Comparison of Chondrocyte Quality dissociated from Cartilage by Crude and Recombinant type Collagenase.

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Abstract

❖ Due to enlarging proportion of elderly population in modern society, diseases related to old age became a huge social issue. Cartilage is susceptible to aging-caused damage. To address this issue, intensive researches have been performed to find treatments for regressed or damaged cartilage. Treatment which utilizes regenerated chondrocyte is considered to be a solid method to restore damaged cartilage. Collecting pure, qualified chondrocyte from patient is a crucial step for successful treatment. Mostly, Collagenase, derived from *Clostridium histolyticum*, is utilized to isolate chondrocyte. However crude collagenase contains various neutral proteases and these residues can cause cellular damage during cell dissociation. Therefore, we produced recombinant collagenase using *Escherichia coli*. Although, cell yield and viability of chondrocyte wasn't different, adhesion and proliferation rate of chondrocyte dissociated by recombinant collagenase has increased. In addition, chondrocyte genes and surface CD markers showed similar pattern to that of healthy chondrocyte. In result, recombinant collagenase is found to be more suitable for harvesting qualified chondrocyte than crude one.

Introduction

❖ Rapid growth of elder population became a huge concern in modern society. Indeed, vulnerability of aged person to degenerative diseases aggravates social problems in matter of health issues. Recent study suggests that these diseases mainly contribute to the poor quality of life in old population. Furthermore, according to National Health Interview Survey (NHIS), in 2010–2012, about 52.5 million adults in the USA had doctor-diagnosed arthritis, and by 2040, the number of US adults with doctor-diagnosed arthritis is projected to increase 49% to 78.4 million. In such clinical circumstance, many researchers are struggling to overcome these problems by applying tissue regeneration technique for treatments. Various tissue regeneration techniques are being studied to overcome arthritis and degenerative cartilage disease. We are focusing on therapeutic research through autograft-treatment. However, recovering high quality chondrocytes from patient's cartilage tissue is not only crucial, but also challenging step for a successful autograft. In general, commercialized collagenase is well known to contain a lot of neutral protease which causes side effects like cell membrane proteins degradation during tissue recovery process. To address this problem, we performed an experiment using high-purity recombinant collagenase in order to optimize the condition of chondrocyte recovery from cartilage.

Material and Methods

❖ Collagenase potency assay

1 mM of FALGPA peptide were mixed with crude or recombinant collagenase at 25˚C, pH 7.5. The amount of dissociated FALGPA product was measured by reading absorbance value at 345 nm every minute for 20 minutes.

❖ Recovery of chondrocyte from cartilage

Donor animals were handled according to protocols approved by the Animal Care and Concern Committee (National Institute of Animal Science, Seoul, South Korea). Bovine chondrocyte was collected from the cartilage of hind legs from 24 to 26 month old Korean cattle (body weight of 550-600-kg). Collected cartilage was minced and digested with commercial crude collagenase 1.30 unit/mL (Liberase TM, Roche) or recombinant collagenase 1.30 unit/mL (KITECH-BMTC). Enzyme unit was determined by the result of potency assay as written above.

❖ Cell culture

Recovered chondrocyte was cultured in chondrocyte culture medium (Promocell) at 37˚C in 95% humidity, 5% CO_2 atmosphere.

❖ Adhesion and proliferation

In order to confirm adhesion and proliferation ratio of seeded chondrocytes, each cultured cells were maintained for 3, 7 and 10 days, respectively. After then cells were detached with Trypsin-EDTA, the number of cells were counted under microscope observation.

❖ Real-time PCR

Real-time PCR was performed with SYBR green and carried out under following condition: Pre-denaturation (95 ℃, 10 min), followed by 40 cycles of denaturation (95℃, 33 sec), annealing at each gene-specific primer Tm (℃), and extension (72 ℃, 33 sec) steps.

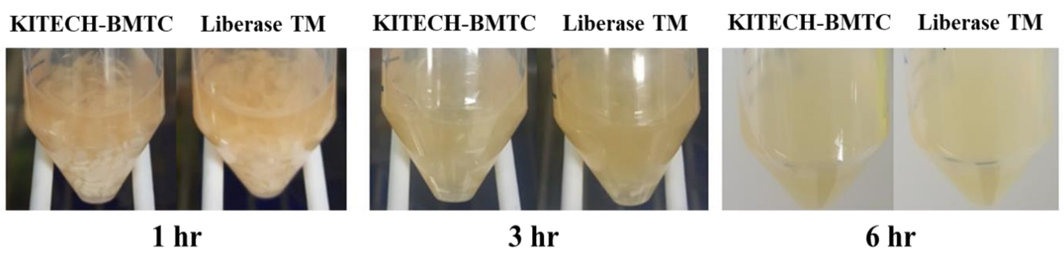
❖ Cell surface CD marker analysis

Detached cells were washed twice using PBS containing 3% FBS and incubated overnight with interested CD marker antibody. Cells were washed twice, then secondary antibody was added subsequently for 1 hour. After appropriate washing steps, FACS analysis was performed to measure protein expression.

Results

❖ Cartilage dissociation and recovery rate of chondrocytes

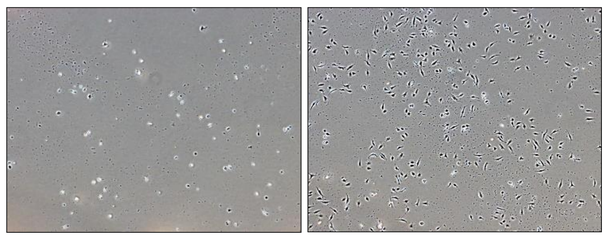
- The pieces of cartilage incubated with crude or recombinant collagenase were completely dissociated at 6 hours with each collagenase.



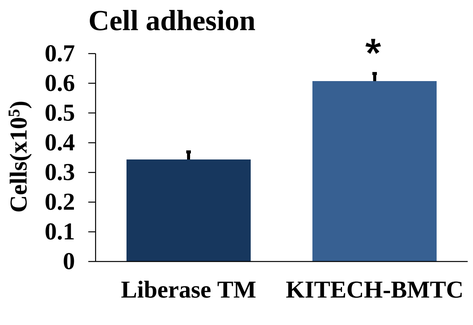
	KITECH-BMTC	Liberase TM
Live (10^6)	23.00 (±2.26)	24.00 (±9.37)
Dead (10^6)	1.42 (±0.5)	1.46 (±0.42)
Total (10^6)	24.42 (±2.41)	25.46 (±9.74)
Viability (%)	94.21 (±1.91)	94.12 (±0.96)
Live Cells (10^6) /cartilage weight (g)	10.75 (±1.06)	10.91 (±4.26)

- Live, dead and total number of recovered chondrocytes from each collagenase was not significantly different and viability was also similar. In addition, number of recovered live chondrocytes per gram of cartilage tissue didn't show big difference between two groups.

❖ Cell adhesion

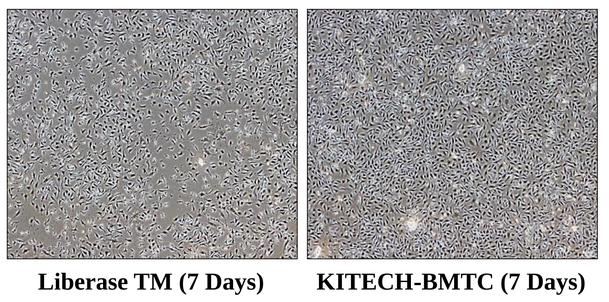


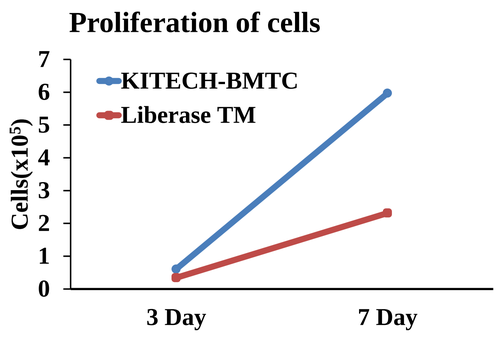
Liberase TM (3 Days) KITECH-BMTC (3 Days)

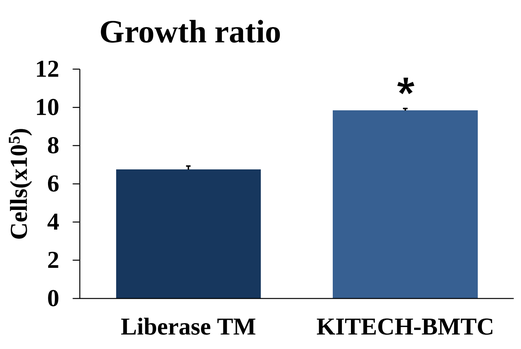


- Cells adhesion showed significant difference after seeding for 3 days. Cells dissociated with KIRECH-BMTC collagenase were about two times more than Liverase TM.

❖ Proliferation

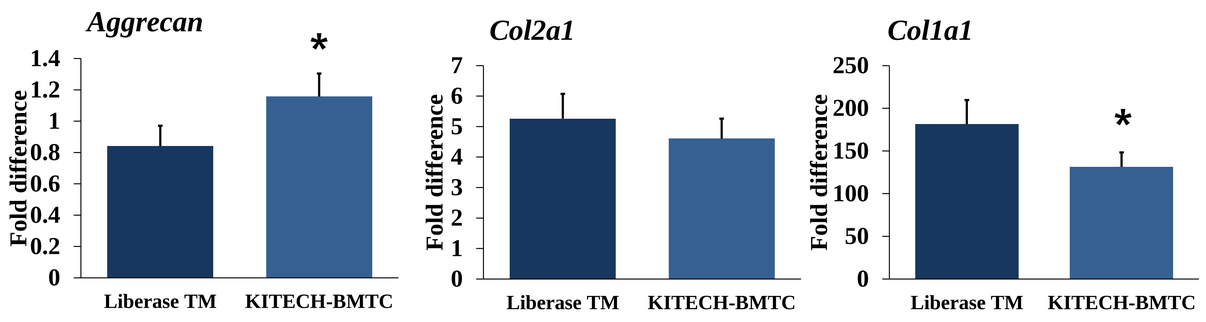






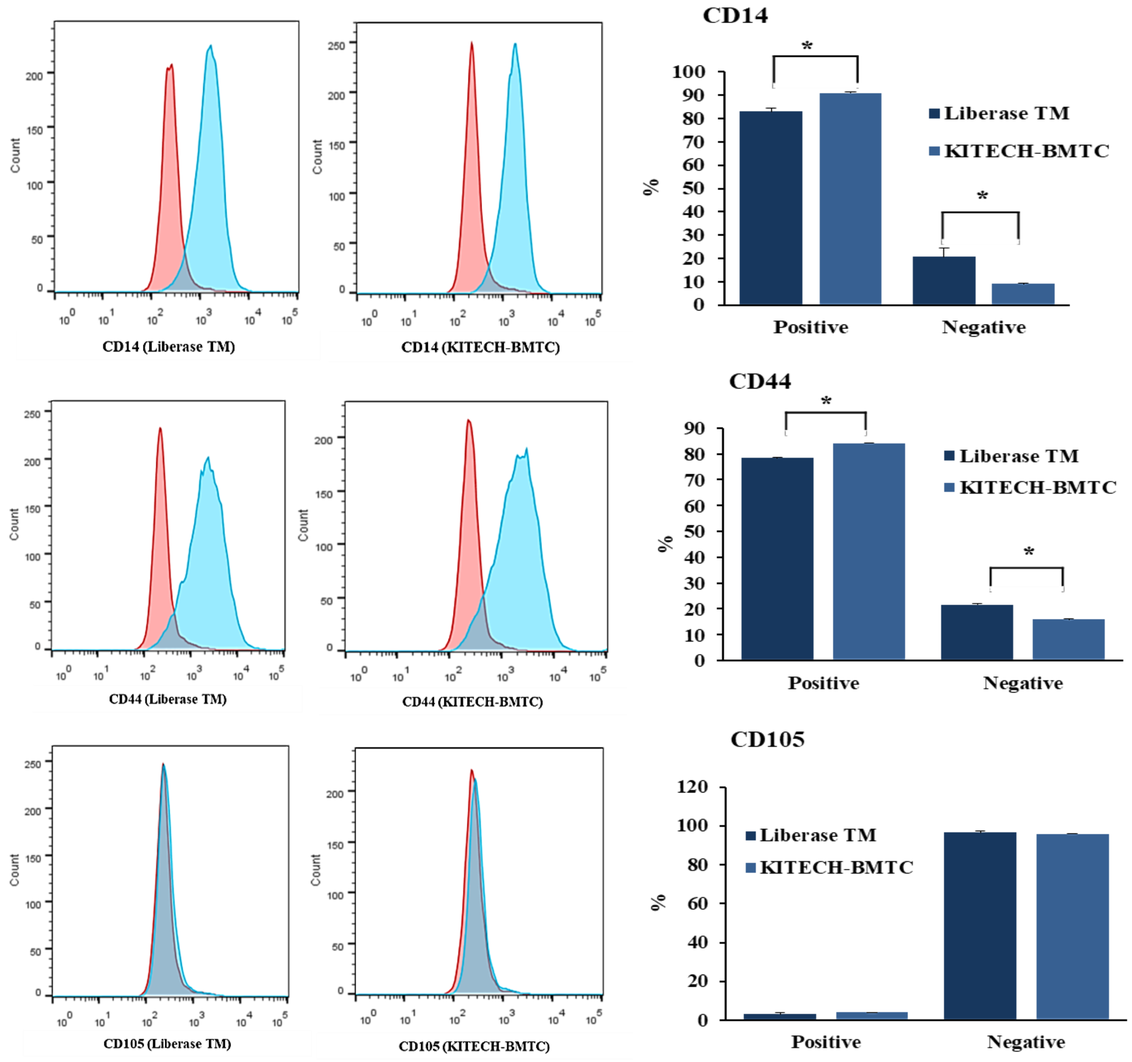
- KITECH-BMTC collagenase also showed significant increase in proliferation rate during culture period. The total number of cells in day 7 culture showed twice more than Liberase TM collagenase. Growth ratio which was calculated based on the number of cells attached to the surface after subculture also showed a significant difference.

❖ Chondrocyte marker gene expression



- *Aggrecan* and *Col2a1* (*Collagen type 2 alpha 1*) are well known chondrocyte marker genes. On the other hand, *Col1a1* (*Collagen type 1 alpha 1*) which determines de-differentiated condition of chondrocyte are considered to be a fibroblast marker gene. The results represent that *aggrecan* expression of chondrocytes recovered with KITECH-BMTC collagenase was significantly higher than that of Liberse TM, but not in *Col2a1* expression. In the case of *Col1a1*, Liberase TM was found to be significantly higher than KITECH-BMTC collagenase.

❖ Cell surface CD marker analysis



- Chondrocyte dissociated with KITECH-BMTC collagenase showed significantly more CD14 (Chondrocyte differentiation marker) and CD44 (Hyaluronan receptor) protein expression than Liberase TM. While CD105 (Mesenchymal stem cell marker) was not the case.

Conclusion

- Although, cell yield and viability of chondrocyte wasn't different, adhesion and proliferation rate of chondrocyte dissociated by recombinant collagenase has increased.
- In addition, chondrocyte genes (*Aggrecan*, *Col1a1*) and surface CD markers (CD14, CD44) showed similar pattern to that of healthy chondrocyte.
- Recombinant collagenase is found to be more suitable for harvesting qualified chondrocyte than crude one.

Reference

- He Huang et al. (2019). Current Tissue Engineering Approaches for Cartilage Regeneration. DOI: http://dx.doi.org/10.5772/intechopen.84429
- Knudson CB et al. (2004). Hyaluronan and CD44 modulators of chondrocyte metabolism. Clin Orthop Relat Res. S152-62.
- Cleary MA et al. (2016) Expression of CD105 on expanded mesenchymal stem cells does not predict their chondrogenic potential. Osteoarthritis Cartilage. 24(5):868-72