รายงานวิจัยฉบับสมบูรณ์

โครงการ

ความผิดปกติของการพัฒนาเซลล์สร้างกระดูกในผู้ป่วยเบาหวานชนิดที่ 2 และความสัมพันธ์กับระดับของ soluble RAGE ในเลือด

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เดือน ปี ที่เสร็จโครงการ มิถุนายน 2556

สัญญาเลขที่ MRG5480270

รายงานวิจัยฉบับสมบูรณ์

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สำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยเชียงใหม่

บทคัดย่อ

รหัสโครงการ : MRG5480270

ชื่อโครงการ : ความผิดปกติของการพัฒนาเซลล์สร้างกระดูกในผู้ป่วยเบาหวานชนิดที่ 2 และความสัมพันธ์กับระดับของ soluble RAGE ในเลือด

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ระยะเวลาโครงการ : 2 ปี

บทคัดย่อ:

บทนำ: การศึกษาในสัตว์ทดลองได้แสดงให้เห็นถึงการหมุนเวียนของกระดูก การสร้างกระดูกและการพัฒนาของเซลสร้างกระดูกลดลงในเบาหวานชนิดที่สองซึ่งอาจเป็นผลมาจากการสะสมของแอดวานซ์ไกลเคชั่นเอนโพรดักส์ (advanced glycation end product, AGE) ในเนื้อกระดูก นอกจากนี้ยังมีการศึกษาซึ่งแสดงถึงผลของสารในเลือดจำเพาะซึ่งจับกับแอดวานซ์ไกลเคชั่นเอนโพรดักส์ (receptor of advanced glycation end product, RAGE) ต่อการป้องกันภาวะแทรกซ้อนเรื้อรังของเบาหวาน แต่การศึกษาในผู้ป่วยเบาหวานชนิดที่สองถึงการพัฒนาเซลสร้างกระดูกและความสัมพันธ์ของ AGE การพัฒนาเซลสร้างกระดูกนี้ AGE ยังไม่สามารถสรุปได้แน่ชัด การศึกษานี้จึงมีเป้าหมายเพื่อศึกษาถึงการพัฒนาเซลสร้างกระดูกในผู้ป่วยเบาหวานชนิดที่สองและความสัมพันธ์ระหว่างการพัฒนาของเซลสร้างกระดูกและระดับของ RAGE ในเลือด

ระเบียบวิธีการวิจัย: การศึกษานี้เป็นการศึกษาแบบ cross-sectional ซึ่งรวบรวมผู้ป่วยเบาหวานและอาสาสมัครที่ไม่เป็นเบาหวานที่มีอายุใกล้เคียงกัน ตัวอย่างเลือดจะถูกเก็บเพื่อปั่นแยกเซลต้นกำเนิดเนื้อเยื่อเกี่ยวพันและตรวจระดับ RAGE การตรวจสอบการพัฒนาเป็นเซลสร้างกระดูกจะทำโดยการตรวจระดับอาร์เอ็นเอของยีนที่จำเพาะต่อเซลสร้างกระดูกซึ่งได้แก่ alkaline phosphatase (ALP) , collagen type 1 (COL1) และ osteocalcin (OCN) โดยใช้วิธีการ real-time PCR รวมทั้งยังตรวจสอบการสะสมแคลเซียมโดยการย้อม alizarin red-S นอกจากนี้ยังทำการตรวจสอบการแสดงออกของยีน RAGE และ BAX โดยใช้วิธีการ real-time PCR

ผลการศึกษา: การศึกษานี้ได้รวบรวมผู้ป่วยเบาหวานชนิดที่สองจำนวน 55 คนและอาสาสมัครที่ไม่เป็นเบาหวานจำนวน 21 คน อายุ เพศ และการทำงานของไตไม่มีความแตกต่างกันระหว่างทั้งสองกลุ่ม ระดับน้ำตาลในเลือดหลังอดอาหารมีความแตกต่างกันอย่างมีนัยสำคัญ (148.6±70.4 vs. 102.5±11.9, p <0.0001) ระหว่างทั้งสองกลุ่ม ผู้ป่วยเบาหวานจะมีการพัฒนาของเซลเนื้อเยื่อเกี่ยวพันเบื้องต้นเป็นเซลสร้างกระดูกลดลงเมื่อเทียบกับผู้ที่ไม่เป็นเบาหวาน (7.4% vs 86.7%, p<0.0001) เซลของผู้ป่วยเบาหวานจะมีการแสดงออกของยีน ALP, COL1 และ OCN น้อยกว่าผู้ที่ไม่เป็นเบาหวานถึง 12, 40 และ 15 เท่าตามลำดับ นอกจากนี้ยังไม่มีการสะสมแคลเซียมจากการย้อม alizarin red-S อีกด้วย ระดับ RAGE ในเลือดของผู้ป่วยเบาหวานและผู้ที่ไม่เป็นเบาหวานไม่มีความแตกต่างกัน (471.9±233.8 pg/mL vs 481±213.5 pg/mL, p=0.977) แต่จากการศึกษาการแสดงออกของยีน RAGE ในเซลพบว่าผู้ป่วยเบาหวานจะมีการแสดงออกของยีนที่สูงกว่าอย่างมีนัยสำคัญเมื่อเทียบกับผู้ที่ไม่เป็นเบาหวานที่มีอายุและมีระดับ RAGE ใกล้เคียงกัน นอกจากนี้ยังพบว่าระดับ RAGE ในเลือดไม่มีความสัมพันธ์กับระดับการแสดงออกของยีน RAGE ดังนั้นจึงแสดงให้เห็นว่าเซลของผู้ที่เป็นและไม่เป็นเบาหวานมีการตอบสนองต่อระดับ RAGE ในเลือดที่แตกต่างกัน การศึกษานี้ยังพบว่าเซลของผู้ป่วยเบาหวานจะมีการแสดงออกของ BAX สูงกว่าผู้ที่ไม่เป็นเบาหวานอย่างมีนัยสำคัญและระดับการแสดงออกของ RAGE และ BAX ก็มีความสัมพันธ์กันอย่างมาก ระดับน้ำตาลในเลือดหลังอดอาหาร ระดับการแสดงออกของยีน RAGE และ BAX ต่างก็มีความสัมพันธ์กับการพัฒนาของเซลกระดูกที่เสื่อมลงในผู้ป่วยเบาหวาน แต่ระดับน้ำตาลในเลือดหลังอดอาหารเป็นปัจจัยที่มีผลต่อการพัฒนาของเซลกระดูกที่เสื่อมลงในผู้ป่วยเบาหวานโดยไม่ขึ้นกับปัจจัยอื่น ๆ

สรุปผลการศึกษา: ผู้ป่วยที่เป็นโรคเบาหวานชนิดที่สองจะมีการเสื่อมลงของการพัฒนาเซลสร้างกระดูก ระดับน้ำตาลในเลือดหลังอดอาหารเป็นปัจจัยที่อิสระที่มีผลต่อการเสื่อมลงของการพัฒนาเซลสร้างกระดูกนี้ ระดับ RAGE ในเลือดไม่มีความแตกต่างกันในผู้ที่เป็นและไม่เป็นเบาหวานในขณะที่ระดับการแสดงออกของยีน RAGE สูงกว่าอย่างมีนัยสำคัญในผู้ป่วยเบาหวานจึงแสดงให้เห็นว่าเซลต้นกำเนิดของผู้ป่วยเบาหวานชนิดที่สองมีความไวต่อการถูกกระตุ้นของ RAGE มากกว่าผู้ที่ไม่เป็นเบาหวาน นอกจากนี้การศึกษานี้ยังแสดงให้เห็นว่าระดับของ RAGE ในเลือดไม่สามารถใช้เป็นตัวบ่งชี้ถึงการทำงานเซลสร้างกระดูกในผู้ป่วยโรคเบาหวานชนิดที่สองได้

คำหลัก: แอดวานซ์ไกลเคชั่นเอนโพรดักส์ สารจำเพาะซึ่งจับกับแอดวานซ์ไกลเคชั่นเอนโพรดักส์ การพัฒนาของเซลสร้างกระดูก เซลเนื้อเยื่อเกี่ยวพันเบื้องต้น โรคเบาหวานชนิดที่สอง

Abstract

Project Code : MRG5480270

Project Title : Loss of osteogenic differentiation of peripheral blood-derived mesenchymal stem cells in type 2 diabetes and its correlation with soluble RAGE

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Project Period : 2 years

Abstract:

Introduction: Multiple preclinical studies in type 2 diabetes demonstrated a state of low bone turnover with bone formation defect and impaired osteoblast differentiation in type 2 diabetes, which partly related to an accumulation of advanced glycation end products (AGEs) in extracellular matrix of the bone. However, current evidence showing a defect in osteoblast differentiation and its correlation to AGEs in type 2 diabetic patients is still lacking. A decoy receptor of AGEs, soluble receptor of advanced glycation end product (RAGE), was shown to correlate with chronic diabetic complications. However, it remained to be elucidated whether soluble RAGE is a predictor of bone formation defect in type 2 diabetes. Therefore, this study aims to elucidate osteoblast differentiation in type 2 diabetes and its correlation to serum RAGE level.

Material and Method: The present study is a cross-sectional study included diabetic patients and age-match non-diabetic control. Peripheral blood was taken for isolating mesenchymal stem cells (PBMSC) and measuring serum RAGE level. Osteoblast differentiation was determined by osteoblast-specific gene expression and mineralization. RAGE and BAX expression were used to demonstrate RAGE activation and apoptosis. The osteoblast-specific gene expression, including alkaline phosphatase (ALP), collagen type 1 (COL1) and osteocalcin (OCN), as well as RAGE and BAX were determined by real-time PCR, while mineralization was demonstrated by alizarin red S staining.

Result: This study included 55 diabetic and 21 non-diabetic individuals. Age, gender, glomerular filtration rate (GFR) and osteoporosis prevalence were comparable between both groups but fasting blood sugar (FBS) was significantly higher in diabetic group (148.6±70.4 vs. 102.5±11.9**,** p <0.0001). The MSC-isolated from diabetic patients showed significantly lower differentiation potential toward osteoblast (7.4% vs 86.7%, p<0.0001). The MSC-isolated from diabetic group expressed ALP, COL1 and OCN lower than those of non-diabetic group by 12, 40, and 15 fold, respectively, as well as showed negative alizarin red-S staining . The serum RAGE were similar in both diabetic and non-diabetic group (471.9±233.8 pg/mL vs 481±213.5 pg/mL, p=0.977). Interestingly, RAGE expression were significantly higher in MSC-isolated from diabetic group, as well as the RAGE expression did not correlate to serum RAGE, suggesting different RAGE activation threshold between diabetic and non-diabetic individuals. In consistent to RAGE expression, BAX expression were also significantly higher in diabetic group. Furthermore, the expression of RAGE and BAX were strongly correlated. FBS, RAGE and BAX expression were correlated to an impaired osteogenic differentiation with univariate analysis but only FBS showed correlation to the differentiation impairment in multivariate analysis.

Conclusion: Type 2 diabetic patients showed an impaired differentiation toward osteoblast. FBS was an independent risk factor for osteogenic differentiation defect. The serum RAGE was not different in diabetic and non-diabetic individuals while RAGE expression were significantly higher in diabetic group, indicating that the cells in diabetic individuals had higher sensitivity for RAGE activation. In the other hand, it can be implied that serum RAGE was not an appropriate surrogate marker to determine osteoblast function in type 2 diabetes.

Keywords : advanced glycation end product (AGE), receptor of advanced glycation end product (RAGE), osteoblast differentiation, mesenchymal stem cells, type 2 diabetes

บทสรุปผู้บริหาร (Executive Summary)

1. ความสำคัญและที่มาของปัญหา

Type 2 diabetes is a major health problem in Thailand, affect approximately 7 % of Thai population. It involves an abnormal glucose metabolism and consequent chronic hyperglycemia, leading to many chronic micro- and macrovascular complications, as well as associated with other health problem including osteoporosis. Type 2 diabetic population had an increased risk of fragility fracture for 20-70% despite preserved bone mineral density, suggesting an adverse effect of diabetes on bone quality. In addition, multiple studies showed a state of low bone turnover with bone formation defect in type 2 diabetes. The impairment of bone quality and bone formation may be caused by an accumulation of advanced glycation end products (AGEs) in extracellular matrix of the bone which leads to the alteration of biomechanical properties of the bone toward fragility as shown in diabetic animal models and in *in vitro* ribosylation of the human bone, as well as the deterioration of osteoblast differentiation and the stimulation of osteoblast apoptosis as shown in diabetic animal models in the human osteoblast exposing to exogenous AGEs. Despite the fact that the AGEs accumulated at accelerated rate in type 2 diabetes, current evidence showing a defect in osteoblast differentiation and its correlation to AGEs in type 2 diabetic patients is still lacking. RAGE is a decoy receptor of AGEs. In preclinical studies, an increase of RAGE either prevented or restored diabetic complications. However, the role of serum RAGE in human remained to be elucidated.

This study aims to determine whether there is a defect in osteoblast differentiation in type 2 diabetes by measuring the development of osteoblast phenotypic markers of the PB-MSC. Furthermore, this study aims to determine the correlation between RAGE, which is a negative regulator of AGE, and osteoblast differentiation in type 2 diabetes. To date, the study proposed in this proposal will be the first to demonstrate whether there is a defect in osteoblast differentiation in type 2 diabetic patients, as well as the correlation of this defect and RAGE.

2. วัตถุประสงค์

The purpose of the present study is to evaluate differentiation potential toward the osteoblast of the peripheral blood-derived mesenchymal stem cells (PB-MSC) and its correlation to serum RAGE in patients with type 2 diabetes comparing to non-diabetic

individuals. We tested the hypotheses that 1) the patients with type 2 diabetes have the PB-MSC containing lower potential to differentiate toward the osteoblast than that of non-diabetic individuals, 2) the serum RAGE correlates to the osteoblast differentiation in type 2 diabetes and its potential role in predicting osteoblast development in diabetic patients.

3. ระเบียบวิธีวิจัย

Material and Method

The present study is a cross-sectional study, performed at Maharaj Nakorn Chiang Mai hospital, Chiang Mai University. An informed consent was obtained in each patient to participate in the study. Inclusion criteria were patients with type 2 diabetes with or without fractures and age-matched non-diabetes patients without fractures (as a control group). Patients without fracture was those denied history of fracture, had normal skeleton on physical examination and/or had no evidence of fractures in thoracolumbar spine radiography. Exclusion criteria were as follows: patients who use thiazolidinedione, steroid, immunosuppressive medications, anti-resorptive agents or anabolic therapy for osteoporosis, patients with elevated serum creatinine higher than 1.4 in female and 1.5 in male, patients with metastases cancer or hematologic malignancy. Blood was drawn from all enrolled patients to isolate the PB-MSC and determine serum RAGE level. FBS and serum creatinine were done simultaneously as part of yearly physical check-up with addition of HbA1c in diabetic patients.

The isolated PB-MSC was characterized by cell surface markers expression (CD34, CD 90, CD105, Stro-1) by real-time PCR. The isolated PB-MSC was cultured in osteogenic-inducing medium and then tested for osteoblast-specific markers, including alkaline phosphatase (ALP), collagen type 1 (COL1) and osteocalcin (OCN) expression by real-time PCR and mineralization by alizarin red staining. Furthermore, RAGE and BAX expression in PB-MSC were analyzed by real-time PCR to determine RAGE activation and apoptosis in the cells. The serum RAGE levels will be assessed by ELISA technique.

Statistic analysis

All descriptive data were reported in Mean ± SD except determine otherwise. Independent T-test was used to compare age of both groups while Mann-Whitney U test was used to compare all other continuous parameters. Fisher-exact test was used to compare binary parameters. Pearson correlation was used to show correlation between parameters. Linear regression analysis was used to demonstrate factors correlation to

osteoblast differentiation. Statistic significant was determine as p<0.05. All statistic was done by using SPSS version 16.

4. แผนการดำเนินงานวิจัยตลอดโครงการในแต่ละช่วง 6 เดือน

Activity	Duration (month)			
	Months 1-6	Months 7-12	Months 13-18	Months 19-24
1. Enrollment of the patients and clinical evaluation	←	—	—	→
Experimental study 2.1 Peripheral blood stem cell isolation and measure osteogenic activity	←	—	—	→
2.2 Measure serum soluble RAGE			←	→
2.3 Measure RAGE and BAX expression			←	→
3. Data analysis			←	→
4. Manuscript preparation				← →
5. Manuscript and full report submission				← →

5 ผลงาน/หัวข้อเรื่องที่คาดว่าจะตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

ผลการศึกษา: This study included 55 diabetic and 21 non-diabetic individuals. Age, gender, glomerular filtration rate (GFR) and osteoporosis prevalence were comparable between both groups but fasting blood sugar (FBS) was significantly higher in diabetic group (148.6±70.4 vs. 102.5±11.9, p <0.0001). The MSC-isolated from diabetic patients showed significantly lower differentiation potential toward osteoblast (7.4% vs 86.7%, p<0.0001). The MSC-isolated from diabetic group expressed ALP, COL1 and OCN lower than those of non-diabetic group by 12, 40, and 15 fold, respectively, as well as showed negative alizarin red-S staining . The serum RAGE were similar in both diabetic and non-diabetic group (471.9±233.8 pg/mL vs 481±213.5 pg/mL, p=0.977). Interestingly, RAGE

expression were significantly higher in MSC-isolated from diabetic group, as well as the RAGE expression did not correlate to serum RAGE, suggesting different RAGE activation threshold between diabetic and non-diabetic individuals. In consistent to RAGE expression, BAX expression were also significantly higher in diabetic group. Furthermore, the expression of RAGE and BAX were strongly correlate. FBS, RAGE and BAX expression were correlate to an impaired osteogenic differentiation with univariate analysis but only FBS showed correlation to the differentiation impairment in multivariate analysis.

สรุปผลการศึกษา: Type 2 diabetic patients showed an impaired differentiation toward osteoblast. FBS was an independent risk factor for osteogenic differentiation defect. The serum RAGE was not different in diabetic and non-diabetic individuals while RAGE expression were significantly higher in diabetic group, indicating that the cells in diabetic individuals had higher sensitivity for RAGE activation. Therefore, serum RAGE was not a good surrogate marker for osteoblast differentiation in type 2 diabetes.

ชื่อเรื่องที่คาดว่าจะตีพิมพ์ : Impaired osteogenic differentiation and enhanced cellular RAGE sensitivity of the peripheral blood-derived mesenchymal stem cell in type 2 diabetes.

J Bone Miner Res: Impact factor 6.043

ระยะเวลาดำเนินงานตลอดโครงการ 2 ปี ผลงานนี้คาดว่าจะตีพิมพ์ในวารสารวิชาการนานาชาติ

วัตถุประสงค์ของงานวิจัย

The purpose of the present study is to evaluate differentiation potential toward the osteoblast of the peripheral blood-derived mesenchymal stem cells (PB-MSC) and its correlation to serum receptor of advanced glycation end-products (RAGE) in patients with type 2 diabetes comparing to non-diabetic individuals. We tested the hypotheses that 1) the patients with type 2 diabetes have the PB-MSC containing lower potential to differentiate toward the osteoblast than that of non-diabetic individuals, 2) the serum RAGE correlates to the osteoblast differentiation in type 2 diabetes and its potential role in predicting osteoblast development in diabetic patients.

ระเบียบวิธีการวิจัย

Material and Method

The present study is a cross-sectional study, performed at Maharaj Nakorn Chiang Mai hospital, Chiang Mai University. An informed consent was obtained in each patient to participate in the study. Inclusion criteria were patients with type 2 diabetes with or without fractures and age-matched non-diabetes patients without fractures (as a control group). Patients without fracture was those denied history of fracture, had normal skeleton on physical examination and/or had no evidence of fractures in thoracolumbar spine radiography. Exclusion criteria were as follows: patients who use thiazolidinedione, steroid, immunosuppressive medications, anti-resorptive agents or anabolic therapy for osteoporosis, patients with elevated serum creatinine higher than 1.4 in female and 1.5 in male, patients with metastases cancer or hematologic malignancy. Blood was drawn from all enrolled patients to isolate the PB-MSC and determine serum RAGE level. FBS and serum creatinine were done simultaneously as part of yearly physical check-up with addition of HbA1c in diabetic patients.

The isolated PB-MSC was characterized by cell surface markers expression (CD34, CD 90, CD105, Stro-1) by real-time PCR. The isolated PB-MSC was cultured in osteogenic-inducing medium and then tested for osteoblast-specific markers, including alkaline phosphatase (ALP), collagen type 1 (COL1) and osteocalcin (OCN) expression by real-time PCR and mineralization by alizarin red staining. Furthermore, RAGE and BAX expression in PB-MSC were analyzed by real-time PCR to determine RAGE activation and apoptosis in the cells. The serum RAGE levels will be assessed by ELISA technique.

Statistical analysis

All descriptive data were reported in Mean ± SD except determine otherwise. Independent T-test was used to compare age of both groups while Mann-Whitney U test was used to compare all other continuous parameters. Fisher-exact test was used to compare binary parameters. Pearson correlation was used to show correlation between parameters. Linear regression analysis was used to demonstrate factors correlation to osteoblast differentiation. Statistic significant was determine as $p<0.05$. All statistic was done by using SPSS version 16.

ผลการวิจัย

This study included 72 patients that were 55 diabetic and 21 non-diabetic patients. The mean age of the patients was 62.53±8.20 years with the mean FBS 135.9±63.53 mg/dL. Age, gender, GFR and osteoporosis prevalence were comparable between diabetic and non-diabetic group. In contrast, serum FBS and creatinine in diabetic group were significantly higher than those in non-diabetic group (Table 1). FBS and HbA1c in diabetic group were 148.6±70.4 and 7.8±1.6, respectively (Table 1). The patients in diabetic group were diagnosed with diabetes for 10.7 years. In consistent to a long period of diabetes, 56.4% and 18.2% of diabetic patients had microvascular and macrovascular complications of diabetes (Table 1).

Table 1 Demographic data and diabetic-related data of the patients.

Parameters	DM (n=55)	Non-DM (n=21)	Significant
Age (years)	63.0±8.1	61.3±8.5	p=0.437
Gender (% female)	63.6	81.0	p=0.177
FBS (mg/dL)	148.6±70.4	102.5±11.9	p<0.0001
HbA1c (%)	7.8±1.6	NA	NA
DM duration (years)	10.7±6.9	NA	NA
Serum creatinine	0.99±0.27	0.78±0.33	p=0.023
GFR	78.2±22.2	78.1±35.1	p=0.642
Diabetic microvascular complications	56.4%	NA	NA
Diabetic macrovascular complications	18.2%	NA	NA
Osteoporosis (% osteoporosis)	27.3%	33.3%	p=0.778

Serum RAGE and peripheral blood mononuclear cells (PBMCs)

Serum RAGE were 471.9±233.8 pg/mL in diabetic group and 481±213.5 pg/mL in non-diabetic group, which were not significantly different between groups (Table 2). Peripheral blood mononuclear cells were isolated from 70.9% of peripheral blood of diabetic and 85.7% of non-diabetic group (Table 2). The mononuclear cells isolated from

both groups showed strong expression of CD90, CD105 and Stro-1 with either weak or no expression of CD34, indicating that these cells were mesenchymal stem cells. Osteoblast differentiation was determined by the expression of osteoblast specific genes including alkaline phosphatase (ALP), collagen type 1 (COL1) and osteocalcin (OCN), as well as calcium deposition by alizarin-S red staining. The MSC-isolated from diabetic patients showed significantly decrease in the potential of differentiation toward osteoblast, only 7.4% expressed the osteoblast specific markers. In contrast, 86.7% MSC-isolated from non-diabetic patients expressed the osteoblast-specific genes. With age and serum RAGE match, MSC isolated from diabetic group expressed RAGE (81.8% vs 7.7%, p<0.001) and BAX (77.3% vs 7.7%, p<0.001) more than those in non-diabetic group (Table 2).

Table 2 Serum RAGE and gene expression profile in diabetic and non-diabetic group.

Parameters	DM	Non-DM	Significant
Serum RAGE (pg/mL)	471.2±233.8 (N=51)	481.2±213.1 (N=16)	p=0.977
MSC isolation (% of isolation)	70.9% (39/55)	85.7% (18/21)	p=0.243
Osteoblast differentiation (% of differentiation)	7.4% (2/27)	86.7% (13/15)	p<0.0001
RAGE expression * (% of increase expression)	81.8% (18/22)	0% (0/12)	P=0.001
BAX expression * (% of increase expression)	77.3% (17/22)	0% (0/12)	P=0.004
*Compare expression between DM and non-DM group with age and serum RAGE match			

Osteoblast differentiation

MSC isolated from non-diabetic group showed significantly higher differentiation potential toward osteoblast comparing to those from diabetic group (Figure 1A and 1B). MSC isolated from non-diabetic group demonstrated higher expression of ALP, COL1 and OCN by 13, 41 and 15 folds (Figure 1B). Moreover, MSC isolated from non-diabetic group showed positive calcium deposition by alizarin-red S staining, indicating potential differentiation toward mature osteoblasts (Figure 2A-B).

Figure 1 Osteoblast specific gene expression in MSC

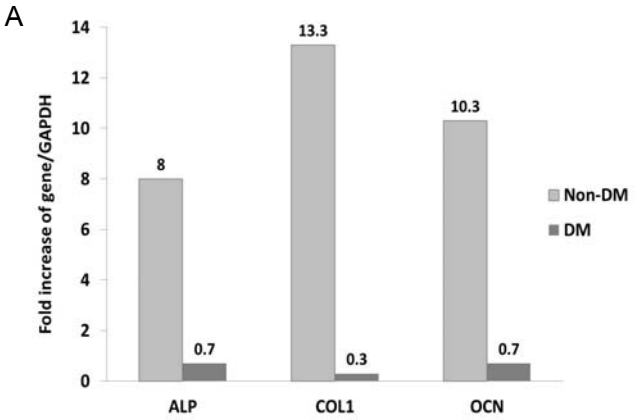

Figure 1A showed osteoblast specific expression in DM and non-DM group

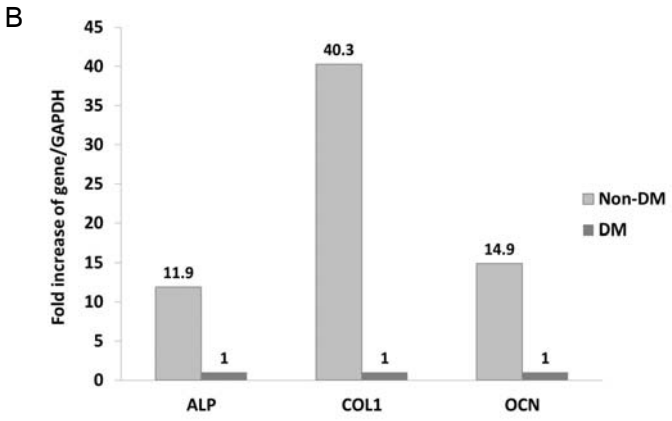

Figure 1B compared the expression of osteoblast specific expresssion between DM and non-DM groups

Figure 2 Alizarin red-S staining

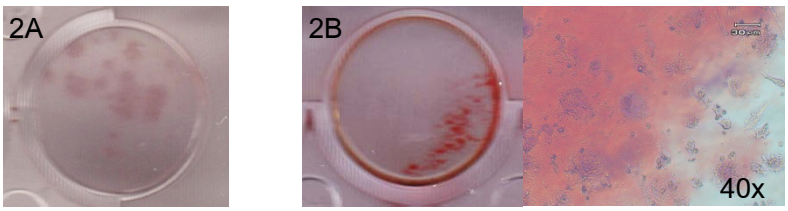

Figure 2A and 2B Positive alizarin-red S staining in non-DM patients

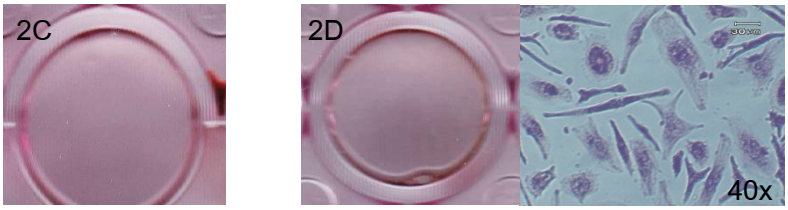

Figure 2C and 2D Negative alizarin-red S staining in DM patients

Factors influences osteoblast differentiation

MSC isolated from 42 patients were completely analyzed for osteoblast specific gene expression. Sixteen patients (38.1%) showed an increase expression of osteoblast-specific genes, which 3 (18.8%) and 13 (81.3%) were in diabetic and non-diabetic group, respectively. Osteoblast differentiation did not related to age, gender and serum RAGE. However, FBS and diabetes were significantly determine an impaired osteoblast differentiation (Table 3). Despite serum RAGE did not related to the osteoblast differentiation, RAGE expression in MSC inversely related the differentiation toward osteoblast (Table 3). Moreover, BAX, an apoptosis marker, strongly inverse relation to the osteoblast differentiation potential (Table 3). With multivariate analysis, FBS is an independent factor for osteoblast differentiation, suggesting that diabetes itself directly influence differentiation potential toward osteoblast and then bone formation in diabetic patients.

Table 3 Factors influence osteoblast differentiation

Parameters	OB differentiation (n=15)	OB non-differentiation (n=22)	Significant
Age (years)	62.1±8.3	63.7±7.7	p=0.525
Gender (% female)	80	66.7	p=0.485
FBS (mg/dL)	104.4±13.3	153.8±92.0	P=0.011
DM (% DM diagnosis)	13.3	92.6	p<0.0001
Creatinine	0.78±0.29	0.97±0.23	p=0.051
GFR	81.1±3.3	78.9±2.0	p=0.478
Osteoporosis (% diagnosis)	26.7	25.9	P=0.958
Microvascular complications (% complication)	13.3	51.9	p=0.02
Macrovascular complications (%complication)	6.7	18.5	p=0.40
Serum RAGE (pg/mL)	484.6±229.3	541.8±238.8	p=0.243
RAGE expression (% increase expression)	0	85	p<0.0001
BAX expression (% increase expresssion)	0	80	p<0.0001

Serum RAGE and RAGE activation

The average mean of serum RAGE in all individuals was 474.1±227.5 pg/mL. The serum RAGE level weakly correlated to FBS (Figure 3A) and creatinine but age, gender and GFR. Consistent to diabetic and non-diabetic group, the serum RAGE level between osteoblast differentiation and none differentiation groups was not different (484±229.3 vs 541.8±238.8, p=0.243). Because serum RAGE was comparable in all groups, RAGE expression was analyzed to determine whether the activation of RAGE was similar.

Interestingly, RAGE RNA level was markedly higher in either diabetic or non-osteoblast differentiation group than those in either non-diabetic or osteoblast differentiaiton group (Table 2 and Table 3). In addition, serum RAGE level did not correlate to RAGE RNA level. Therefore, serum RAGE did not directly determine RAGE activation in the MSC (Figure 3B).

Figure 3 showed correlation between FBS and serum RAGE, as well as serum RAGE and RAGE activation.

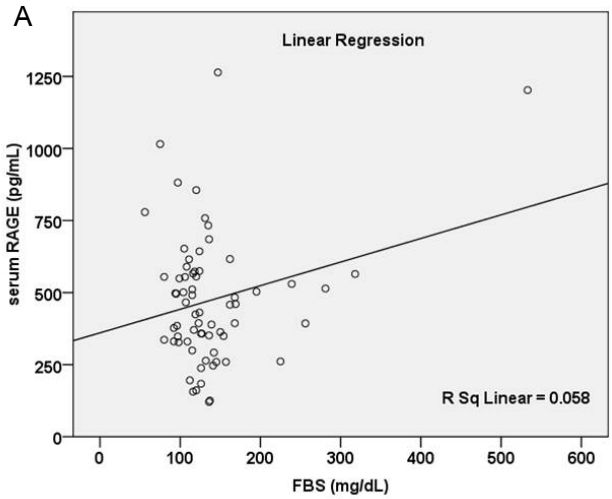

Figure 3A correlation between serum RAGE and FBS

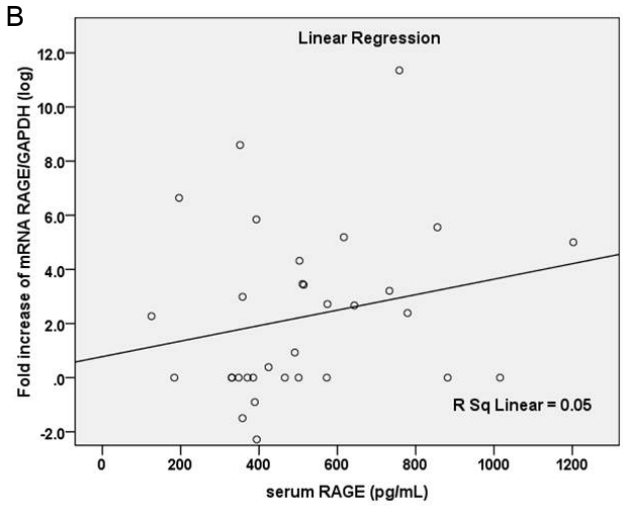

Figure 3 B Correlation between serum RAGE and RAGE expression.

(RAGE RNA level was shown in $\log_{10}$ scale)

RAGE activation and apoptosis in MSC-isolated from diabetic patients

Because MSC-isolated from diabetic group showed poorer osteogenic differentiation and higher RAGE expression, the enhancement of apoptosis by RAGE activation was hypotheses as a potential mechanism for impaired osteogenic differentation in diabetes. In consistent to RAGE expression, the MSC isolated from diabetic patients showed higher expression of BAX comparing to non-diabetic patients with age and serum RAGE match (Figure 4A). Moreover, the increment of RAGE and BAX expression were strongly correlation (Figure 4B). By the fact that 1) serum RAGE was comparable in all groups, 2) serum RAGE was not correlate to RAGE activation and 3) RAGE and BAX activation were higher in either diabetes or non-ostegenic differentiation group, these evidence suggested that the MSC of diabetes patients had higher cellular sensitivity for RAGE activation which may lead to higher apoptosis and impaired osteogenic differentiation in diabetes. It is noteworthy to state that only 2 MSC isolated from the patients showed the the expression of osteoblast specific markers, indicating the differentiation potential toward osteoblast. With age and serum RAGE match to non-diabetic patients, RAGE and BAX were not highly expressed in these 2 MSC isolation, suggesting an essential role of RAGE activation and apoptosis increment for an impairment of differentiation toward osteoblast.

Figure 4 RAGE and BAX expression in MSC comparing between non-diabetes and diabetes with age and serumRAGE match.

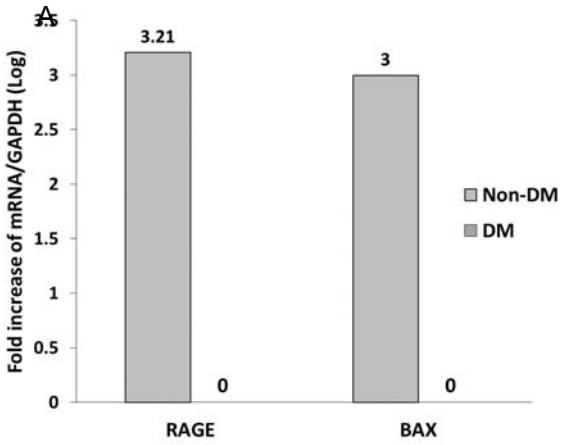

Figure 4A showed RAGE and BAX expression comparing between DM and non-DM groups with age- and serum RAGE match.

RAGE and BAX mRNA level showed in $\log_{10}$ scale.

B

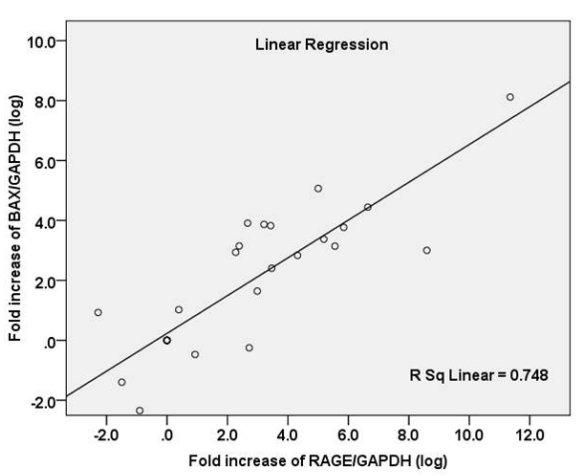

Figure 4B showed correlation between RAGE and BAX expression comparing between DM and non-DM groups with age- and serum RAGE match.
Both RAGE and BAX mRNA level showed in $\log_{10}$ scale.

สรุปผลและอภิปรายผล

สรุปผลการศึกษา

This study showed that patients with type 2 diabetes had an impaired differentiation toward osteoblast. Only 7.4% of the MSC-isolated from diabetic patients expressed osteoblast-specific genes while 86.7% in non-diabetic individuals showed the expression of those genes. The MSC-isolated from diabetic group expressed ALP, COL1 and OCN lower than those of non-diabetic group by 12, 40, and 15 fold, respectively, as well as showed negative alizarin red-S staining. Serum RAGE was comparable between diabetic and non-diabetic individuals, as well as between osteoblast differentiation and no differentiation group. In contrast, RAGE expression was significantly higher in diabetic group and did not correlate to serum RAGE, suggesting that the MSC in diabetic individuals had higher sensitivity for RAGE activation. FBS, RAGE and BAX expression were correlate to an impaired osteogenic differentiation with univariate analysis but only FBS showed correlation to the differentiation impairment in multivariate analysis.

อภิปรายผลการศึกษา

AGEs are chemical modifications of proteins by reducing sugar generated during Millard reaction[1-2]. The Milliard reaction is accelerated in the present of hyperglycemia;

therefore, the ACEs accumulation in diabetes is higher than that in non-diabetes[3-4]. AGEs interact with their receptors, RAGE, resulting in the activation of several inflammatory signaling pathway and cellular dysfunction[5-7]. Multiple studies showed that AGE-RAGE interaction affected on both microvascular and macrovascular complications of diabetes[8-13].

Type 2 diabetic population had an increase risk of fragility fracture for 20-70%[14-18] despite preserved bone mineral density, suggesting an adverse effect of diabetes on bone quality. In addition, multiple studies showed a state of low bone turnover with bone formation defect in type 2 diabetes[19-21]. Several studies suggested that the impairment of bone quality and bone formation may cause by an accumulation of advanced glycation end products (AGEs) in extracellular matrix of the bone that leads to the alteration of biomechanical properties of the bone toward fragility[22-25] as well as the deterioration of osteoblast differentiation[26-30]. However, evidences showing a defect in osteoblast differentiation and its correlation with AGEs remain to be clarified. Our study showed that patients with type 2 diabetes had an impaired differentiation toward osteoblast. Only 7.4% of the MSC-isolated from diabetic patients expressed osteoblast-specific genes while 86.7% in non-diabetic individuals showed the expression of those genes. The MSC-isolated from diabetic group expressed ALP, COL1 and OCN lower than those of non-diabetic group by 12, 40, and 15 fold, respectively, as well as showed negative alizarin red-S staining. Therefore, our study confirmed a decline in differentiation toward osteoblast in human with type 2 diabetes. This impairment may entail an impaired bone quality and increased fracture risk found in patient with type 2 diabetes.

Serum RAGE functions as a decoy AGEs, neutralizing AGEs-RAGE signals [5,31]. Several preclinical studies suggested a protective effect of serum RAGE for chronic micro- and macrovascular complications of diabetes[32-35]. However, in human, serum RAGE level in diabetes was still controversial. Several Japanese groups showed a decline in serum serum RAGE in both type 1 and type 2 diabetes[36-37], while others showed an increase level in both types of diabetes[38-40]. Our study demonstrated that serum RAGE in both diabetic and non-diabetic group was comparable but highly variable. Because of this high variability, serum RAGE in the previous study possibly was shown either in a decreasing or enhancing level. Furthermore, in this study, we also showed that serum RAGE was comparable in both individuals with osteoblast differentiation and without differentiation.

Because AGE-RAGE activation was documented for leading to osteoblast differentiation defect and inducing apoptosis[26-29], we elucidated the RAGE activation and apoptosis in the MSC comparing diabetic and non-diabetic individuals by measuring RAGE and BAX expression by real-time PCR. With age and serum RAGE match, RAGE expression was significantly higher in diabetic group, suggesting that the MSC in diabetic individuals had higher sensitivity for RAGE activation. Furthermore, the RAGE expression level did not correlate to serum RAGE. Because of the serum RAGE was high variability and indiscriminate among groups, as well as uncorrelated to the level of RAGE activation, this serum RAGE is conceivable not an appropriate marker for osteoblast differentiation in type 2 diabetes. In consistent to RAGE expression, BAX expression was also higher in diabetic patients comparing to age and serum RAGE-matched non diabetic individuals. Moreover, the level of RAGE and BAX expression strongly correlated, suggesting that RAGE activation may increase apoptosis marker in the MSC-isolated from patients with type 2 diabetes. Our results was in consistent to several previous *in vitro* studies involving primary culture osteoblast and mesenchymal stem cell which showed the adverse effects of AGEs to attenuate osteoblast differentiation and enhance osteoblast apoptosis[26-29]. FBS, RAGE expresssion and BAX expression were correlate to an impaired osteogenic differentiation with univariate analysis but only FBS showed correlation to the differentiation impairment in multivariate analysis. Therefore, our study demonstrated that FBS was as an independent risk factor for osteogenic differentiation defect in type 2 diabetes.

Because serum RAGE level was indiscriminate between groups with or without osteoblast differentiation, and uncorrelated to RAGE activation, we could implied that serum RAGE was not an appropriate surrogate marker to determine osteoblast function in type 2 diabetes. This study also found a different cellular sensitivity to AGEs for stimulating RAGE and its downstream signals including an apoptosis pathway, as shown by a higher RAGE expression in diabetes comparing to age and serum RAGE-matched non-diabetes. This could be implied that a difference cellular sensitivity for RAGE activation between diabetic and non-diabetic individuals may lead to an enhanced apoptosis of the cells in diabetes which leaded to an impaired osteogenic differentiation in diabetes. However, the mechanism underlined the higher cellular sensitivity to AGEs in diabetes remains to be further elucidated.

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ผลงานที่ได้รับจากโครงการนี้

ผลงาน	รายละเอียด
1. **รูปแบบผลงานวิจัย**	การนำไปใช้ประโยชน์
☐ ต้นแบบผลิตภัณฑ์ ☐ กระบวนการใหม่ ☐ เทคโนโลยีใหม่ ☒ องค์ความรู้ 1. Impaired osteogenic differentiation in human with diabetes 2. Enhanced cellular RAGE sensitivity in diabetes	<u>เชิงสาธารณะประโยชน์</u> การเผยแพร่ผลงานวิจัยโดยการจัดประชุม/สัมมนา/ฝึกอบรม ☐ ก. ดำเนินการแล้ว ☒ ข. อยู่ระหว่างดำเนินการ การนำเสนอผลงานวิจัย “Impaired osteogenic differentiation in type 2 diabetes” ในการประชุมเมธีวิจัยอาวุโส ณ. อเวย์สวนสวรรค์รีสอร์ท อ. แม่ริม จ. เชียงใหม่ วันที่ 19 กรกฎาคม 2556 ☒ ค. มีแผนที่จะเสนอผลงานวิจัย มีแผนจะนำเสนอผลงานวิจัย “Impaired osteoblast development in type 2 diabetes and its relation to RAGE oversensitivity” ในการประชุม “World Diabetes Congress” ณ. เมืองเมลเบิร์น ประเทศออสเตรเลีย วันที่ 2-6 ธันวาคม 2556
	การเผยแพร่ผลงานวิจัยโดยการตีพิมพ์ผลงาน ☒ ก. มีแผนนำเสนอผลงานวิจัยโดยการตีพิมพ์ในวารสารนานาชาติ กำลังดำเนินการในการทำ manuscript

ภาคผนวก

บทคัดย่อในการนำเสนอผลงานวิจัย “Impaired osteogenic differentiation in type 2 diabetes”
ในการประชุมเมธีวิจัยอาวุโส ณ. อเวย์สวนสวรรค์รีสอร์ท อ. แม่ริม จ. เชียงใหม่
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Abstract:

Introduction: Multiple preclinical studies in type 2 diabetes demonstrated a state of low bone turnover with bone formation defect and impaired osteoblast differentiation in type 2 diabetes, which partly related to an accumulation of advanced glycation end products (AGEs) in extracellular matrix of the bone. Furthermore, a decoy receptor of AGEs, soluble receptor of advanced glycation end product (RAGE), was shown as a protective factor of chronic diabetic complications. However, current evidence showing an effect of AGEs in human is still lacking. Therefore, this study aims to elucidate osteoblast differentiation in type 2 diabetes as well as to explore a role of serum RAGE as a predictor of bone health in diabetes.

Material and Method: The present study is a cross-sectional study included diabetic patients and age-match non-diabetic control. Peripheral blood was taken for isolating mesenchymal stem cells (PBMSC) and measuring serum RAGE level. Osteoblast differentiation was determined by osteoblast-specific gene expression and mineralization. RAGE and BAX expression were used to demonstrate RAGE activation and apoptosis. The osteoblast-specific gene expression, including alkaline phosphatase (ALP), collagen type 1 (COL1) and osteocalcin (OCN), as well as RAGE and BAX were determined by real-time PCR, while mineralization was demonstrated by alizarin red S staining.

Result: This study included 55 diabetic and 21 non-diabetic individuals. Age, gender, glomerular filtration rate (GFR) and osteoporosis prevalence were comparable between both groups but fasting blood sugar (FBS) was significantly higher in diabetic group (148.6±70.4 vs. 102.5±11.9, $p < 0.0001$). The MSC-isolated from diabetic patients showed significantly lower differentiation potential toward osteoblast (7.4% vs 86.7%, $p<0.0001$). The MSC-isolated from diabetic group expressed ALP, COL1 and OCN lower than those of non-diabetic group by 12, 40, and 15 fold, respectively, as well as showed negative alizarin red-S staining. The serum RAGE were similar in both diabetic and non-diabetic group (471.9±233.8 pg/mL vs 481±213.5 pg/mL, $p=0.977$). Interestingly, RAGE expression

were significantly higher in MSC-isolated from diabetic group, as well as the RAGE expression did not correlate to serum RAGE, suggesting different RAGE activation threshold between diabetic and non-diabetic individuals. In consistent to RAGE expression, BAX expression were also significantly higher in diabetic group. Furthermore, the expression of RAGE and BAX were strongly correlated. FBS, RAGE and BAX expression were correlated to an impaired osteogenic differentiation with univariate analysis but only FBS showed correlation to the differentiation impairment in multivariate analysis.

Conclusion: Type 2 diabetic patients showed an impaired differentiation toward osteoblast. FBS was an independent risk factor for osteogenic differentiation defect. The serum RAGE was not different in diabetic and non-diabetic individuals while RAGE expression were significantly higher in diabetic group, indicating that the cells in diabetic individuals had higher sensitivity for RAGE activation.

Keywords : advanced glycation end product (AGE), receptor of advanced glycation end product (RAGE), osteoblast differentiation, mesenchymal stem cells, type 2 diabetes

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