Application of recombinant collagenase to tissue dissociation and cell recovery

Jeong Ho Lim[1,2], Duk Hwan Choi[2], Jung Hun Park[1], Chi Min Choi[1,3], Woo-Jong Lee[1,3,*]

1 Biomedical Manufacturing Technology Center, Korea Institute of Industrial Technology, Yeongcheon, 38822, Republic of Korea.
2 Connext Co., Ltd, Yeongcheon, 38822, Republic of Korea.
3 Department of Medical Biotechnology, Yeungnam University, Gyeongsan, 38541, Republic of Korea.

Abstract

❖ **Purpose**

Collecting pure and qualified cells from human donor is a critical but challenging process that hinders successful tissue-engineering and cell biology researches. Mostly, collagenase, derived from *Clostridium histolyticum*, is utilized to isolate cells from tissue. However, crude collagenase contains various neutral proteases which can cause cellular damage during tissue dissociation. Therefore, we produced recombinant collagenase from *Escherichia coli* through bioprocessing technology.

❖ **Materials and methods**

Collagenase potency assay of recombinant collagenase was performed using FALGPA peptide as a substrate (synthetic short polypeptide specifically recognized by collagenase). Based on potency assay, proper unit of recombinant collagenase was treated to lung, kidney and spleen tissue from mice to recover immune cells and bovine cartilage to obtain chondrocytes. Three commercialized enzymes were used as a control representing crude collagenase. To compare cell quality, genetic and CD markers were measured by real time PCR and flow cytometry.

❖ **Results**

Comparing to control collagenases, immune cells recovered by recombinant collagenase showed fewer numbers, while population of T cell, B cell, NK cell, NKT cell, myeloid and mononuclear phagocyte was similar in both collagenases. However, population of immune cells from spleen obtained by recombinant collagenase showed more even distribution compare to crude collagenase derived ones. Chondrocytes, on the other hand, showed similar recover rate with both cases but ones gained by recombinant collagenase exhibited healthy chondrocyte-like characteristics in regard of genes (*Aggrecan*, *Col1a1*) and surface CD markers (CD14, CD44).

❖ **Conclusions**

Although tissue dissociation capacity of crude collagenase in lung, kidney and spleen was confirmed to be superior to the recombinant one, distribution of recovered immune cells was mostly similar in both cases. In particular, unlike other two tissues, spleen-originated immune cells with recombinant collagenase showed more even distribution. Since splenic immune cells are used as a reference for immune cells extracted from organs, it is advantageous to present a clear standard using recombinant collagenase. In addition, chondrocytes recovered by using our recombinant collagenase were determined to maintain their original characteristics better than crude collagenase-isolated ones.

Material & Method

❖ **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.

❖ **Dissociation of tissues and recovery of the cells**

Lung, kidney, spleen and cartilage were using crude or recombinant collagenase . The protocol was followed the method of crud collagenase manufacturer.

❖ **Adhesion and proliferation**

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

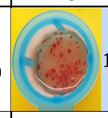
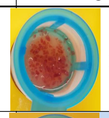
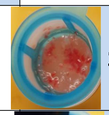
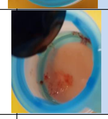
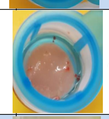
➢Collagenase activity

	Crude			Recombinant
Manufacturer (Collagenase type)	Sigma-Aldrich (Collagenase type I)	Gibco (Collagenase type IV)	Roche (Collagenase G-H blend)	KITECH-BMTC (Collagenase G-H blend)
Unit/mg	0.64 unit/mg	0.34 unit/mg	1.31 unit/mg	1.64 unit/mg

- **Crude type showed low enzyme activity due to residual non-specific neutral protease in cocktail while pure enzyme mixture performed higher activity. Notably, KITECH-BMTC showed highest performance.**

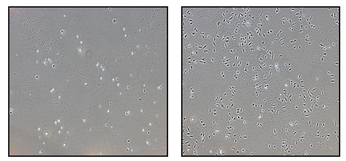
- Collagenase type I is extracted with minimized processing.
- Type IV are processed to moderate tryptic activity in order to mitigate membrane/recepter membrane damage.
- Collagenase G and H is purified collagenase extracted from *Clostridium histolyticum* (crude) or *Escherichia coli* (recombinant), both enzymes are blended to be used.

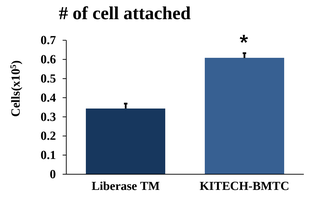
➢Dissociation of tissues using different type of collagenase

	Sigma-Aldrich Collagenase type I	Gibco Collagenase type IV	Roche Liberase TM	KITECH-BMTC Collagenase
Kidney (cells/organ)	 16 (±2)	-		 12 (±2)
Lungs (cells/organ)	-	 2.3 (±3)		 2.2 (±2)
Spleen (cells/organ)	-	 66 (±4)		 42 (±3)
Cartilage (cell/gram)	-	-	 11 (±1)	 11 (±4)

Unit (× 10 ^{6} cell)

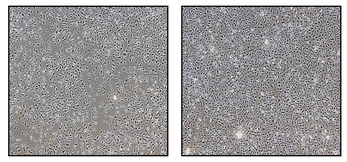
➢Cell adhesion

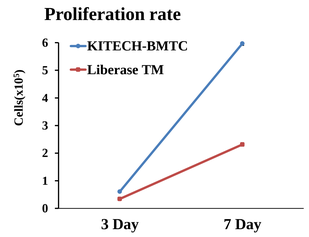




- **KITECH-BMTC dissociated chondrocytes showed significantly higher cell adhesion rate (~2 times) after 3 days of seeding.**

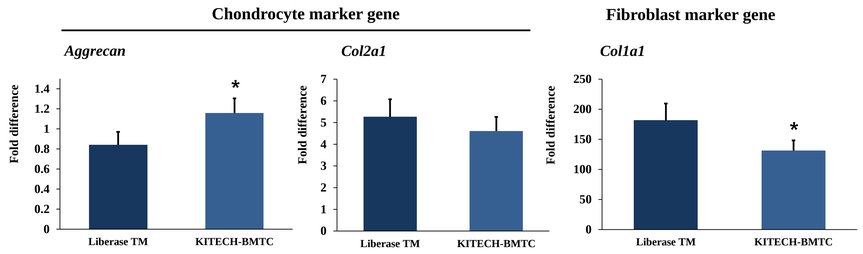
➢Proliferation





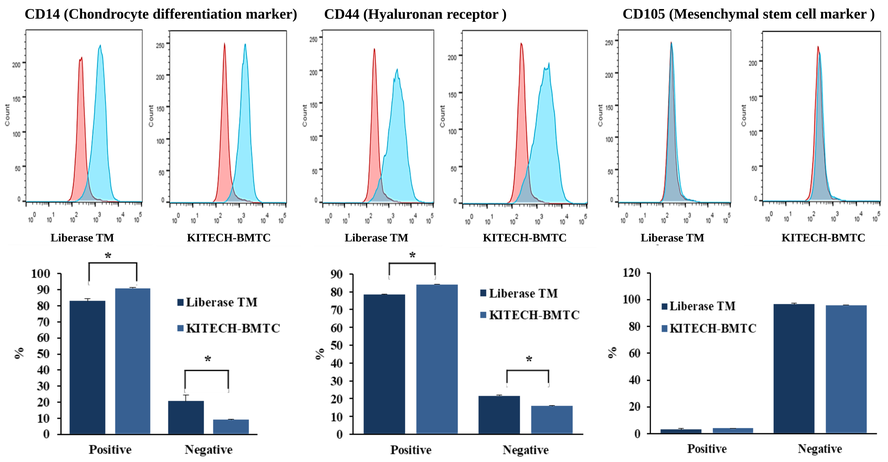
- **KITECH-BMTC collagenase also showed significant increase in proliferation rate during culture period. The total number of cells in day 7 culture showed twice more than Liberase TM collagenase. Growth ratio which was calculated based on the number of cells attached to the surface after subculture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

➢ Chondrocyte marker gene expression



- **Expression level of *aggrecan* gene in chondrocytes recovered with KITECH-BMTC collagenase was significantly higher than that of Liberse TM, but was similar in *Col2a1* gene expression.**
- **In the case of *Col1a1*, Liberase TM was found to exhibit significantly higher expression pattern which indicates potential de-differentiation condition of isolated chondrocytes.**

➢Cell surface CD marker analysis (Chondrocyte & Spleen)



- **Chondrocyte dissociated with KITECH-BMTC collagenase showed significantly more CD14 and CD44 protein expression than cells isolated with Liberase TM. While CD105 was not the case.**

Cell Type (Spleen) \ Marker	T cell			B cell
	CD4 – CD8 +	CD4 + CD8 –	CD4 – CD8 –	TCRβ – CD19 +
Gibco Collagenase IV	12.9%	16.2%	0.45%	29.2%
KITECH-BMTC Collagenase	11.6%	**20.1%**	**14.5%**	**31.7%**

- **There was no significant difference between two enzymes in terms of the distribution of immune cells recovered from Kidney and lung.**
- **In the other hand, immune cells extracted from spleen using KITECH-BMTC collagenase showed more even distribution.**

Conclusion

- **Although, yield and viability of dissociated chondrocytes wasn't much different, adhesion and proliferation rate of chondrocytes 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.**
- **When recombinant collagenase was used for spleen dissociation, it was confirmed that immune cell distribution was less lopsided then when crude collagenase was used.**
- **All things considered, using recombinant collagenase is found to be more suitable for harvesting qualified chondrocyte and spleen-derived immune cell than using 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