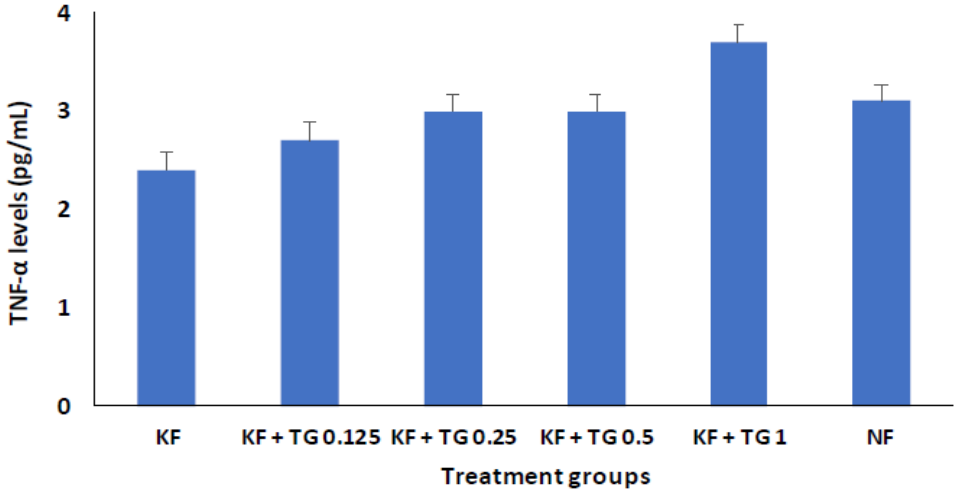

FIGURE 3. The TNF-α levels on keloid fibroblast culture supernatant after tagitinin C treatment.

DISCUSSION

VEGF is a cytokine proangiogenesis that plays an important role in normal conditions as well as wound healing, including pathology. VEGF angiogenic peptides have various isoforms, and VEGF is also a vascular permeability factor that promotes neovascularization and growth cells.[15] The lower VEGF expression in normal fibroblasts indicated that the production of VEGF by normal fibroblasts was affected by the condition of the cell. VEGF increases granulation tissue and promotes keratinocyte migration.[16] Keratinocytes also participate in abnormal wound healing processes, leading to the formation of keloid scars with increased proliferation.[17]

Increased expression in keloid fibroblasts was caused by local hypoxic conditions, which also contributed to the increased expression of VEGF in keloid fibroblasts with the accumulation of high HIF-1.[18] Hypoxia can induce cytokine expression and the production of growth factors released by macrophages, keratinocytes, and fibroblasts. Cytokines that are produced in response to hypoxia include PDGF, TGF-β, VEGF, TNF-α, and endothelin-1, which are promoters of

cell proliferation, migration, chemotaxis, and angiogenesis.[19] HIF-1, the major transcription factor in response to hypoxia, is a heterodimeric molecule of 2 subunits, HIF-1α and HIF-1β. HIF-1α has an oxygen-dependent degradation domain, and its expression and activity are regulated by the cellular oxygen concentration, while HIF-1β is constitutively expressed. HIF-1α binds to DNA on hypoxia response elements (HREs) in promoters of more than 60 target genes, such as VEGF and MMPs, many of which are involved in keloid formation.[20]

Fibrosis in phase keloid tissue caused by increased exogenous and endogenous VEGF in keloid fibroblasts. Exogenous VEGF will increase by VEGF receptors, whereas endogenous VEGF will increase with increased transcription factor VEGF and IGF in tissue keloid fibroblasts.[4] VEGF is a pro-angiogenic growth factor that increases keloid tissue angiogenesis, chronic inflammation occurs and persistently increases fibroblast proliferation.

This study showed that tagitinin C significantly decrease the VEGF expression of keloid fibroblast in a dose-dependent manner (FIGURE 2 and TABLE 1). At the highest concentration of tagitinin C (1 μg/mL), the VEGF levels reached 75.7 ± 3.6pg/mL. At the lowest concentration of tagitinin C (0.125 μg/mL) the VEGF levels (142.2 ± 2.0pg/mL) was still significantly lower than the VEGF levels of keloid fibroblast (222.9 ± 3.6 pg/mL) indicating its effecthas been observed.

This studys howed that tagitinin C might inhibit the proliferation of keloid fibroblast cells through the inhibition of the VEGF expression. It might inhibit the formation of a transcription factor of endogenous VEGF in keloid fibroblasts. VEGF is abundant in the dermis underlying keloids. *In vitro* studies showed that VEGF is expressed at high levels in keloid fibroblasts derived from normal skin fibroblasts.[21] Therefore, administration of tagitinin C could decrease VEGF production to effectively inhibit the formation of new extracellular matrix via inhibition of the VEGF pathway, signaling on the proliferation of keloid fibroblast tissue. Investigation of other growth factors can be performed to find another path that can be traversed by tagitinin C in the inhibition of keloid fibroblast proliferation. Derivatives such as platelet growth factor (PDGF) also play a role in increasing collagen production and synthesis of new extracellular matrix. PDGF receptors are also produced greatly in keloid fibroblasts.[22]

The effect of tagitinin C on TNF-α expression in the keloid fibroblast cultures was also investigated in this study. Tumor necrosis factor-alpha, a proinflammatory cytokine, is an important mediator during the inflammatory phase of wound healing. Excessive amounts of the TNF-α are closely associated with an increase in inflammatory diseases such as chronic wounds. TNF-α stimulates the secretion of active MMP-2 and type IV collagenase in organ culture, which affects the thickness of human skin. The excessive amounts of TNF-α causes the activation of NF-κB, which stimulates MMPs during wound healing. It also stimulates angiogenesis to tumor metastasis by inhibiting caspase 8 during the apoptosis process that closely connected with the potential increase in keloids.[6]

TNF-α expression of the keloid fibroblast control (2.4±0.5pg/mL) compared to the normal fibroblast (3.1±1.1pg/mL) was not significantly different (TABLE 1; $p>0.05$). It was indicated that the keloid process does not affect the TNF-α expression. Fibroblast cell proliferation during keloid process was not associated with the TNF-α expression. Furthermore, the tagitinin C administration did not affect the TNF-α expression. The TNF-α levels in keloid

fibroblast cells culture after exposure of various concentration (0.125 – 1 µg/mL) of taginitin C were not significantly different (p>0.05).

CONCLUSION

Tagitinin C decreases the VEGF expression in keloid fibroblasts. However, it has no effect on the TNF-α expression.

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