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Attenuation of Transforming Growth Factor-β Expression by Alteplase in Anterior Lens Capsule Fibrosis Model with Fibrin Reaction In Vitro

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Abstract

Background: Underlying pathophysiology of posterior capsule opacification, a frequent visual disturbance postop complication of cataract surgery, is multifactorial. Several cytokines and growth factors, such as Transforming Growth Factor-β (TGF-β), Fibroblast Growth Factor 2 (FGF-2), and Hepatocyte Growth Factor (HGF), have been found to play roles in the formation of PCO. Multiple advancements have been made including researches in therapeutic agents. Alteplase, a fibrinolytic agent, has been studied to decrease fibrin formation, which is a mechanism causing PCO. This study aimed to investigate the effect of alteplase on TGF-β expression in an anterior lens capsule fibrosis model resembling PCO.
Methods: An experimental study was performed using cultured anterior lens capsule tissue taken during cataract surgery. Primary cell isolation culture and lens epithelial lens characterization were conducted. Following identification of lens epithelial cell and 90% confluency rate on cell culture, lens epithelial cells were divided into four groups and then scratched to induce inflammatory reaction resembling PCO process after cataract surgery. Doses of 25 mcg/ml, 50 mcg/ml, and 100 mcg/ml were then applied to each group of cell cultures with the other group serving as negative control. Using a fluorescein microscope, measurements to asses the influence of alteplase were taken 7 days after scratching by analyzing the intensity of fluoresce using FITC antibody of TGF-β.
Results: The attenuation effect of alteplase to TGF-β expression was observed in all treatment groups; 25 µg/ml (15.5716x106 ±2.56558x10^6), 50 µg/ml (12.7364x106 ±1.67067x10^6), and 100 µg/ml group (7.8422x106 ±2.24941x106). The Tukey HSD posthoc tests found significant decrease between control group and each treatment group (p=0.019, p=0.000, and p=0.000; respectively).
Conclusion: Alteplase within measure dose range holds potential effect in inhibiting fibrosis formation in PCO through attenuation of TGF-β

Keywords: posterior capsule opacification; anterior lens capsule fibrosis model; alteplase; TGF-β

1. Introduction

Cataract is the leading cause of blindness across the world and second highest cause of moderate to severe visual disturance (up to 48% overall visual disturbances cases worldwide) (Jonas *et al.*, 2014). Cataract has affected 283 million people from a total of 593 million South-east Asian population, and WHO estimates the number will increase following aging and increasing number of population along with improving life expectancy (Masood, 2017). Until today there is no means to prevent cataract and cataract extraction surgery is the only definitive management for the disease.

Complications of cataract surgery consist of intra- and postoperative; in which posterior capsular opacification (PCO) as one of postoperative complications exists as the most frequent complication. This condition occurs as the posterior capsule experiences secondary haziness due to migration, proliferation, and differentiation of lens epithelial cells. Incidence of PCO was found

significantly high up to 40% following cataract extraction procedure with intraocular lens implantation, especially in patients possessing diabetes mellitus and uveitis risk factors (Sinha *et al.*, 2013).

Underlying pathophysiology of PCO is multifactorial; several cytokines and growth factors, such as Transforming Growth Factor-β (TGF-β) (main), Fibroblast Growth Factor 2 (FGF-2), and Hepatocyte Growth Factor (HGF), along with Matrix Metalloproteinases (MMPs) have been found to play roles in the formation of PCO (Sinha *et al.*, 2013). Many studies has looked into therapeutic agents i.e heparin in the irrigation solution and subconjunctival mitomycin-C as an alternative to standard method of costly Nd:YAG Laser (Haripriya *et al.*, 2017; Wormstone *et al.*, 2004).

A recombinant tissue plasminogen activator (r-tPA), alteplase, had been studied to degrade fibrin formation through anterior chamber administration. Previous research of alteplase application on lens capsule cultured epithelial cells having undergone scratching method to simulate fibrosis model on anterior lens capsule reported decreasing expression of Plasminogen Activator Inhibitor-1 (PAI-1), whereas other study learned that inhibition of PAI-1 would subsequently attenuate the expression of TGF-β, mainly considered primary cytokine in PCO inflammation process (Acheampong & Ford, 2012; Omori *et al.*, 2016; Dotan *et al.*, 2013). These findings lead to futher investigation whether administration of alteplase will induce a fibrinolytic process, thus inhibiting the formation of anterior lens capsule fibrosis. This study aimed to investigate the effect of alteplase on TGF-β expression in an anterior lens capsule fibrosis model resembling PCO.

2. Materials and Methods

The primary reagents included type I collagenase (Roche USA), alpha modified eagle medium (α-MEM, Gibco-Life Technologies, USA), nonessential amino acid (NEAA, Sigma, USA), trypsin (Gibco - Life Technologies, USA), fetal bovine serum (FBS, Biowest, USA), basic fibroblast growth factor (bFGF, Gibco- Life Technologies, USA), anticoagulant citrate phosphate dextrose acid (CPDA), $CaCl_2$ (sigma). Primary FITC conjugated antibodies for immunochemistry of TGF-β vimentin was purchased from BIOSS (USA).

Study design

This is an in vitro study using human LEC (HLEC), conducted at the Stem Cell Research and Development Center, Universitas Airlangga. All experiments conformed to the local ethics review board, Dr. Soetomo Hospital. Following in vitro wound scratch assays on fibrin coated dishes, HLEC were divided into 4 groups consisting of FBS 10% control group and treatment groups of alteplase at 25, 50 and 100 mcg/ml. Analysis of TGF-β was evaluated on the 7th day after scratching and treatment.

Isolation of HLECs

Human lens epithelial cells were isolated from a patient suffering from senile cataract aged 54 years old without ocular abnormality. Tissue was placed in 60 mm culture petri containing 2 mL of 0.2 mg/mL collagenase type I and incubated for 30 minutes at 37oC. Collagenase was removed and 4 ml of culture media (alpha-modified eagle medium; α-MEM + 10% fetal bovine serum; FBS + gentamicin reagent fluid + 5ng/mL, bFGF) was added to the petri, stored in an incubator at 37oC, while 5% CO2 was stored for 48 hours. Culture media were changed every 3 days prior to 90%

confluency, which were subcultured by warm trypsination techniques. This method was based on the protocol developed by Ibaraki (Ibaraki, 2002).

Fibrin Coated Dish preparation

Fibrin coated dishes were made according to protocol established by Komaratih et al (2019) with a modification of using blood from the subject. Fibrin made from 9 ml of blood taken with 10 ml spuit containing 1 ml of acid citate dextrose was placed in sterile tubes and left overnight in -4°C. The following day, the blood was centrifuged at 40 g for 10 minutes to obtain the plasma; 500 µl of this was placed into each well, with the additional mixture of calcium chloride in 1:10 ratio to create fibrin at the bottom of the well. Isolated HLEC were then placed on top of the fibrin layer (Komaratih *et al.*, 2019).

Characterization of HLECs

Cells were characterized with diamidinophenylindole (DAPI) and fluorescein isothiocyanate (FITC) staining with vimentin to identify mesenchymal cells, and p63 to identify epithelial cells.

Alteplase preparation

Alteplase preparation was performed made using extrapolation from cultured human corneal endothelial cells study as described previously by Yoeruek et al (2008). Alteplase was dissolved in culture medium until it reached 25, 50 and 100 mcg/ml (Yoeruek *et al*., 2008).

In vitro scratch assay

HLEC were seeded at a density of 5×103 cells/well (6 wells for each group) into 24 wells. Afterwards, the cells were scratched using yellow pipette tips perpendicular vertically to the petri meridian and washed with culture medium to remove loose and dead cells.

TGF-β expression assessment

Using a fluorescein microscope, measurements to assess the effect of alteplase were performed 7 days after scratching by analyzing the intensity of fluoresce in stained cells of FITC antibody of TGF-β using inverted immunofluorescence microscope (Olympus), whereas the level of expression was analyzed using ImageJ software and presented as pixel of positive cells.

3. Results

HLEC isolation

This study managed to isolate HLECs successfully at 90% confluency in 7 days. Supplementation of bFGF and NEAA allowed cells proliferation promotion while maintaining its phenotype and viability. Cells were placed in a fibrin coated dish and characterized using DAPI to

identify cells with a nucleus and FITC staining with vimentin and p63 to identigy mesenchymal and epithelial cells (Figs. 1 and 2).

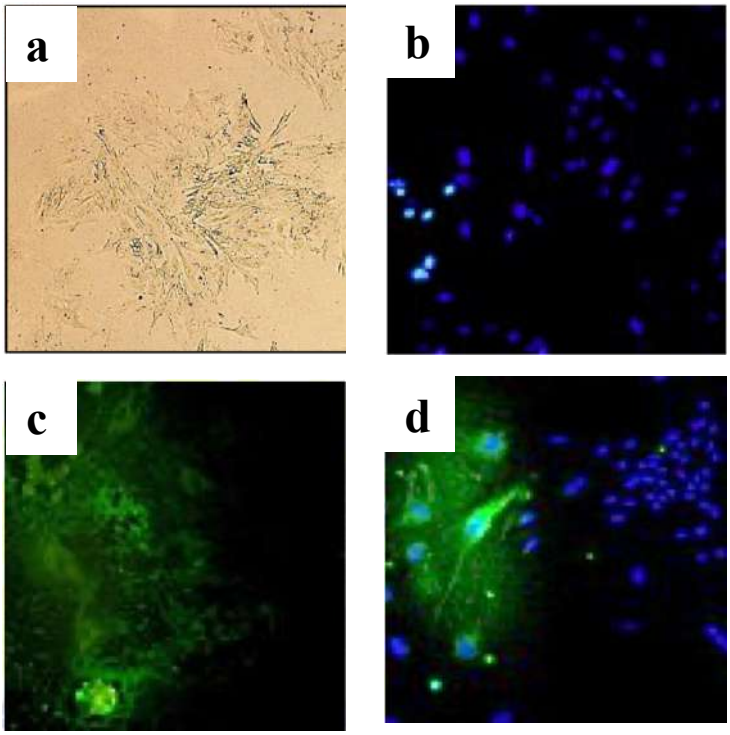

Fig. 1 Series of vimentin staining a) contrast phase; b) nucleus showing blue fluorescence with DAPI staining; c) green fluorescence showing positive vimentin FITC; d) merge (positive vimentin cells)

TGF-β antibody assessment

The attenuation effect of alteplase to TGF-β expression was observed in all treatment groups; 25 µg/ml (15.5716x106 ±2.56558x10^6 pixels), 50 µg/ml (12.7364x106 ±1.67067x10^6 pixels), and 100 µg/ml group (7.8422x106 ±2.24941x10^6 pixels) (Table 1). Further Tukey HSD posthoc tests found significant decrease between control group and each treatment group (p=0.019, p=0.000, and p=0.000; respectively). TGF-β antibody expression levels using immunofluorescence gradually decreased as dose of alteplase increased. The lowest level of expression was found in 100 µg/ml group (Figure 3).

Table 1. TGF-β expression

Group	N	Mean (pixel)	SD (pixel)	P (Oneway ANOVA)
Control	6	21.1161 x 10^6	4.53395 x 10^6	0.000
Alteplase 25 µg/ml	6	15.5716 x 10^6	2.56558 x 10^6	
Alteplase 50 µg/ml	6	12.7364 x 10^6	1.67067 x 10^6	
Alteplase 100 µg/ml	6	7.8422 x 10^6	2.24941 x 10^6	

4. Discussion

Wound healing process consists of several phases: homeostasis, inflammation, proliferation, and remodelling. The first phase, homeostasis, starts within seconds, lasting only up to several hours following injury before decreases rapidly afterwards and the process continues to the next phase. Platelet and fibrin have been well-known for their involvement during this phase. Fibrin formation is regulated by fibrinolysis which takes place in less than a minute and becomes stable in 30 minutes (Kattula *et al.*, 2018; Guo & DiPietro, 2010; Meng *et al.*, 2013).

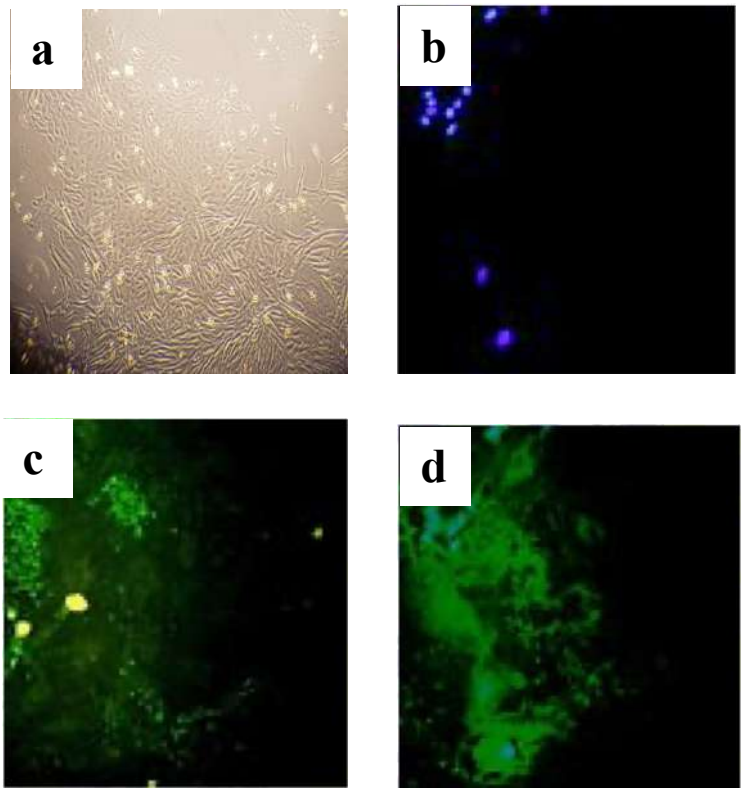

Fig. 2 Results a) contrast phase; b) DAPI staining showing blue fluorescent nucleus; c) positive p63 FITC shown in green fluorescent; d) merge (positive p63 cells)

The occurrence of PCO is higher in cataract surgery patients carrying risks for intraocular inflammation, which might cause fibrin formation due to presence of LEC lesions. The lesion attracts inflammatory mediators such as IL-1 and PGE2, that can alter the balance of blood aqueous barrier, and interfere with the balance of plasminogen, tPA, and thrombin subsequently. During its formation, it increases the expression of PAI-1 to inhibit the activity of tPA and uPA (both serve as fibrinolysis inducer). Fibrosis mechanism related with PCO manifests as excessive production of extracellular matirx (ECM) secretion and remodelling process, where EMT with its major inflammatory cytokine of TGF-β is an important component of this process. Meng et al (2013) in their study observed that TGF-β induces EMT promoting exccess synthesis and deposition of ECM proteins such as fibronectin and type I collagen in the Human Lens Epithelial Cells (HLECs), and

mTOR is activated by TGF-β -induced EMT. TGF-β functions through 2 pathways, Smad dependent which has Smad 1 to 5 components and Smad independent pathways which consists of Rho-GTPase, phosphatidilinositol-3-kinase and MAPK (Wormstone *et al.*, 2004; Dotan *et al.*, 2013; Chen *et al.*, 2014; Song, 2010).

Fibrinolysis occurs from the degradation of fibrin, as carried out by uPA or tPA generated endogenously or exogenously by recombinant techniques (thus named r-tPA), one of which is alteplase. Alteplase has been studied extensively and proven useful in the fields of neurology and cardiovascular disease as an emergency intravenous therapy to induce fibrinolysis in cases with occlusion of blood vessels. Prior study of alteplase in ophthalmology revealed it decreased PAI-1 levels significantly in an in vitro experimental study using cultures of HLECs (Song, 2010; Nishi, 2012; Ghatak *et al.*, 2018; Wulandari *et al.*, 2019).

In this study, we utilized cellular injury and fibrin to resemble clinical conditions during cataract surgery. This study replicated the procedures used in study by Stamm et al (2016) to scratch a culture dish in order to represent mechanical injury that occurs clinically. To that end, this study followed a mechanical injury model to form a discontinuity of tissue as seen in the LEC sequence in the anterior lens (Stamm *et al.*, 2016).

This study, which was carried out on the 7^{th} day post-treatment following a graph of TGF-β formation in the wound healing process, indicates that alteplase was able to decrease TGF-β expression levels. The expression significantly decreased after administration in all dose group (25, 50, and 100 mcg/ml) compared to the control group. The attenuation effect was shown to be dose-dependent, with its lowest expression was observed in highest dose group (100 mcg/ml). This finding is in line with previous study by which saw a significantly higher rate of PCO fibrosis at 90 days postop in the control group (41 subjects) compared to 44 treatment group subjects receiving 10 mcg intracameral alteplase at the conclusion of cataract surgery. The study also reported significantly better visual acuity in treatment group compared to control group, although the mechanism of how the treatment caused improvement had not been described (Heiligenhaus *et al.*, 1998).

The finding of TGF-β expression decrease supports previous study which concluded that inhibition of PAI-1 attenuates TGF-β -dependent EMT transition and differentiation in their study of human alveolar epithelial cells (Omori *et al.*, 2016). Flevaris et al (2017) investigated the activity of PAI-1 and also found its controlling effect to TGF-β in their murine cardiac injury model research (Flevaris *et al.*, 2017). Prior ophthalmology experimental study conducted by Wulandari et al (2019) observed significant decrease of PAI-1 expression following alteplase treatment in HLECs culture, similar to this study (Wulandari *et al.*, 2019). The action of alteplase in decreasing PAI-1 might further suppress TGF-β expression although precise mechanism underlying the link between TGF-β and PAI-1 still has not been established yet.

This study suggested that the administration of alteplase alleviates EMT by decreasing its TGF-β expression. It has a crucial role in the process of EMT and fibrosis. Currently, pharmacological PCO prophylaxis has not been achieved, despite advancements developed in recent decades to prevent PCO using various therapeutic agents. The result of present study demonstrated that alteplase may be potential agents for prevention and management of PCO. However, further investigations are needed to study other signaling pathways involved in EMT regulation in HLECs (Wormstone *et al.*, 2014; Sinha *et al.*, 2013).

5. Conclusion

Alteplase significantly reduces the expression of TGF-β, an important inflammatory mediator of fibrosis process with reaction to fibrin.

6. Acknowledgement
None

7. Financial Support
None

8. Conflict of Interest
Nil

9. Ethical Standard
Ethical approval was obtained from Institutional Ethical Committee of Medical Faculty Universitas Airlangga, Surabaya – Indonesia (No 0491/LOE/301.4.2/VI/2021). All procedures performed were in accordance with the ethical standards and with the 1964 Helsinki Declaration and its later amendments. Before the subject recruitment, an explanation of general research information was carried out to the subject for their consent.

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