

# 学位論文の要旨

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学位論文名      Advanced Glycation End Product 3 (AGE3) Suppresses the Mineralization of Mouse Stromal ST2 Cells and Human Mesenchymal Stem Cells by Increasing TGF- $\beta$  Expression and Secretion

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## 論文内容の要旨

### INTRODUCTION

Both diabetes mellitus (DM) and osteoporotic fractures are important healthcare problems, because they affect quality of life and increase morbidity and mortality. Previous studies have shown that patients with type 2 DM (T2DM) have a 1.4- to 4.7-fold increased risk of fracture, although they have normal or slightly increased bone mineral density (BMD). These findings suggest that fracture risk in T2DM is not reflected by BMD but is related to bone fragility independent of BMD.

Hyperglycemia in DM accelerates the formation of advanced glycation end products (AGEs), which result from a chain of chemical reactions in plasma or tissue that follow nonenzymatic browning reactions between reducing sugars and free reactive amino groups of proteins. The cellular interactions of AGEs are mediated by the receptor for AGE (RAGE). We previously showed that AGEs suppressed the mineralization of mouse stromal ST2 cells.

Transforming growth factor (TGF)- $\beta$  is a multifunctional polypeptide that regulates a variety of cellular functions. Previous animal studies have shown that reducing TGF- $\beta$  signaling leads to increases in functional parameters of bone quality. Moreover, another study has shown that inhibition of TGF- $\beta$  type 1 receptor kinase has anabolic and anti-catabolic effects on bone. These reports suggest that enhancement of its signal causes bone quality deterioration. In addition, a number of reports suggest that AGEs can cause diabetic complications in the kidneys, nerves, and retina by increasing TGF- $\beta$  levels. However, whether AGEs adversely affect osteoblastic mineralization and differentiation during diabetic bone disorder by enhancing TGF- $\beta$

signals is unknown. We therefore examined the roles of TGF- $\beta$  in the AGE-induced suppression of mineralization of ST2 cells and human mesenchymal stem cells (hMSCs).

### **MATERIALS AND METHODS**

AGE3 was prepared by incubating 50 mg/mL BSA with 0.1 M glycolaldehyde at 37 °C for 7 days under sterile conditions in 0.2 M sodium phosphate buffer (pH 7.4) containing 5 mM DTPA. After the incubation period, low-molecular-weight reactants and aldehydes were removed by using a PD-10 column and dialyzing against PBS. Mouse ST2 cells were cultured under 5% CO<sub>2</sub> at 37 °C in  $\alpha$ -minimum essential medium (MEM) supplemented with 10% fetal bovine serum and antibiotics. To induce osteoblastic differentiation, ST2 cells were cultured in  $\alpha$ -MEM supplemented with 5 mM  $\beta$ -glycerophosphate, 100  $\mu$ g/mL ascorbic acid, and 100 ng/mL BMP-2 after reaching confluence. hMSCs were cultured in MSC basal medium. To induce osteoblastic differentiation, hMSCs were cultured in MSC basal medium supplemented with  $\beta$ -glycerophosphate, 100  $\mu$ g/mL ascorbic acid, and dexamethasone after reaching confluence. The mineralization of ST2 cells and hMSCs was assessed in 6-well or 12-well plates by using von Kossa staining and Alizarin Red staining on days 7, 14 and 21. Total RNA was collected on days 1, 3, 5, and 7, and Osteocalcin (OCN), Osterix (OSX) and Runx2 mRNA expressions were measured by real-time PCR. The concentration of TGF- $\beta$ 1 in whole-cell lysates or culture medium was determined by using commercially available diagnostic kits on days 1, 3, 5 and 7. Cell proliferation was evaluated by BrdU-ELISA on day 3 and the apoptotic cell death was analyzed in an ELISA for DNA fragments at 3 day following the manufacturer's protocols.

### **RESULTS AND DISCUSSION**

AGE3 significantly ( $P < 0.001$ ) inhibited mineralization in both cell types, whereas transfection with siRNA for the RAGE significantly ( $P < 0.05$ ) recovered this process in ST2 cells. AGE3 increased ( $P < 0.001$ ) the expression of TGF- $\beta$  mRNA and protein, which was partially antagonized by transfection with RAGE siRNA. Treatment with a TGF- $\beta$  type I receptor kinase inhibitor, SD208, recovered AGE3-induced decreases in osterix (OSX) ( $P < 0.001$ ) and osteocalcin (OCN) ( $P < 0.05$ ) and antagonized the AGE3-induced increase in Runx2 mRNA expression in ST2 cells ( $P < 0.001$ ). Moreover, SD208 completely and dose-dependently rescued AGE3-induced suppression of mineralization in both cell types. In contrast, SD208 intensified AGE3-induced suppression of cell proliferation as well as AGE3-induced apoptosis in proliferating ST2 cells.

Accumulating evidence indicates that the TGF- $\beta$  signal suppresses osteoblast differentiation in vitro. We showed that AGE3-TGF- $\beta$  signaling has negative effects on, and impairs osteoblastic differentiation in, both cell types at the maturation stage. Moreover, our findings that SD208-induced inhibition of TGF- $\beta$  signaling increased the mineralization of ST2 cells and hMSCs agree with previous animal studies and suggest that AGEs augment these inhibitory effects of TGF- $\beta$  on osteoblastic differentiation.

We found that siRNA-induced inhibition of RAGE recovered AGE3-induced suppression of mineralization and antagonized AGE3-induced increases in TGF- $\beta$  protein in ST2 cells. These results demonstrate that, by binding to RAGE, AGE3 at least contributed to the increased expression of TGF- $\beta$  protein and suppressed mineralization of ST2 cells. AGEs have multiple receptors, including RAGE, CD36, macrophage scavenger receptors, and AGER1, 2, and 3. The presence of multiple AGE receptors may explain why RAGE siRNA had only a partial effect on the cells in our study.

Both Runx2 and OSX are essential transcription factors for osteoblast differentiation and control the expressions of bone-related genes such as OCN. We found that the AGE3–TGF- $\beta$  signal increased Runx2 mRNA expression in ST2 cells on day 14 after treatment with AGE3. Runx2 is known to have contradictory effects during the early, compared with late, stages of osteoblastic differentiation. During early differentiation, Runx2 directs multipotent mesenchymal cells to the osteoblast lineage. In contrast, after becoming immature osteoblasts, Runx2 inhibits their further maturation and transition into osteocytes, keeping osteoblasts in an immature stage. Therefore, the suppression of OCN and OSX expression and the AGE3-associated enhancement of Runx2 expression at the late osteoblastic differentiation stage likely would act in concert to inhibit the differentiation of preosteoblasts to mineralizing mature osteoblasts and seems to be in accord with the AGE3-induced suppression of mineralization of ST2 cells and hMSCs.

Our results suggest that the TGF- $\beta$  signal antagonizes the detrimental effects of AGE3 by increasing cell number and reducing apoptosis in proliferating ST2 cells. These findings contrast with those we obtained by using confluent cells, in which the TGF- $\beta$  signal augmented the detrimental effects of AGE3 on osteoblast differentiation. Therefore, our results also indicate that TGF- $\beta$  has biphasic and contradictory effects in ST2 cells, depending on their maturation stage. These findings suggest that we should consider the extent of cell maturation when we target TGF- $\beta$  signals to treat diabetic osteoporosis. Animal studies using diabetic and nondiabetic wild-type and RAGE knockout mice are needed to demonstrate the role of TGF- $\beta$  in diabetic bone loss and to ascertain the relevance of our findings in the clinical context.

## **CONCLUSION**

We found that, after cells become confluent, AGE3 partially inhibits the differentiation and mineralization of osteoblastic cells by binding to RAGE and increasing TGF- $\beta$  expression and secretion. They also suggest that TGF- $\beta$  adversely affects bone quality not only in primary osteoporosis but also in diabetes-related bone disorder, and that TGF- $\beta$  adversely affects not only kidney, retina, and nerves, but also the bone, as part of diabetic complications.