The Addition of Hyaluronic Acid into Platelet-Rich Fibrin Lysate in Restoration of Senescent Human Dermal Fibroblasts Activities

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ABSTRACT

Senescent human dermal fibroblasts had reduced capacity in proliferation and collagen synthesis. It is due to unresponsiveness against transforming growth factor-β1 (TGF-β1) stimulation. Either platelet-rich fibrin (PRF)-lysate or hyaluronic acid (HA) can restore TGF-β1 signaling pathway. To determine whether HA addition to PRF lysate has a better activity than PRF-lysate alone in restoring senescent human dermal fibroblasts (HDFs) activities. HDF isolated from six different human skins was divided into normal HDFs and senescent HDFs which are induced by serum starvation. The senescent groups were then given 50% PRF-lysate and various levels of HA. Amelioration of TGF-β1 signaling was measured by cellular proliferation index and collagen deposition. Addition of HA into PRF-lysate resulted in a significant increase in proliferation index and collagen deposition index than PRF-lysate alone. The best level of HA for this mixture ranged from 20.83 mM to 41.67 mM. HA in PRF lysate is an excellent candidate material for treating clinical signs related to senescent human dermal fibroblasts.

Key words: Collagen deposition, hyaluronic acid, platelet-rich fibrin lysate, proliferation index, senescent human dermal fibroblasts

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INTRODUCTION

Failure of chronic ulcer healing (Harding et al., 2005; Clark, 2008) and wrinkle lines in the photoaged skin (Naru et al., 2005; Yin B et al., 2013) are both resulting from the presence of senescent fibroblasts. Senescent fibroblasts either isolated from chronic ulcers (Cowin et al., 2001; Kim et al., 2003) or from photo-aged skin (Quan et al., 2004) are unresponsive towards transforming growth factor (TGF)-β1 stimulation due to down-regulation of membrane-TGF-β1 receptor gene expressions. Impaired TGF-β1 signaling in human dermal fibroblasts (HDFs) leads to reduced proliferation and collagen synthesis (Czuwara-Ladykowska et al., 2001). Decreasing of dermal collagen content effect on fibroblasts mechanical forces to shrink and to cause further hiding of the remaining TGF-β1 receptors (Quan et al., 2013).

Previous studies showed that the administration of platelet-derived growth factor (PDGF) restores gene expression of TGF-β1 receptors (Czuwara-Ladykowska et al., 2001; Pan et al., 2010) where high levels of PDGF can be obtained from the secretion factors of platelets (Anitua et al., 2009). Moreover, the high concentration of platelets can be obtained from platelet-rich fibrin (Dohan et al., 2009) and a previous study showed that 50% PRF lysate (PRF lysate)
can restore the amelioration of chronically UVA-irradiated human dermal fibroblasts as a model of UV-induced senescent HDFs (Wirohadidjojo et al., 2016).

Furthermore, hyaluronic acid (HA) has the capability to restore fibroblasts microenvironment mechanical forces indicated by fibroblasts elongation, and this event is associated with up-regulation of TGF-β signaling pathway. Structural properties of the dermal extracellular matrix play a significant role in modulating fibroblasts function (Quan et al., 2013). In this study, the effect of HA addition into PRF-lysate in proliferation index and collagen deposition of senescent HDFs is reported.

**Material and Methods**

**Isolation and culture of human dermal fibroblasts**

Human dermal fibroblasts were obtained from human skin from 6 different individuals with informed consent. After mechanical removal of epidermal tissue from the skin, dermal parts were cut in 2-4 mm³ size, placed into a culture flask, immersed in a small amount of growth medium consisting of high glucose Dulbecco’s minimal essential medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS-Gibco) and 1% penicillin/streptomycin (Gibco), then incubated at 37°C until tissues were attached on the bottom of the flask. The medium was then exchanged with ten mL of fresh medium every 72 hours until 60% of the HDFs were outgrowing. The human dermal fibroblasts were then harvested and replicated until tertiary passage.

**Induction of cellular senescence**

Senescence of HDFs were artificially driven by culturing HDFs from these individuals in DMEM containing 1% fetal bovine serum for 48 hours. This treatment resembled previous studies (Alcorta et al., 1996 and Ramirez et al., 2001). Two hundreds µL of 5x10³ HDFs/mL suspension in DMEM+10% FBS was placed in all 96 well plates, incubated for 24 hours in 37°C and 5% CO2. The medium was then aspirated, rinsed with PBS and filled with DMEM containing 1% FBS-Gibco for 48 hours.

**Collection of platelet-rich fibrin (PRF) lysate**

PRF was isolated from peripheral blood of a volunteer by a standard protocol (Dohan et al., 2009). Isolation of PRF lysate was done by incubating PRF for 24 hours with 4°C temperature, using applied method from the study by He et al, 2009.

**Hyaluronic acid**

Hyaluronic acid (Z fill deep®, New-Ulm, Germany) which consisted of hyaluronic acid 23 mg/ml (Molecular weight 3 Mio. Daltons). Based on previous study, the best level of HA in stimulation of senescent HDFs is 20.83 mM (Sansan et al., 2015). Therefore, this experiment started to add 20.83 mM HA into 50% PRF-lysate.

**Experiments**

**Treatments**

Senescent HDFs were treated with 200µL of 50% PRF lysate, 20.83 mM HA; 41.67 mM HA; 83.33 mM HA and addition of those levels of HA into 50% of PRF lysate (50% PRF lysate+20.83mM HA; 50% PRF lysate+41.67 mM HA and 50% PRF lysate+83.33 mM HA) diluted in DMEM supplemented with 1% FBS. The negative control consisted of DMEM plus 1% FBS. As a control, normal fibroblasts were cultured in growth medium (DMEM plus 10% FBS). All of the cells were cultivate for 72 hours in 37°C in 5% CO2.

**Measurement**

**Proliferation Index**

Cellular viability was measured using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The medium was removed, and the cells were washed with PBS. 200 µL complete medium plus 50 µL of (5 mg/mL) MTT (MP biomedical-France) was added to each well. The plates were wrapped with aluminum foil and incubated at 37°C in 5% CO2 for 4 hours. The medium and MTT then removed and the remaining MTT-formazan was dissolved by adding 200 µL DMSO (Dimethyl sulfoxide). The optical density resulting from the formazan in each well was measured at 570 nm using a spectrophotometer.

Determination of proliferation rate = \( \frac{\text{The OD of experimental groups}}{\text{The OD of paired normal Fibroblasts}} \times 100\% \)

**Cellular collagen deposition rate**

The collagen deposition assay was based on an insoluble collagen of Sirius red assay following Taskiran et al., 1999. After treatment, those well were briefly washed with PBS, and the cells were fixated with Bouin solution for one hour. The wells were then washed off with tap water and allowed to dry at room temperature overnight. Two
hundred microliters of 0.1 % Sirius red in saturated picric acid (Sigma-Aldrich, USA) was added to each well for one hour. The un-bound Sirius red was washed for four times, using 200 µL 0.1 N HCL. The Sirius red bound to collagen was dissolved using 200 µL of 0.5 N NaOH, and the optical density was read using a spectrometer at 570 nm. Collagen deposition rate among experimental groups was calculated based on following formula:

\[
\text{Collagen deposition} = \frac{\text{The OD of experimental groups}}{\text{The OD of Normal Fibroblasts}} \times 100\%
\]

**Statistics**

All the results are shown as average ± standard error. Analysis and comparison among groups was conducted with ANOVA test followed by LSD for post hoc test or Friedman depending on the normality of data distributions. \( P < 0.05 \) was considered significant.

**RESULTS**

Fig.1: Characteristic of senescent HDFs induced by serum starvation
(A) Proliferation Index, (B). Collagen Deposition; NF= normal fibroblasts; SF=starved fibroblasts; DM=dilution medium; *\( p<0.05 \)

On this figure, serum starvation of HDFs in dilution medium for 48 hours significantly inhibits (\( p<0.05 \)) the cellular proliferation index and collagen deposition. They also presented larger and more polygonal cells morphology.

Fig. 2: The effect of hyaluronic acid on senescent HDFs
(A) proliferation index and (B) collagen deposition.
NF=normal fibroblasts; SF=starved fibroblasts; DM=dilution medium; HA=hyaluronic acid; *\( p<0.05 \).

This figure shows that HA could elevate proliferation index and collagen deposition of senescent HDFs significantly (\( p<0.05 \)) compared to dilution medium (negative control). The highest proliferation was observed in medium containing 20.83 mM HA (Fig. 2A). On the other hand, the most significant fibroblasts collagen deposition was observed in medium containing 83.33 mM HA (Fig. 2B).
Fig. 3: The effect of hyaluronic acid and 50% PRF lysate on senescent HDFs (A) Proliferation index and (B) collagen deposition.
NF= normal fibroblasts; SF= starved fibroblasts; DM= dilution medium; HA= hyaluronic acid; PL= platelet lysate;
*p<0.05

In Figure 3, there is a higher elevation of senescent HDFs proliferation index and collagen deposition cultured in 50% PRF lysate compared to those cultured in HA. However, this difference is not statistically significant (P>0.05). Senescent HDFs cultured in 50% PRF lysate appeared to be more compact and denser compared to those cultivated in HA.

Fig. 4: The effect of addition of HA into 50% PRF lysate on senescent HDFs (A) proliferation index and (B) collagen deposition.
SF= Starved fibroblasts; HA= hyaluronic acid; PL= PRF lysate;
*p<0.05

The figure above shows that addition of HA into PRF-lysate gave a significantly better proliferation index and collagen deposition index than PRF-lysate alone. The highest proliferation index appeared on senescent HDFs cultured in 50% PRF lysate + 20.83 mM HA (Fig. 4A). However, the highest collagen deposition was observed in senescent HDFs cultured in 50% PRF lysate+41.67 mM HA (Fig. 4B).

The figure below shows that the best medium to restore senescent HDFs activities (proliferation index and collagen deposition) were the combination of 50% PRF lysate + HA. This medium had significantly different values for both normal fibroblasts and starved fibroblasts cultured in dilution medium (negative control), 20.83 mM HA, or 50% PRF lysate. We can also find the best and densest fibroblasts morphology in this medium combination.
Responsive to the stimulatory function (Eckes et al., 2008). Hyaluronic acid (HA) binds to CD44, a cell surface glycoprotein (David et al., 2015). Hyaluronan (HA) is a ubiquitous, multifunctional cytokine playing a crucial role in regulating pre-collagen synthesis. HA-mediated Smad 2/3 phosphorylation was a ubiquitous linker between different signaling pathways. HA-β1 initiates its cellular proliferation index and collagen deposition, possibly due to the dilution medium ability to decrease overall cellular function. Senescent HDFs are described as larger and polygonal in shape compared with compact, spindle shape of normal skin fibroblasts (Agren et al., 1999). A similar event was also found in this study. Senescent HDFs showed a larger and more polygonal morphology (Fig. 1).

Senescent HDFs displayed a decreased proliferative response to growth factors due to impaired intracellular signaling. Recently, increasing evidence suggests that senescent fibroblasts are unresponsive to the stimulatory action of transforming growth factor beta 1 (TGF-β1). It has been shown that some of the mechanisms that might be responsible for HDFs unresponsiveness to TGF-β1 include the decrease of TGF-β type II receptor expression and abnormalities in the downstream signaling pathway involving MAPK and the early Smad pathway (Kim et al., 2003). TGF-β- Smad pathway is the fibroblast’s main regulator for the proliferation as well as synthesis of type I procollagen (Capelo et al., 2005). In senescent HDFs, reduced TGF-β signaling and CTGF/CCN2 expression contribute to decreased collagen production (Quan et al., 2006; Quan et al., 2010).

Incubation of platelet concentrate at 4°C for 24 hours produces the high concentration of PDGF-BB, epidermal growth factor and TGF-β1 (He et al., 2009). Those growth factors effectively stimulate both collagen and hyaluronic acid production by dermal fibroblasts (Kim et al., 2014). Growth factors, such as PDGF-BB and TGF-β are key regulators of cellular functions, including proliferation, migration, and differentiation. Growth factor signaling is modulated by context-dependent cross-talk between different signaling pathways. PDGF-BB-mediated Smad 2 phosphorylation was dependent on the kinase activities of both TGF-β type I receptor (TβRI) and PDGF-β receptor (PDGFRβ) (Porsch et al., 2014). TGF-β is a ubiquitous, multifunctional cytokine that play a crucial role in regulating pre-collagen synthesis. TGF-β initiates its cellular action by binding to specific cell surface receptor complexes typically composed of TGF-β type I (TβRI) and TGF-β type II (TβRII). Binding of TGF-β to TβRII activates the intrinsic serine/threonine kinase activity of TβRI, which phosphorylated transcription factors Smad 2 and Smad 3 (Quan et al., 2004). On the surface of dermal fibroblasts membrane, the receptors for PDGF-BB and TGF-β both physically interact and ameliorate each other’s signaling and stability (Porsch et al., 2014). Other responsible factor for content shortage of collagen in senescent fibroblast is the expression of gene MMP-1 to degrade collagen type I. Study showed that platelets can release tissue inhibitors of MMP (Villeneuve et al., 2009). Improvement of collagen deposition in senescent HDFs can be obtained by inhibition of MMP-1 activity by MMP tissue inhibitors in PRF lysate (Wirohadidjojo et al., 2016).

TGF-β signaling pathway is also influenced by mechanical force and pivotal to dermal fibroblast function (Eckes et al., 2006). The addition of exogenous, monomeric hyaluronic acid to cultured fibroblasts is reported to trigger TGF-β signaling and collagen production (David-Raoudi et al., 2008; Mast et al., 1993). Some of these responses are mediated by binding of HA to CD44, a cell surface glycoprotein (David-Raoudi et al., 2008). Another study found that injection exogenous hyaluronic acid stimulates proliferation of fibroblasts (Quan et al., 2013). This stimulation is suggested due to...
to the increase of local mechanical forces, characterized by fibroblast elongation/spreading, furthermore it is also mediated by up-regulation of type II TGF-β receptor and connective tissue growth factor (Eckes et al., 2006; Quan et al., 2013). Similar events were also found in this experiment, fibroblasts elongation presented after treatment with combination of 50% PRF lysate with hyaluronic acid (Fig.5). Importantly, fibroblasts elongation is associated with up-regulation of the TGF-β signaling pathway (Quan et al., 2013). Thus, this study found that structural properties of fibroblasts played a significant role in modulating senescent fibroblasts function. Fibroblasts proliferation can be driven by numerous mechanisms, including increased mechanical force (Eckes et al., 2006).

HA in PRF lysate is a potent candidate material for senescent human dermal fibroblasts treatment. It is assumed that HA and growth factors from PRF lysate act synergistically to repair proliferation and collagen deposition of senescent human dermal fibroblasts by ameliorating TGF-β1 signaling. Hyaluronic acid was shown to interact with growth factors, thereby protecting them from degradation by proteases (Locci et al., 1995). Therefore, due to hyaluronic acid specific properties correlated with growth factors, HA addition into human growth factors obtained from PRF lysate might be particularly well suited to help improve proliferation index and collagen deposition of senescent human dermal HDFs. This combination may be utilized to increase the positive outcome of the restoration of senescent human dermal fibroblasts activities.

CONCLUSIONS
The addition of HA into PRF lysate is a good candidate material for treating clinical signs related to senescent human dermal fibroblasts.

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