치의과학 석사학위논문

Age-related changes to macrophage phenotype expression during bone wound healing in a mouse model of critical sized calvarial defect

마우스 두개관 손상 모델에서 증령에 따른 대식세포의 발현 변화

2021 년 8 월

서울대학교 대학원
치의과학과 분자유전학 전공
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이 논문을 치의과학 석사학위논문으로 제출함

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김재형의 석사학위논문을 인준함

2021 년 6 월

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Abstract

Age-related changes to macrophage phenotype expression during wound healing in a mouse model of critical sized calvarial defect

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Bone diseases or defects present major clinical problems especially in the elderly despite its capacity of regeneration. Given that macrophages play critical roles in wound healing, we investigated the age-related changes to macrophage phenotypes during an early period of bone healing in a mouse model of critical sized cranial bone defect. In this model, micro CT imaging at 2-week intervals showed less amounts of bone formation in the aged (15~18-month old) than in the young (10-week old). Confirmed by RT-qPCR and RNA sequencing, the aged highly expressed pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) at all the time points checked in this study, compared to the young mice. Also, the levels of arginase1 in the aged were sustained much higher for up to 7 days. Transcriptomic analyses revealed that the dynamic regulation along wound healing processes tended blunt in the aged than in the young, especially for the expression of M2 signature genes. Furthermore, the aged increased to express the gene sets of secretory factors but decreased the Reactomes of innate immune system and interleukin 4 and interleukin 13 signaling, suggesting that the aged highly expressed secretory factors yet executed with lesser efficiency than the young did. Taken together, findings in this study indicates that the healing process in the aged is of stagnated features while the young progressed in a dynamic manner and suggest that macrophage phenotype switching during bone wound healing would be dysregulated. Based on these features, new strategies of therapeutics could be developed for the aged.

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Keywords: aging, bone defect, wound healing, macrophage, transcriptomic analysis

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Introduction

Bone wound healing is a complex but highly organized process that involves interactions between various immune cell types and the extracellular micro-environment (Vi et al., 2015; Baht et al., 2018). Macrophages play a key role during the full process of tissue repair and or regeneration (Chazaud et al., 2014; Dort et al, 2019). Macrophages are derived from myeloid lineage cells and execute a variety functions upon the micro-environments which leads macrophage to distinct phenotypes.

Upon injury, macrophages recruit to wound site from peripheral blood next to neutrophils that quickly release inflammatory cytokines and chemokines (Sinder et al., 2015). Pro-inflammatory, classically activated macrophages, named as M1, are associated with the first phases of acute inflammation. M1 macrophages can be induced by induced by interferon, lipopolysaccharide or tumor necrosis factor and secrete reactive oxygen and nitrogen intermediates, and inflammatory cytokines (IL-1, TNF, IL-6), and chemokines (CXCL9, CXCL10), executing phagocytosis and cleaning. When the inflammatory response by M1 macrophage is over, the M2 macrophage begins anti-inflammation process (Atri et al., 2018). M2 macrophage is induced by IL-4 and IL-13 (Chazaud et al., 2014) and highly express YM1, arginase 1, CCL24 and CCL17 (Gordon et al., 2010; Stein et al., 1992). M2 macrophages have been made to further classify such as M2a, M2b, M2c, and M2d (Martinez et al., 2008). M2a macrophages are induced by IL-4 and IL-13 through activation of the STAT6 pathway via receptor IL-4Ra. The role of M2a is tissue repair and antifungal response (Chistiakov et al., 2015; Wang et al, 2020). M2b macrophages are immune complexes and IL-1R or TLR agonists and mainly participates in the regulation of immune responses (Zhang et al., 2010; Wang et al., 2020). M2c macrophages are induced by IL-10. Mer receptor tyrosine kinase and

exhibits potent anti-inflammatory and phagocytic effects (Lu et al., 2013; Lee et al., 2019). M2d macrophages, a TLR agonist and belongs to a newly identified macrophage induced by IL-6 (Gundra et al., 2013; Girgis et al., 2014).

In the elderly, including healthy people, higher levels of circulating pro-inflammatory cytokines are found (Rea et al., 2018; Singh et al., 2011), which is related to the predisposition to the extents of chronic inflammatory conditions such as osteoporosis, Alzheimer's disease, type 2 diabetes, atherosclerosis, and Parkinson's disease (Furman et al., 2019). In experimental animal models, dysregulation of systemic inflammation negatively affects bone formation and fracture healing outcomes (Lin et al., 2019). It was suggested that delayed and incomplete healing of bone defects might results from changes in the activity of macrophages with age-related changes (Linehan et al., 2015) and the dysregulated expression of chemokines and cytokines in aged macrophages compared to young (Gibon et al., 2016; Hebb et al., 2018). However, the specific factors or signaling pathways remain to be elucidated. In this study, we investigated the age-related changes to macrophage phenotypes during an early period of bone healing through transcriptomic analyses.

Materials & Methods

Animals and bone defect model

C57BL/6 mice (10-week-old or 15-month-old) were purchased from OrientBio (Seoul, Korea). All the procedures were approved by the Institutional Review Board of Seoul National University (authorization number SNU-200512-3). The mice were subjected to anesthesia using isofurane and Zoletil50. The scalp was incised, and the periosteum was spread on both sides. Using a 3-mm diameter trephine bur, a circular bone defect was made. The periosteum and skin were sutured for each layer.

Live micro-CT

We examined bone healing in the young and the aged using live micro-CT (n=6). every 2 weeks until 8 weeks. The μ-CT imaging system (Quantum GX, perkin elmer, America) was equipped with a 5-μm focal spot X-ray tube as a source and a flat panel detector. A cylindrical ROI with the dimensions of the primary injury site was calculated, based on the diameter of the drill needle and the width of the bone at the injury site.

Total RNAs extraction, quantitative real-time PCRs, & transcriptome sequencing

Both the mice were sacrificed after 1 day (n=9), 4 days (n=5~9), or 7 days (n=5~9), and the samples around wound bed were harvested. Total RNAs were extracted using Trizol reagent (Qiagen, Hilden, Germany). cDNA was synthesized using TB Green Ex Taq II (TAKARA, Japan). Quantitative real-time PCRs (RT-qPCR) were performed on a Real-time PCR system using SYBR Premix Ex TaqTM according to the manufacturer's instructions. Mouse primer sequences used in this study list in Table 1. The relative levels of the target gene mRNAs were normalized to those of glyceraldehyde-3phosphate dehydrogenase (GAPDH). Transcriptome sequencing of each group (n=4) was put to Macrogen (Seoul, Korea).

Statistics

Statistical analysis was performed using repeated analysis of variance (ANOVA) using SPSS software (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

Results

Bone repair in critical sized defects

To validate the suitability of the animal model to our study, time-course images were reconstructed from 0 time to 8 weeks post-op by using a live micro-CT, and bone volumes were measured (Figure 1A). The amount of bone formation at the site of defect was determined by subtracting volume $_{0\text{-time}}$ from volume $_{x\text{-week}}$, and the differences in amounts were compared between the young (10-week old) and the aged (15~18-month old) mice (Figure 1B). Bone tissue was gradually formed at the defect sites in both groups. However, larger amounts of bone formation were significantly observed in young mice (p <0.05), indicating that this model could be suitable to investigate the underlying events of age-related alteration in bone repair. Based on this feature, this animal model has been adapted in following experiments.

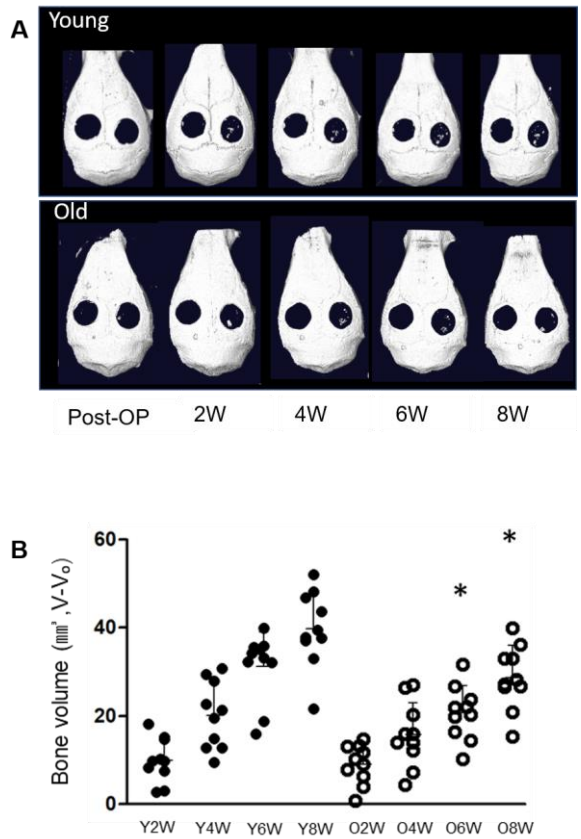

Figure 1. (A) Reconstructed, time-course micro-CT images around the bone defects in the young and aged mice. Representative ones were shown (n=8). (B) Amounts of new bone formation. *Significantly deferent from the young group (ANOVA, $p<0.05$).

Expression of monocyte/macrophage-related genes

Expression of monocyte/macrophage-related genes was analyzed by RT-qPCRs from individual samples which were filled in wound beds (n=5~9 mice each group). Markers of monocyte-macrophage lineage such as adgre1 (F4/80), cd68, and cd86 were considerably expressed in all the groups. Adgre1 expression increased time-dependently in both the mice, however, the aged mice expressed much lower levels of adgre1 transcripts at all the time points examined with large individual variations than the young mice did (p <0.05) (Figure 2). Another marker cd68 expression also increased along with time in both the mice. On the contrary to adgre1, cd68 was expressed at higher levels in the old than in the young (p <0.05). On the other hand, expressions of itgam (also known as macrophage-1 antigen, Mac1 or cd11b), a component of Integrin $\alpha_M\beta2$, were declined in both the mice. Its levels in the young tended slightly lower than those in the old at all the time points examined. Given that integrin $\alpha_M\beta2$ is expressed on the surface of many leukocytes involved in the innate immune system, including granulocytes, monocytes, macrophages, and natural killer cells, the level of itgam would reflect the recruitment activity of several leukocytes.

Pro-inflammatory cytokines, TNF-α and IL-1β, were expressed at much greater levels on day 1 in the aged mice (p <0.05, Figure 3). The levels of TNF-α and IL-1β transcripts and their differences between the young and the old decreased along with time. TGF-β1, a representative anti-inflammatory cytokine, was highly expressed in both the mice, of which levels were increased with time (Figure 4). On day1, old mice expressed TGF-β 1 at a greater level than young mice did (p <0.05). Later on, due to large individual variations, the differences between the young and the old were not significant. Expression patterns of IL-10 were similar to those of TGF-β 1.

It was notable the expression profiles of macrophage phenotype markers, nos-2 (M1) and arg-1 (M2), in the young and the old (Figure 5). Young mice highly expressed nos-2, a representative M1 marker up to on day 4, and the levels were

declined on day 7 (p <0.05). Meanwhile, in old mice nos-2 was expressed and sustained at some lower extents. Contrastively, arg-1, a representative M2 marker was expressed in old mice at much higher levels than in young mice (p <0.05). These features suggest the age-related dysregulation of macrophage phenotype switching during the bone healing.

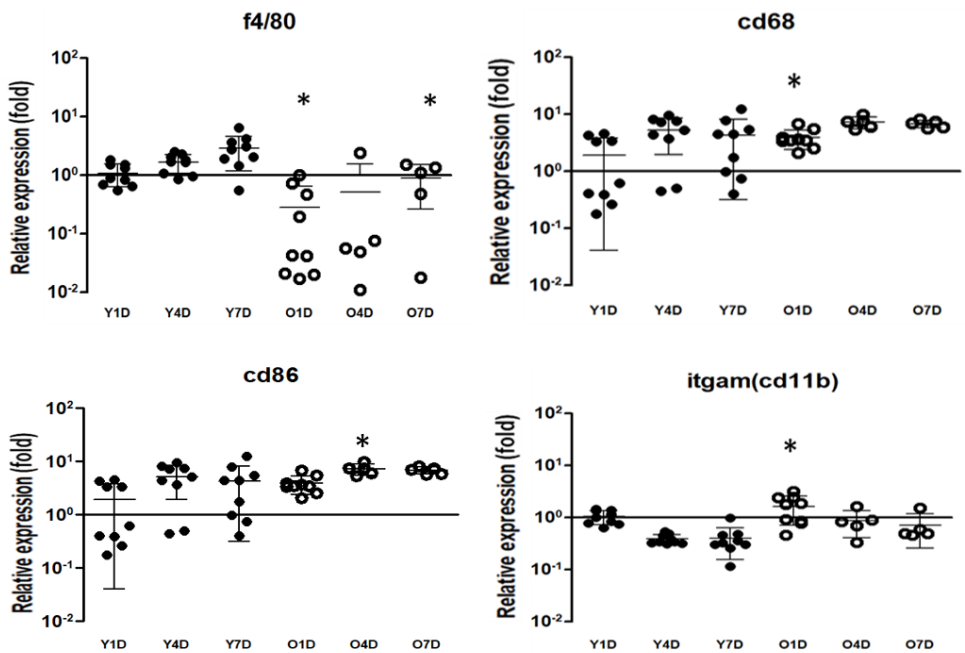

Fig 2. Expression of monocyte/macrophage-related genes in individual wound beds of critical-sized bone defects (n=5~9). Relative amounts of each genes were shown to the levels in the 1-day young group. Individual values are presented, and horizontal bars are indicated to average ± SD. *Significantly deferent from the young group (ANOVA, p<0.05).

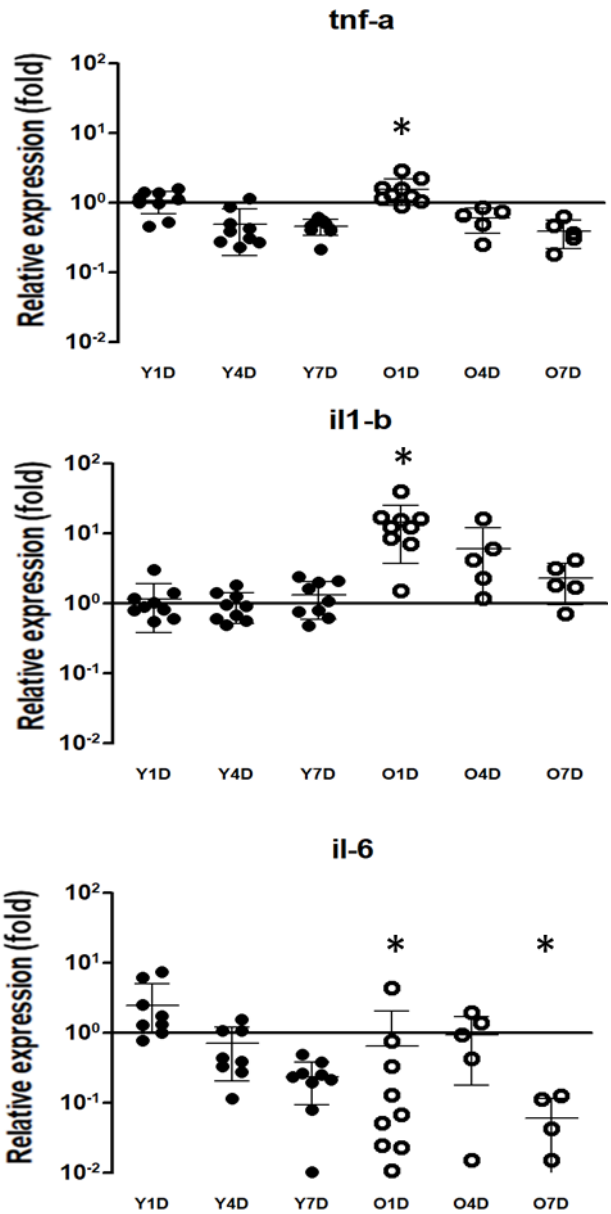

Fig 3. Expression of classical M1 macrophage markers in individual wound beds of critical-sized bone defects (n=5~9). Relative amounts of each genes were shown to the levels in the 1-day young group. Individual values are presented, and horizontal bars are indicated to average ± SD. *Significantly deferent from the young group (ANOVA, p<0.05).

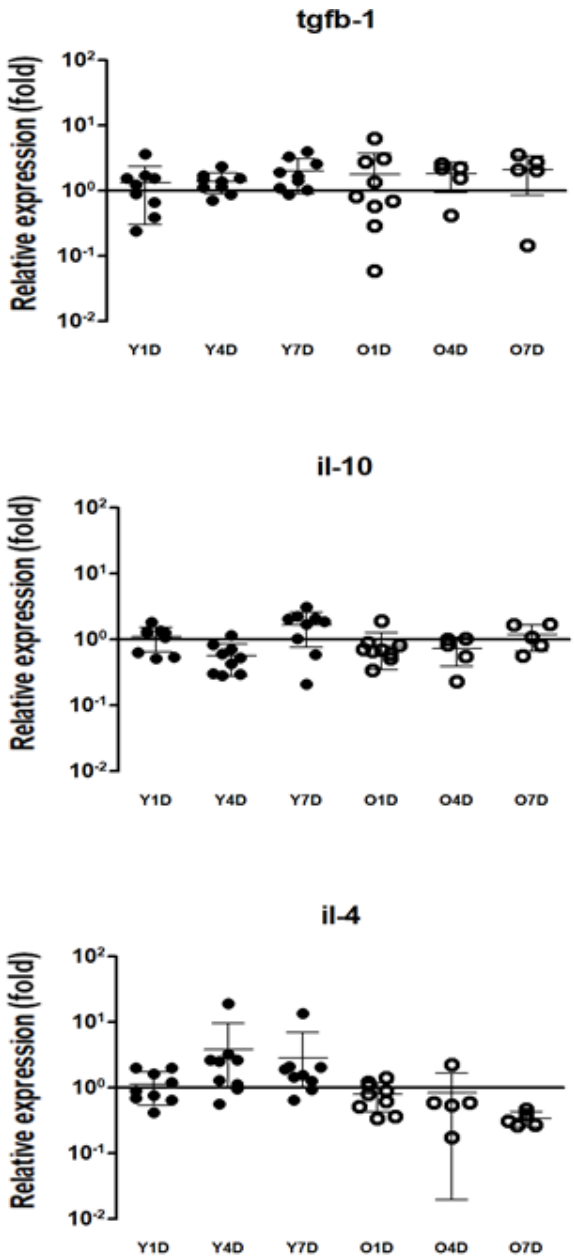

Fig. 4 Expression of M2 macrophage markers in individual wound beds of critical-sized bone defects (n=5~9). Relative amounts of each genes were shown to the levels in the 1-day young group. Individual values are presented, and horizontal bars are indicated to average ± SD. *Significantly deferent from the young group (ANOVA, $p<0.05$).

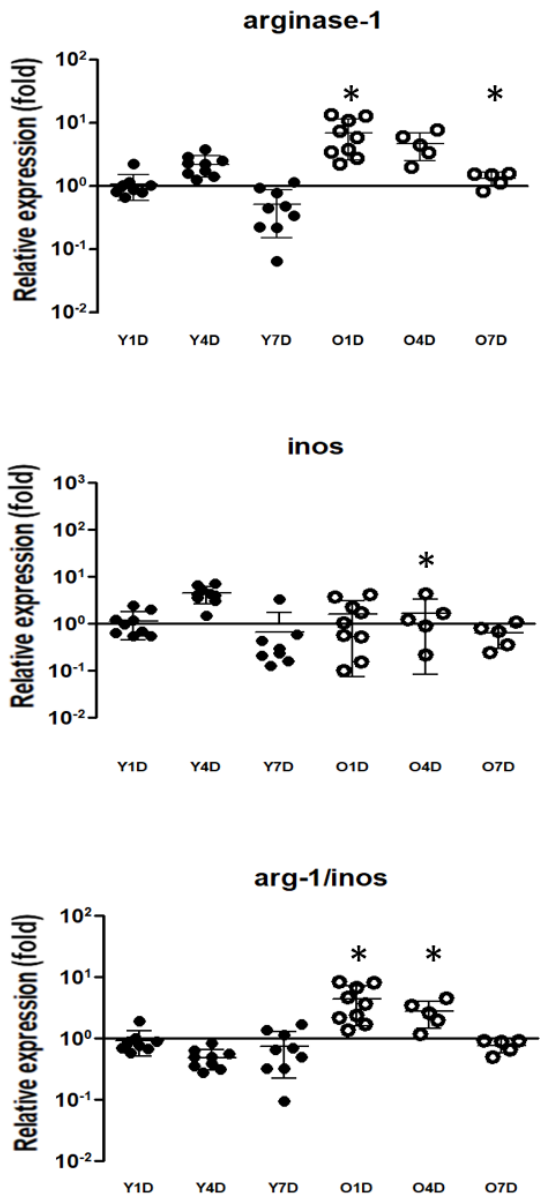

Fig 5. Expression of M1 phenotype marker (iNOS) and M2 phenotype marker (arginase-1) in individual wound beds of critical-sized bone defects (n=5~9). The expression ratio of arginase-1 to iNOS (M2/M1) was calculated. Relative amounts of each genes were shown to the levels in the 1-day young group. Individual values are presented, and horizontal bars are indicated to average ± SD. *Significantly deferent from the young group (ANOVA, $p<0.05$).

Transcriptomic analysis (RNA sequencing)

It was examined larger transcriptomic repertoire of macrophage phenotype markers during bone wound healing through RNA sequencing. Principal component analyses of top 100 transcripts in full transcriptomes from 4- and 7-day wound beds of both the mice showed that the difference in a time-course expression was at much lesser extents in the old mice than in the young mice (Figure 6A). A dendrogram with heatmap also confirmed the similarity of O7 to O4 and the distinctiveness of Y7 to Y4 (Figure 6B). These features suggest that the healing process in the old mice was somewhat stagnated, while the young mice progressed in a dynamic manner. Narrowing transcriptomic repertoire, macrophage in vivo signature genes were selected based on literature and analyzed. M1 and M2 gene signatures *in vivo* were shared, consistent with previous reports. As confirmed by principal component analyses, the time-course difference of M2 gene signature in the old mice was not far apart, while that of M1 gene signature in the old was as distinct as in the young (Figure 7A). A dendrogram for M2 genes showed a dissimilarity of O7/O4 to Y7/Y4 (Figure 7B). Gene set enrichment analyses revealed that expression in old mice were higher enrichment in the gene sets of Secretory Factors, Matrisome, Matrisome-Associated, and Cytokine, Cytokine Receptor, Interaction (Figure 8A). However, the old mice decreased the Reactomes of Innate Immune System (related to M2b switching), Interleukin 4 and Interleukin 13 Signaling (related to M2a switching), Cytokine Signaling in Immune System and Signaling by Interleukins, compared to young mice (Figure 8B). These results indicate that the old mice highly expressed secretory factors yet executed with lesser efficiency than the young mice did.

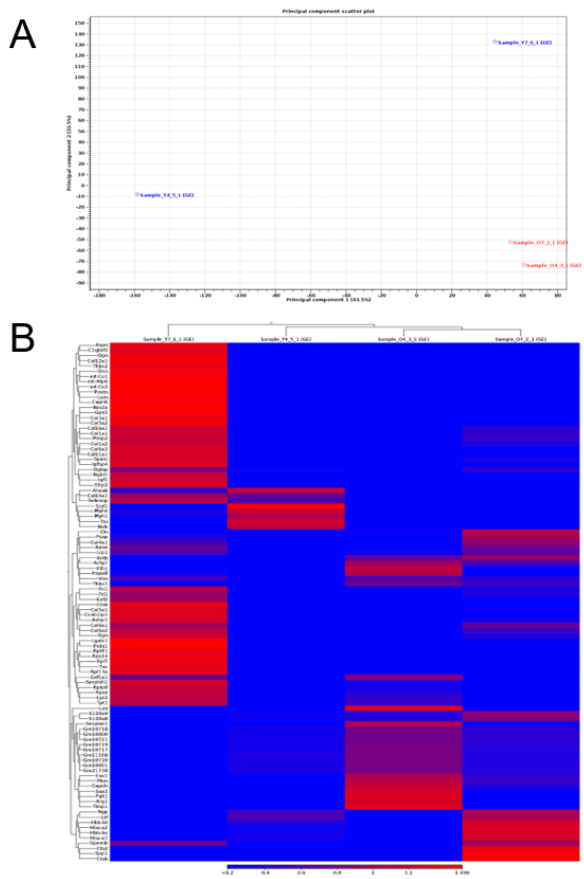

Figure 6. Scatter plot of principal component 1 versus PC 2 scores. Each point is represented by symbol denoting its analytic cluster young and old 4 days, 7 days. (A) High concordance between the transcriptoms of freshly isolated macrophages derived from calvarial defects. Heat map depicts the mean expression levels of the top 100 gene markers for calvarial macrophages.(B)

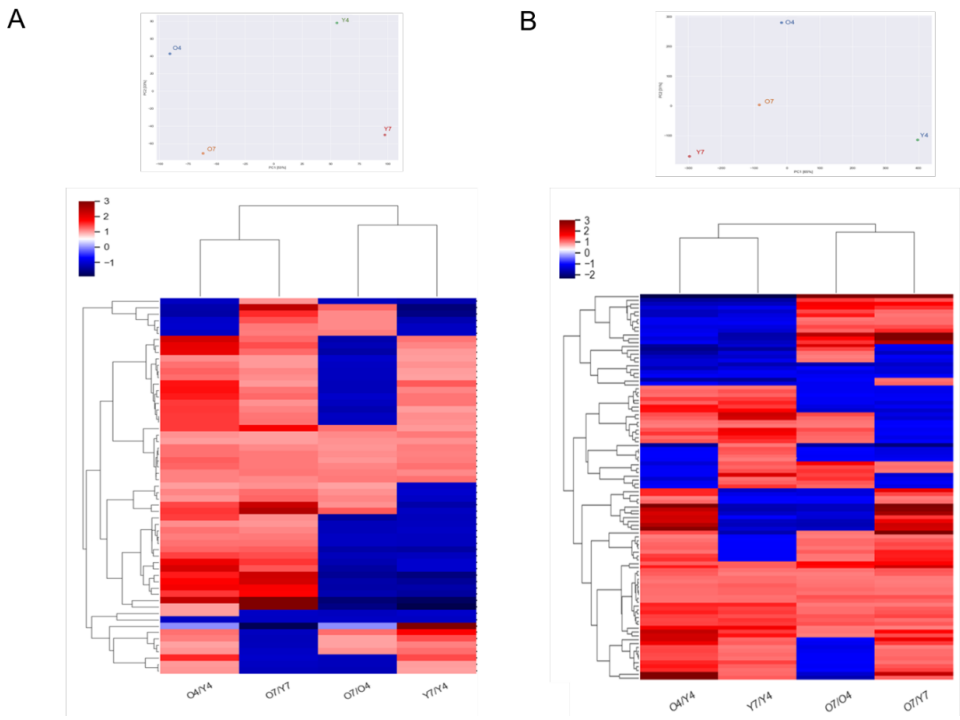

Figure 7. Scatter plot of principal component 1 versus PC 2 scores. Hierarchal clustering demonstrates O4/Y4, O7/Y7, O7/O4, Y7/Y4 macrophages treated. (A,B)

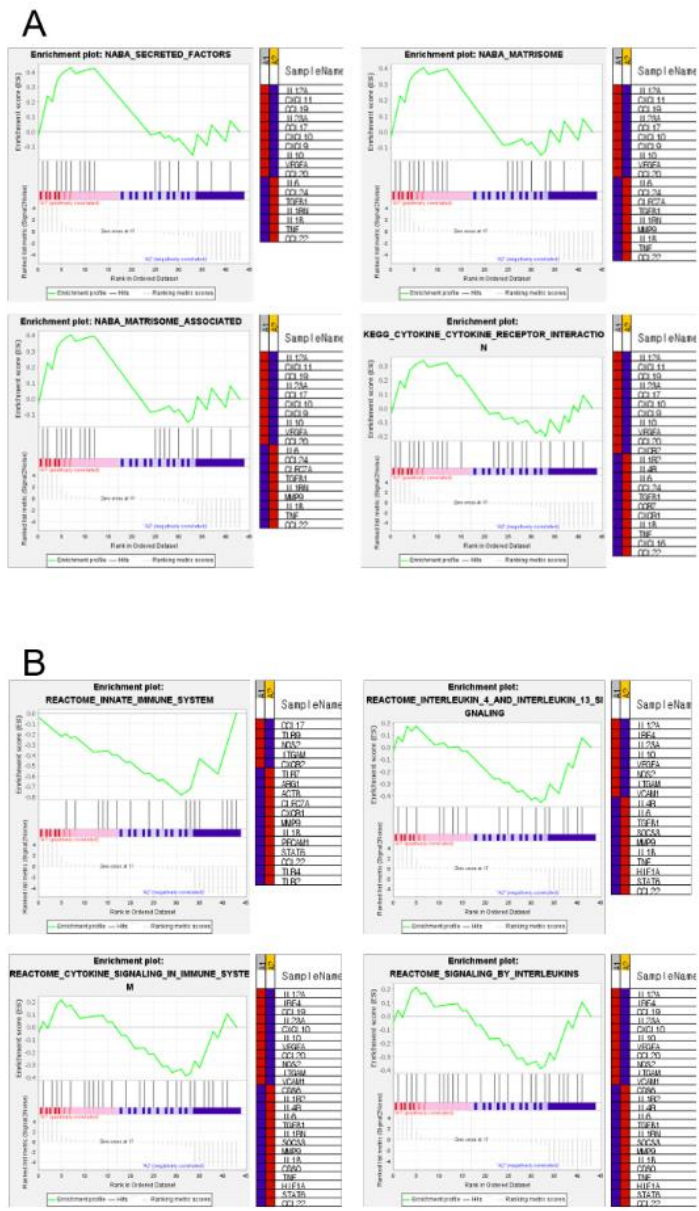

Figure 8. GSEA enrichment plot (score curves). Gene set enrichment analysis (GSEA) was biological process, gene sets in GSEA molecular signatures database. The green curve corresponds to the ES (Enrichment Score) curve, which is the running sum of the weighted enrichment score obtained from GSEA software. (A,B)

Discussion

Bone is one of the few tissues that can be continuously remodeled and often regenerated after injury (Wallace et al., 2020). Despite this remarkable intrinsic capacity, bone healing continues to present major clinical problems especially the elderly (Schilckewei, et al., 2019; Meinberg et al., 2019). Thus, the new therapeutic strategy of bone regeneration should be developed for the elderly. To do so, in this study, we first validated a model to investigate the underlying cellular molecular events. It was observed that new bone tissue was formed in both the mice and that the increase in bone volume was slower in the aged than in the young (Fig. 1), indicating a suitable model.

An aged macrophage phenotype can be detrimental to bone wound healing. Using RT-qPCR, we confirmed that inflammatory cytokines were higher in aged mice and persisted (Fig. 3), consistent with previous reports that aged mice have a more M1, pro-inflammatory gene signature young mice (Wynn et al., 2013; Reidy et al., 2019; Tabula et al., 2020). In addition, The difference was observed in the gene expression signatures of early inflammatory and immune cells that infiltrated wound site in young and aged mice. Despite the levels of cd68 were slightly higher in the aged at day 1, expression of F4/80, indicating recruitment of monocyte/macrophage-lineage cells, were much lower levels in the aged mice than in the young ones (Fig. 2).

M2 macrophages initiate down-regulation of the inflammatory response with production of TGF-β1, IL-4 and IL-10. Also these macrophages promote tissue repair and regeneration through the production of growth factors (Johnson et al., 2014; Mosser et al., 2021). We observed that the levels of anti-inflammatory cytokines were slightly higher in the young than in the aged (Fig. 4). In addition, individual variations were large in the aged, especially at day 1. According to the principal component scatter plot graphs (Fig. 6 & 7), the difference between young 4 days and young 7 days was large, while little difference between 4 days and 7 days in the aged. These feature suggest that the transition from M1 to M2 was dysregulated in the aged, resulting in bone healing delayed. Specifically, gene set enrichment analyses revealed that expression in aged mice were higher enrichment

in the gene sets of Secretory Factors, Matrisome, Matrisome-Associated, and Cytokine, Cytokine Receptor, Interaction. However, the aged mice decreased the Reactomes of Innate Immune System (related to M2b switching), Interleukin 4 and Interleukin 13 Signaling (related to M2a switching), Cytokine Signaling in Immune System and Signaling by Interleukins, compared to young mice (Fig. 8). These features in this study suggest that the aged mice highly expressed secretory factors despite executed with lesser efficiency than the young did

Summary

In this study, it was investigated the age-related changes to macrophage phenotypes during bone healing of critical sized cranial bone defects in the C57BL/6 mice 10-week old (young) or 15~18-month old (aged). The aged ones highly expressed pro-inflammatory cytokines (il1β, il6, tnf) at all the time points, and the levels of arginase1 in the aged were also sustained much higher. Transcriptomic analyses of macrophage-related genes revealed that the dynamic regulation along wound healing processes tended blunt and stagnated in the aged, compared to the young, especially for the expression of M2 signature genes. Furthermore, the aged increased to express the gene sets of secretory factors but decreased the Reactomes of innate immune system and interleukin 4 and interleukin 13 signaling, suggesting that the aged highly expressed secretory factors yet executed with lesser efficiency than the young did. Taken together, findings in this study indicates that the inflammatory process in the aged is of stagnated features, while the young progressed in a dynamic manner. Macrophage phenotype switching during bone wound healing was dysregulated. These features can lead to a delayed bone wound healing. Based on these features, new strategies of therapeutics could be developed for the aged.

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Table 1. Sequence of Primers used in RT-qPCRs

Molecules	Primer sequence used in quantitative PCR	Accession No. (Gene bank)
GAPDH	5’-GGTCGGTGTGAACGGATTTG-3’ 5’-TGTAGACCATGAGTTGAGGTCA-3’	NM-008084.2
Arginase 1	5’-ACAAGACAGGGCTCCTTT-3’ 5’-TGAGTTCCGAAGCAAGCC-3’	NM-007482.3
Cd11b	5’-ATGTGAGCCCCATAAAGCAG-3’ 5’-ATCTTCGCAGCATTCTCCC-3’	NM-001082960.1
Cd68	5’-GGACCTACATCAGAGCCCGA-3’ 5’-TGAATGTCCACTGTGCTGCC-3’	NM-001291058.1
Cd86	5’-CAGCACGGACTTGAACAACC-3’ 5’-TCCACGGAAACAGCATCTGA-3’	NM-019388.3
IL-1β	5’-TTTGAAGTTGACGGACCCCA-3’ 5’-GGGAAAGACACAGGTAGCT-3’	NM-0083613.4
IL-6	5’-TCTCTGGGAAATCGTGGAAATGA–3’ 5’-GGTACTCCAGAAGACCAGAAGG–3’	NM-031168.2
IL-10	5’-CCCAGAAATCAAGGAGCATT-3’ 5’-TCACTCTTCACCTGCTCCAC-3’	NM-010548.2
INOS	5’-CTGGGAGCGCTCTAGTGAAG-3’ 5’-ACTACCCCTGCTTTATGGCG-3’	NM-001313921.1
TGFβ-1	5’-CACCATCCATGACATGAACC-3’ 5’-CAACCCAGGTCCTTCCTAAA-3’	NM-011577.2
TNF-α	5’-CCCATATACCTGGGAGGAGTC-3 5’-TCACAGAGCAATGACTCCAAA-3’	NM-013693.3

국문초록

마우스 두개관 손상 모델에서 증령에 따른 대식세포의 발현 변화

서울대학교 대학원 분자유전학 전공

김재형

(지도교수 우경미)

증령에 따라 뼈 조직 손상 치유가 지연되는 원인을 알아보고자 마우스 두개관 손상 모델을 이용하여 뼈조직 치유과정에서 증령에 따른 대식세포 표현형 전사체 발현 변화를 분석하였다. 10 주령 및 15~18 월령 C57BL/6 수컷 마우스의 두개골에 외과적으로 임계크기 손상을 만들어서, 뼈조직 손상 후 1~7 일 후 창상부위 시료에서 total RNAs 를 추출하였다. 마이크로 씨티 분석으로 나이든 (15~18 개월) 마우스가 젊은 (10 주령) 마우스보다 골 형성이 덜 된 것을 확인하였다. RT-qPCR 결과, 젊은 마우스와 비교했을 때 나이든 마우스에서 전염증성 사이토카인 IL-1β, IL-6, TNFα가 더 높게 발현되었으며, arginase 1 을 비롯한 M2 마커 유전자가 관찰 초기부터 지속적으로 높게 발현이 유지된 것을 확인하였다. 전사체 분석을 통해 젊은 마우스에서 치유과정 중 전사체 발현이 역동적으로 변화한데 반해 노령 마우스는 전사체 발현 변화가 둔화되어 있음을 관찰하였다. 또한, 노령 마우스는 ‘gene sets of secretory factor’를 증가시켰지만 선천 면역의 반응과 interleukin 신호전달 감소를 제시하는 결과를 얻었다. 이와 같은 결과는 나이든 마우스는 뼈조직 손상 치유 중 대식세포 M2 표현형 전환 조절 이상과 분비인자들에 대한 신호

전달 이상을 시사하며, 이는 고령 환자에서 뼈조직 재생 치료전략의 단서를 제공할 수 있을 것이다.

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주요어: 노화, 뼈손상, 치유, 대식세포, 전사체 분석
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