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授与した学位	博　士
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学位授与の要件	医歯薬学総合研究科機能再生・再建科学専攻 （学位規則第４条第１項該当）
学位論文の題目	Positive regulation of S-adenosylmethionine on chondrocytic differentiation via stimulation of polyamine production and the gene expression of chondrogenic differentiation factors （S-アデノシルメチオニンはポリアミン産生および軟骨分化関連因子の遺伝子発現を介して軟骨分化を正に制御する。）
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学位論文内容の要旨

S-adenosylmethionine (SAM) is an intermediate metabolite playing key role in many biological processes such as methylation, polyamine synthesis and transsulfuration pathway. Over 50 years, some studies have focused on SAM and consequently making it available in pharmaceutical market as a dietary supplement and a viable alternative treatment for depression and liver disorders. Along with that, SAM has been found to alleviate symptoms of degenerative cartilage diseases, although its mechanism is not clear. In this study, I hypothesized that polyamines and cellular communication network factor 2 (CCN2) could be involved in the chondroprotective action of SAM. Because it was discovered that internal polyamines following the increase of ornithine decarboxylase (ODC) activity stimulate expression of differentiated phenotype of cultured rabbit costal chondrocytes. Equally important, CCN2 is known as the crucial factor in the proliferation and differentiation of chondrocytes.

To test our theory, the present study involved a thorough investigation of cultured chondrocytes following the application of SAM, with a focus on chondrocytic phenotype markers, polyamine production and regulation of CCN2. Regarding the research model, the human chondrocyte-like cell line-2/8 (HCS-2/8) was selected based on its proven suitability as substitute for human primary articular chondrocyte, which maintained

differentiated chondrocyte phenotype and superior proliferative capacity. To strengthen this result, rat chondrocyte-like cell line (RCS) was also used as the supplementary research model, and they showed the same results. Through alcian blue staining, the application of SAM indicated a noticeable increase on aggrecan accumulation in HCS-2/8 cells. The most improvement reached average increase 10.6% on the 7th day and 31.3% on the 14th day, occurred with the administration of SAM at 10 μg/ml.

Next, to figure out the effect of SAM on cell proliferation, a WST-8 assay and cell counting were conducted, the results of which indicated that the increase of aggrecan accumulation induced by high concentrations of SAM does not depend on the proliferation of chondrocytes. Therefore, the elevated alcian blue staining was caused by enhancive effect of SAM on aggrecan. In addition, SAM promoted gene expression of cartilage-specific matrix markers (aggrecan and type II collagen), Sry-Box transcription factor 9 (SOX9), and chondroitin sulfate biosynthetic enzymes (Chondroitin sulfate synthase and chondroitin sulfate N-acetylgalactosaminyltransferase). Especially, these two enzymes are not only makers but also directly enhance glycosylation of aggrecan. Conversely, the blockade of methionine adenosyltransferase 2A (MAT2A) enzyme catalyzing intracellular SAM biosynthesis, which was caused by either AG-270 inhibitor or siRNA against MAT2A, limited alcian blue staining and gene expressions induced by SAM addition in chondrocytes. Subsequently, the gene expression and protein levels of CCN2 under SAM stimulation were examined because CCN2 is known to stimulate the gene expression and production of cartilage-specific ECM in growth plate and articular chondrocytes. It came out that SAM enhanced both gene expression and protein level of CCN2, which in turn was limited by the MAT2A knockdown. Moreover, the polyamine level in chondrocytes observed through polyamineRED staining was higher in SAM-treated culture than control culture. Equally important, expression of *ODC,* the rate-limiting enzyme of polyamine synthesis, was found 28.2% higher in the SAM-treated group than in the untreated group. HPLC analysis validated the result by showing the internal concentration of spermine and

spermidine were promoted significantly in SAM-treat group as compared with control group. To further confirm the contribution of polyamine synthesis to mechanism of SAM action, we used an inhibitor of the rate-limiting enzyme of polyamine synthesis, which is a-difluoromethylornithine (DFMO), an ODC inhibitor. The results of the alcian blue staining revealed that the impact of polyamine on the production of aggrecan was significant, with a reduction of over 50% under the presence of DFMO. Additionally, the effect of SAM on aggrecan accumulation was completely nullified by DFMO. Moreover, the RT-PCR analysis showed that the expression of the genes of interest was brought down to the basal level by DFMO, regardless of the presence of SAM. which demonstrates that the stimulation of these gene expressions by additional SAM is dependent on polyamine synthesis. All experiments performed on HCS-2/8 cells were conducted using the same methodology on RCS cells and the outcome demonstrated nearly consistent trend.

In conclusion, this study provides insights into the effects of SAM on chondrocyte differentiation. These results suggest that the stimulation of polyamine synthesis and gene expression of chondrogenic differentiation factors, such as CCN2, account for the mechanism underlying the action of SAM on chondrocytes.

論文審査結果の要旨

[Introduction] S-adenosylmethionine (SAM) is considered to be a useful therapeutic agent for degenerative cartilage diseases, although its mechanism is not clear. It was reported that polyamines stimulated the expression of differentiated phenotype of chondrocytes. On the other hand, the cellular communication network factor 2 (CCN2) was reported to play a huge role in the proliferation and differentiation of chondrocytes. Therefore, polyamines and CCN2 could be involved in the chondroprotective action of SAM.

[Methods] The present study was performed by using human and rat chondrosarcoma derived cell (HCS-2/8 and RCS, respectively) following the administration of SAM with a focus on chondrocyte phenotype markers, polyamine production and regulation of CCN2. Regarding the research tools, alcian blue staining, WST-8 assay, cell counting assay, RT-qPCR, Western blotting, PolyamineRED and HPLC assays were mainly used in this study.

[Results] Exogenous SAM enhanced proteoglycan production but not cell proliferation in HCS-2/8 and RCS cells. Moreover, SAM enhanced gene expression of cartilage-specific matrix (aggrecan and type II collagen), Sry-Box transcription factor 9 (SOX9), CCN2, and chondroitin sulfate biosynthetic enzymes. The blockade of the methionine adenosyltransferase 2A (MAT2A) enzyme synthesizing intracellular SAM restrained the effect of SAM on chondrocytes. The spermidine and spermine level in chondrocytes were higher in SAM-treated culture than control culture. Additionally, alcian blue staining and RT-qPCR indicated that the effects of SAM on the production and gene expression of aggrecan were reduced by the inhibition of polyamine synthesis.

[Discussion] These results suggest that the stimulation of polyamine synthesis and gene expression of chondrogenic differentiation factors, such as CCN2, account for the mechanism underlying the action of SAM on chondrocytes.

This study has revealed the mechanism of chondrocyte differentiation by SAM. The results present novel findings and lay the groundwork for future research in the field. This article has already been accepted in International Journal of Molecular Sciences. Therefore, the defense committee hereby accepts this article as a doctoral dissertation in dentistry/ philosophy.