Adrenomedullin 2 improves bone regeneration in type 1 diabetic rats by restoring imbalanced macrophage polarization and impaired osteogenesis

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Research

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Abstract

Background

Both advanced glycation end products (AGEs) and AGE-mediated M1 macrophage polarization contribute to bone marrow mesenchymal stem cell (BMSC) dysfunction, leading to impaired bone regeneration in type 1 diabetes mellitus (T1DM). Adrenomedullin 2 (ADM2), an endogenous bioactive peptide belonging to the calcitonin gene-related peptide family, exhibits various biological activities associated with the inhibition of inflammation and reduction of insulin resistance. However, the effects and underlying mechanisms of ADM2 in AGE-induced macrophage M1 polarization, BMSC dysfunction, and impaired bone regeneration remain poorly understood.

Methods

The polarization of bone marrow-derived macrophages was verified by flow cytometry analysis. In addition, alkaline phosphatase (ALP) staining, ALP activity detection, and alizarin red staining were performed to assess the osteogenesis of BMSCs. Quantitative real-time polymerase chain reaction, enzyme-linked immunosorbent assay, western blotting, and immunofluorescence staining were used to assess polarization markers, PPAR γ /I κ B α /NF- κ B signaling, and osteogenic markers. *In vivo*, a distraction osteogenesis (DO) rat model with T1DM was established, and the tibia samples were collected at different time points for radiological, biomechanical, and histological analyses, to verify the effects of ADM2 in terms of bone regeneration and M2 polarization under diabetic conditions.

Results

ADM2 treatment reversed the M1 macrophage polarization induced by AGEs towards the M2 phenotype, which was partially achieved by the PPAR γ -mediated inhibition of NF- κ B signaling. The PPAR γ inhibitor GW9662 significantly attenuated the effects of ADM2. Besides, ADM2 treatment improved the AGE-impaired osteogenic potential of BMSCs *in vitro*. Furthermore, ADM2 accelerated bone regeneration, as revealed by improved radiological and histological manifestations and biomechanical parameters, accompanied by improved M2 macrophage polarization in diabetic DO rats, and these effects were partially blocked by GW9662 administration.

Conclusions

These results indicate that ADM2 enhances diabetic bone regeneration during DO, by attenuating AGE-induced imbalance in macrophage polarization, partly through PPAR γ /I κ B α /NF- κ B signaling, and improving AGE-impaired osteogenic differentiation of BMSCs simultaneously. These findings reveal that ADM2 may serve as a potential bioactive factor for promoting bone regeneration under diabetic

conditions, and imply that management of inflammation and osteogenesis, in parallel, might be a promising therapeutic strategy for diabetic patients during DO treatment.

Background

Distraction osteogenesis (DO) is widely accepted and applied in orthopedics and traumatology, because of its unique osteogenesis-inducing ability[1–3]. In the process of DO, gradual rhythmic traction is applied using an external fixator to fully induce neo-osteogenesis in the distraction zone[4]. However, bone regeneration is a complex physiological process regulated by multiple factors, and therefore various metabolic disorders tend to impair bone regeneration during DO[5]. In clinical practice, diabetes mellitus (DM)-induced impairment of bone regeneration, characterized by a prolonged mineralization phase, is a relatively common condition, which leads to increased patient discomfort and complications[6–8]. As the number of people with DM is on the rise worldwide, with a predicted increase to a population of 592 million in 2035; there is a high demand for developing novel treatment strategies for accelerating bone regeneration in diabetic patients during DO[9].

DM-induced metabolic disorders have been proven to exert detrimental effects on bone regeneration, leading to a greater risk of poor fracture healing or bone grafting failure[6, 10]. Several physiological conditions have been identified to contribute to DM-induced bone regeneration impairment, including insulin deficiency, accumulation of advanced glycation end products (AGEs), and elevated levels of circulatory homocysteine[11, 12]. AGEs are formed by the non-enzymatic reaction of glucose with proteins under diabetic conditions, and upon interaction with its cell membrane-specific receptor affect cellular functions[13]. Previous studies found that the AGE-receptor of AGEs (RAGE) interaction could regulate various cellular signals, such as mitogen-activated protein kinase, hypoxia-induced factor-1 α , peroxisome proliferator-activated receptor γ (PPAR γ), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which results in M1 macrophage polarization[14–17]. Meanwhile, prolonged inflammation mediated by M1 macrophage contributes to impaired osteogenic potential of bone marrow mesenchymal stem cell (BMSC)[18]. Additionally, AGEs have been shown to exert negative effects on stem cell osteogenic differentiation by modulating DNA methylation and the Wingless/Integrated (Wnt) signaling pathway[19]. Consequently, AGEs could be identified as an important therapeutic target to directly and indirectly attenuate BMSC dysfunction under diabetic conditions.

Adrenomedullin 2 (ADM2), also known as intermedin, is an endogenous peptide belonging to the calcitonin gene-related peptide/calcitonin family and is ubiquitously expressed in various tissues[20]. A previous study has reported that systemic ADM2 levels were significantly decreased in diabetic rats compared to healthy individuals, indicating a relationship between low ADM2 levels and DM-related metabolic disorders[21]. ADM2 has also been reported to play protective roles in the cardiovascular and renal systems via multiple mechanisms, such as anti-inflammation and inhibition of oxidative and endoplasmic reticulum stress[22–24]. Additionally, Pang et al. reported that ADM2 treatment could restore the M1/M2 balance and improve systemic insulin sensitivity in hyperhomocysteinemic mice[25]. Considering the increasing recognition of the vital role of M2 macrophage polarization in bone

regeneration and decreased levels in DM[26], we speculated that ADM2 treatment might indirectly create a pro-regenerative microenvironment for enhanced bone regeneration under DM conditions, by facilitating a dynamic shift from M1 to M2 macrophage polarization. Moreover, the direct effect of ADM2 on the osteogenic differentiation of AGE-exposed BMSCs also remains largely unknown.

In this study, we first investigated the roles of ADM2 in macrophage polarization and osteogenic differentiation of BMSCs under AGEs exposure, and the underlying mechanisms. Furthermore, a diabetic rat DO model was employed to examine the *in vivo* effects of ADM2 on bone regeneration and macrophage polarization.

Methods

Cell culture

Bone marrow-derived macrophages (BMDMs) and BMSCs were isolated from 4-week-old C57BL/6 male mice and Sprague Dawley (SD) male rats, respectively, by flushing the bone marrow from femurs and tibias with phosphate buffer saline (PBS; HyClone, USA). BMDMs were cultured in Dulbecco's Modified Eagle's Medium (HyClone) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin-streptomycin (P/S; Gibco), and 20% conditioned media collected from L929 cells. BMSCs were cultured in Modified Eagle's Medium Alpha (HyClone) supplemented with 10% FBS and 1% P/S. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂.

Macrophage treatment

BMDMs were stimulated with AGEs (200 µg/ml; BioVision, USA) for 48 h in the presence or absence of ADM2 (1 µM; Phoenix Pharmaceuticals, USA). An equal volume of PBS was added to the control group. In addition, GW9662 (2 µM, pretreatment for 2 h; Beyotime) was administrated along with AGEs and ADM2 treatment to verify the molecular mechanism by which ADM2 regulates AGE-induced macrophages.

Flow cytometry analysis

After treatment, BMDMs were fixed with 4% paraformaldehyde (PFA), blocked with 5% bovine serum albumin (BSA), and then incubated with FITC-conjugated F4/80 antibody (eBioscience, USA), APC-conjugated CD206 antibody (eBioscience), and PE-conjugated CD86 antibody (eBioscience) for 30 min. The candidate cells were detected using a BD FACS Caliber flow cytometer and analyzed using FlowJo v10.0 software. F4/80⁺ cells were identified as macrophages, and the expression levels of CD86 and CD206 were detected to evaluate the M1 and M2 polarization states of BMDMs.

Enzyme-linked immunosorbent assay (ELISA)

The media supernatant was collected from the culture of BMDMs and stored at -80 °C. The concentrations of tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) were measured using ELISA kits (Proteintech, China), according to the manufacturer's protocols.

Immunofluorescence staining

BMDMs were fixed with 4% PFA, washed with PBS thrice, blocked with 5% BSA for 1 h, and then incubated with the primary antibody against p65 (Beyotime) at 4 °C overnight. The cells were then incubated with the Cy3-conjugated secondary antibody (Beyotime) at 25 °C for 1 h and then stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The activation and nuclear translocation of p65 were observed using a fluorescence microscope.

Osteogenic differentiation and detection

To determine the effects of ADM2 on the osteogenic differentiation of AGE-induced BMSCs, both alkaline phosphatase (ALP) and mineral deposition were detected. Briefly, BMSCs were inoculated in 24-well plates (5×10^4 /well). Upon 80% confluence, the medium was replaced with osteogenic induction medium (OIM: 20 mM β -glycerophosphate, 1 nM dexamethasone, and 50 μ M L-ascorbic acid-2-phosphate in the complete medium; Sigma-Aldrich, USA) containing AGEs (200 μ g/ml) in the presence or absence of ADM2 (1 μ M), and the medium was replenished every 2 d. ALP staining and activity assays were performed 7 d after osteogenic induction according to the manufacturer's instructions (Beyotime). On the 14th day of differentiation, alizarin red S (ARS; Cyagen Biosciences, China) staining was performed to evaluate mineral deposition. For quantitative analysis of the mineralization, calcium deposition was eluted with 10% (v/v) cetylpyridinium chloride (Sigma-Aldrich), and the OD value was measured at 570 nm.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total cell RNA was extracted using an RNA Purification Kit (EZBioscience, USA) and cDNA was obtained from 500 ng of total RNA using the Reverse Transcription Kit (EZBioscience). Thereafter, qRT-PCR was performed using SYBR Green qPCR Master Mix (EZBioscience). Relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method and GAPDH was used as the reference gene for normalization. The primer sequences are shown in Table. 1.

Western blot analysis

Total cell protein was extracted using RIPA lysis buffer with protease inhibitor and protein phosphatase inhibitor (Solarbio, China) at 4 °C. Protein concentration was determined using a BCA Protein Assay Kit (EpiZyme, China). Equal amounts of protein (30 μ g) were subjected to 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore, USA). After blocking with 5% BSA, the membrane was incubated with primary antibodies at 4 °C overnight. Afterward, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch, USA) at 25 °C for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence reagent (Millipore) and the grayscale of protein bands were semi-quantified using ImageJ software.

The primary antibodies used in this study included anti-PPAR γ (Cell Signaling Technology, USA), anti-I κ B α (Cell Signaling Technology), anti-p65 (Cell Signaling Technology), anti-phosphorylated p65 (Cell Signaling Technology), anti-BMP2 (Abcam, UK), anti-OSX (Abcam), anti-OCN (ABclonal, China), and anti-GAPDH (Cell Signaling Technology).

Induction of Type 1 Diabetes mellitus (T1DM) rat model

All experimental procedures were approved by the Animal Research Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. After fasting for 12 h, a single high dose (65 mg/kg) of streptozotocin (STZ, 10 mg/mL in 0.01 M citrate buffer; Sigma-Aldrich) was intraperitoneally injected to SD rats, weighing 350-400 g, to induce T1DM models. After 7 d of STZ injection, random plasma glucose levels (PGLs) were determined with a glucometer using blood from the tail vein. Rats with PGLs above 16.7 mmol/L were considered as diabetic individuals, and those that failed to reach the target glycemic index were excluded from the study.

Animal surgery and treatment

A total of 36 T1DM SD rats were used in this study and randomly assigned to the DM (n = 12), DM+ADM2 (n = 12) and DM+ADM2+GW9662 (n = 12) groups. Rats injected with an equal volume of citrate buffer were assigned to the non-diabetic control group (n = 12). To establish the DO model, a transverse osteotomy was performed at the midshaft of the right tibia after anesthesia and exposure. Then, a specially designed monolateral external fixator (Xinzhong Company, China) was mounted to fix the proximal and distal segments of the tibia. Thereafter, surgical incisions were closed layer-wise. The periosteum was preserved as much as possible during the procedure. The DO procedures comprised three phases: latency phase for 5 d, distraction phase for 10 d (0.25 mm every 12 h), and consolidation phase for 4 weeks. ADM2 (200 μ g/kg/d) was subcutaneously injected during the consolidation phase to the DM+ADM2 group and DM+ADM2+GW9662 group, and the latter was intraperitoneally administered GW9662 (1 mg/kg/d). Equal-volume PBS was subcutaneously injected at the same time as the DM and control groups.

Digital radiography and micro-computed tomography (CT)

From the beginning of the consolidation phase, X-ray films which were focused on the distraction gaps were acquired weekly. The lengthened tibia specimens were harvested 2 (n = 6) and 4 (n = 6) weeks after distraction. Micro-CT scanning was performed to quantitatively evaluate bone regeneration in the distraction zone. Thereafter, three-dimensional (3D) reconstructions of the regenerated callus were produced using the CTVox software. Parameters including bone mineral density (BMD) and bone volume/tissue volume (BV/TV) of the regenerated bone were analyzed using the CTAn software.

Biomechanical testing

The mechanical characteristics of the fresh tibia specimens (n = 3) were determined using a four-point bending device after 4-week consolidation. During the test, the tibia specimens were loaded in the

anterior-posterior direction with the posterior side in tension. The modulus of elasticity (E-modulus), ultimate load, and energy to failure were recorded and analyzed using Vernier Graphical Analysis software.

Histological and immunohistochemical staining

For histological analyses, after 2 (n = 3) and 4 (n = 3) weeks of consolidation, tibia specimens were fixed in 4% PFA for 24 h, decalcified in 10% ethylene diamine tetraacetic acid (EDTA, pH = 7.4) for 21 d, dehydrated through graded ethanol of increasing concentration, and then embedded in paraffin. Samples were cut into 5 µm-thick longitudinally oriented sections and then subjected to hematoxylin-eosin (H&E), Masson's trichrome, and Safranin O-Fast Green (SO-FG) staining.

For immunohistochemical staining, sections were incubated in 0.3% hydrogen peroxide for 20 min to quench endogenous peroxidase activity. After antigen retrieval in 0.01 mol/L citrate buffer (pH 6.0) at 65 °C for 20 min and blocking with 5% goat serum for 1 h, sections were incubated with anti-OCN antibody (ABclonal) at 4 °C overnight. After incubation with secondary antibodies conjugated with HRP at 25 °C for 1 h, an HRP-streptavidin system was used to detect positive areas followed by counterstaining with hematoxylin.

Immunofluorescent analysis

CD68 and CD86, or CD68 and CD206 double immunofluorescent staining were performed to detect M1 or M2 macrophages, respectively. After 2 weeks of consolidation, tibia specimens (n = 3) were decalcified in 18% EDTA for 3 d after fixation. Subsequently, the samples were dehydrated in 30% sucrose, embedded in optimal cutting temperature compound, and cut into 10-µm thick longitudinally oriented sections. After blocking with 5% BSA for 1 h, bone sections were incubated with primary antibodies overnight at 4 °C, followed by incubation with fluorophore-conjugated secondary antibodies at 25 °C for 1 h. Nuclei were stained with DAPI. A fluorescent microscope was used for observation and image capture. For semiquantitative analysis, the ratios of CD86⁺CD68⁺ / CD68⁺ cells and CD206⁺CD68⁺ / CD68⁺ cells in the distraction area were calculated using Image-Pro Plus software.

The primary antibodies used in this study included anti-CD68 (Abcam), anti-CD86 (Novus, USA), and anti-CD-206 (Abcam).

Statistical analysis

All data are presented as mean ± standard deviation. The statistical differences were analyzed with Student's *t*-test between two groups or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test among groups using GraphPad Prism 8 software. Results were considered statistically significant at a two-tailed *P*-value less than 0.05.

Results

ADM2 reversed AGE-induced M1 macrophage polarization to M2 phenotype

AGEs exposure significantly promoted the expression of genes related to M1 polarization, including *iNOS*, *IL-6*, and *TNF- α* (Figure 1 A). However, ADM2 administration reversed the elevated M1 marker genes expression induced by AGEs, and further promoted the expression of genes related to M2 polarization including *Arg-1*, *MRC1*, and *TGF- β* (Figure 1 A, B). The results of flow cytometry showed a similar trend: AGEs exposure amplified the population of M1 macrophages, while ADM2 administration reversed M1 polarization to M2 phenotype (Figure 1 C, D). Additionally, the results detected by ELISA revealed that AGEs significantly promoted the secretion of pro-inflammatory cytokine *TNF- α* in BMDMs, while ADM2 not only moderated the pro-inflammatory effect of AGEs but also increased the production of the anti-inflammatory molecule *TGF- β* (Figure 1 E).

ADM2 attenuated AGE-induced activation of NF- κ B through PPAR γ /I κ B α pathway

The NF- κ B pathway plays an essential role in M1 macrophage polarization, and PPAR γ could modulate NF- κ B-dependent inflammation by upregulating the expression of I κ B α , a negative regulator of p65[27]. As shown by western blotting, the protein expression of total PPAR γ and I κ B α was significantly suppressed by AGEs, leading to the activation of NF- κ B p65 (Figure 2 A, B), which was also confirmed by immunofluorescence staining for p65 nuclear translocation (Figure 2 C, D). However, ADM2 treatment rescued the expression of PPAR γ and I κ B α and subsequently diminished the activation and nuclear translocation of p65 (Figure 2 A-D). When BMDMs were treated with the PPAR γ antagonist GW9662 along with AGEs and ADM2, the effects of ADM2 on the PPAR γ /I κ B α /NF- κ B pathway were partially abated (Figure 2 A-D), leading to enhanced M1 polarization with respect to gene expression, surface marker expression, and cytokine production (Figure 1 A-E). Therefore, these findings indicated that ADM2 reversed AGE-induced macrophage inflammation via the PPAR γ /I κ B α /NF- κ B pathway.

ADM2 rescued AGE-mediated impairment of osteogenic potential

To investigate the effect of AGEs and the protective potential of ADM2 on the osteogenesis of BMSCs *in vitro*, ALP staining, ALP activity, and ARS staining were performed. As evidenced by the qualitative and quantitative results, the AGE-induced impairments of ALP activity and matrix mineralization were attenuated by ADM2-treatment (Figure 3 A-D). Moreover, we observed that osteogenic genes, including *ALP*, *OCN*, *OPN*, and *OSX*, were significantly upregulated after ADM2 treatment (Figure 3 E). As revealed by western blotting, ADM2 treatment re-upregulated the expression levels of BMP2, OCN, and OSX in BMSCs under AGEs exposure (Figure 3 F, G), suggesting that exogenous ADM2 administration partially rescued the osteogenic potential of BMSCs impaired by AGEs.

ADM2 accelerated bone formation and consolidation during DO in diabetic rats

As shown in Figure 4 A, the experimental detection of mechanical properties of the fresh tibia specimens exhibited improved ultimate load, energy to failure, and elasticity modulus in the DM+ADM2 group and jeopardized parameters in the DM+ADM2+GW9662 group (Figure 4 A). Besides, a series of representative

X-ray images across the time course of DO showed the progression of bone consolidation (Figure 4 B). Opaque callus appearing in the distraction regenerates was jeopardized in the DM group and rescued by ADM2 treatment in terms of volume and continuity of the callus in the middle of the consolidation phase. However, the recovery of bone regeneration was compromised by GW9662 administration. At the end of the consolidation phase, the cortical bone within the distraction area is nearly continuous with abundant callus in the control and DM+ADM2 groups. Nevertheless, the bone regeneration in the DM and DM+ADM2+GW9662 groups remained unsatisfactory, with a certain amount of neo-callus and discontinuous cortical bone. Similar observations were confirmed by micro-CT examination of distraction regenerates at 2 and 4 weeks after the distraction phase (Figure 4 C). The BMD and BV/TV in the distraction gaps were impaired in the DM group and improved by ADM2 treatment (Figure 4 D). However, GW9662 co-administration partially compromised the protective effect of ADM2 (Figure 4 D), indicating that ADM2 induced preferable bone regeneration in diabetic DO model at least partially through PPAR γ activation.

ADM2 administration accelerated mineralized callus formation within the distraction zone

As shown in Figure 5, H&E, Masson's trichrome, and SO-FG staining of the distraction regenerates revealed various amounts of newly formed trabecular bone, cartilaginous tissue, and fibrous-like tissue, parallel with the distraction forces (Figure 5). Distraction regenerates treated with ADM2 exhibited enhanced bone consolidation at 2 and 4 weeks after distraction in comparison with the DM group, which was evidenced by more mature trabecular bone and less fibrous-like tissue in the ADM2 group (Figure 5). However, the ossification process was impeded by GW9662 (Figure 5). In addition, immunohistochemical analysis of distraction regenerates revealed a similar tendency: the expression of OCN in the control and DM+ADM2 groups was more intense than that in the DM and DM+ADM2+GW9662 groups (Figure 5).

ADM2 restored the imbalance of macrophage polarization during DO in diabetic rats.

To explore whether ADM2 promoted M2 polarization within distraction regenerates, we applied double-immunofluorescence to label M1 (CD68⁺CD86⁺) and M2 (CD68⁺CD206⁺) macrophages. As shown in Figure 6, there was minimal detection of M1 macrophages in the control group, but enriched distribution in the DM group (Figure 6 A, B). However, after ADM2 treatment, the ratio of M1 macrophages significantly decreased (Figure 6 A, B). Conversely, the ratio of M2 macrophages was minimal in the DM group, but increased in the DM+ADM2 group (Figure 6 C, D). Moreover, these effects of ADM2 were mostly compromised by GW9662 administration (Figure 6 A-D). Taken together, these results indicate that ADM2 induced macrophage M2 polarization from the M1 phenotype, in distraction regenerates of diabetic DO rats.

Discussion

In this study, we found that ADM2 reversed AGE-induced M1 macrophage polarization to M2 phenotype *in vitro*. In addition, the M2 polarization effect of ADM2 was achieved, at least in part, by the inhibition of

NF- κ B signaling via the activation of PPAR γ . Moreover, we verified the rescue effect of ADM2 on AGE-induced BMSC dysfunction during osteogenic differentiation. *In vivo*, the rescue effects of ADM2 on bone regeneration and M2 macrophage polarization under DM were verified and the involvement of PPAR γ activation in these effects of ADM2 was also investigated. To the best of our knowledge, this is the first study to show that ADM2 can accelerate bone regeneration under diabetic conditions by regulating macrophage polarization and osteogenesis in parallel.

Even with insulin replacement therapy, a high rate of prolonged consolidation is observed in most T1DM patients undergoing DO treatment, and this effect is primarily attributed to impaired bone regeneration[8, 28]. There is considerable evidence to show that BMSCs deteriorate under diabetic conditions and exhibit reduced osteogenic capability[19, 29]. Although the specific mechanism of diabetes-induced BMSC dysfunction is not fully understood, the AGE/RAGE pathway is considered as one of the primary mechanisms. On the one hand, AGEs have been reported to directly interact with RAGE of osteoblast lineage cells and impair osteogenic differentiation by modulating DNA methylation and Wnt signaling[19]. On the other hand, as the knowledge about the cellular mechanisms underlying bone regeneration in DM is expanding, more recent studies have increasingly acknowledged that the osteogenic differentiation of BMSCs is, to a great extent, suppressed by prolonged inflammation under diabetic condition[30]. Since pathologically elevated ratio of M1 macrophages is the fundamental cause of prolonged inflammation, AGE-induced M1 macrophage polarization could be another promising treatment candidate for diabetic bone regeneration in DO[31]. Therefore, the present study established the simultaneous attenuation of AGE-induced M1 polarization and BMSC dysfunction, which intervenes in the indirect and direct factors leading to impaired osteogenesis, as a therapeutic strategy, and verified that ADM2 could indeed improve bone regeneration under diabetic conditions by exerting this dual positive effect (Fig. 7).

The NF- κ B family of transcription factors plays an essential role in inflammation and macrophage M1 polarization induced by various molecules, including AGEs[32]. Although there has been abundant research on the NF- κ B-inhibiting effect of ADM2, the mechanism by which ADM2 affects NF- κ B remains unclear[21, 33, 34]. PPAR γ is a key nuclear transcription factor involved in inflammation and macrophage polarization[35, 36]. A recent study verified that PPAR γ stimulation could inhibit the activation of NF- κ B through upregulation of I κ B α expression at the transcriptional level, which retains the NF- κ B subunits p50/p65 in a cytoplasmic inactive complex[27]. Of note, ADM2 has been reported to exhibit anti-inflammatory and M2 polarization effects; therefore, we hypothesized that ADM2 might exert a positive effect on PPAR γ activation in BMDMs. Indeed, we observed that the ADM2 treatment significantly rescued the expression of PPAR γ and I κ B α , which was downregulated by AGEs. As expected, the activation and nuclear translocation of NF- κ B were also diminished during ADM2-induced M2 macrophage polarization. In the diabetic DO model, ADM2 administration distinctly increased the ratio of M2 macrophages within distraction regenerates. Moreover, the M2 polarization effect of ADM2 *in vitro* and *in vivo* could be at least partially reversed by a PPAR γ antagonist, indicating that ADM2 might facilitate the dynamic shift from M1 to M2 under diabetic conditions through the PPAR γ /I κ B α /NF- κ B pathway. However, various pathways participate in AGE-induced M1 macrophage polarization under DM

conditions, and the comprehensive mechanisms, except for PPAR γ activation, induced by ADM2 are poorly understood[37–39]. In addition, in order to inhibit the pathophysiological process caused by AGE/RAGE interaction, direct interventions upstream also represent a potential therapeutic strategy, including inhibition of AGE formation, downregulation of RAGE expression, and blockage of AGE/RAGE interaction[40–42]. Therefore, other feasible mechanisms by which ADM2 accelerates diabetic bone regeneration are yet to be explored.

Although the osteogenic differentiation process was, to a great extent, suppressed by prolonged inflammation under diabetic conditions, AGE could also directly inhibit the osteogenic potential of adipose-derived stem cells[19]. Hence, even though the prolonged inflammation under diabetic conditions could be relieved by ADM2 treatment, the AGE-induced direct osteogenesis impairment still needs to be retrieved for full restoration of bone regeneration under diabetic conditions. In this study, we verified that ADM2 could at least partially rescue the AGE-impaired osteogenic capacity of BMSCs. This discovery, along with the improvement effect of ADM2 on M2 polarization, provided theoretical support for ADM2 facilitating diabetic bone regeneration during DO. Based on previous studies, the inhibitory effect of AGEs on osteogenic differentiation is closely related to DNA methylation and downregulation of the Wnt pathway[19]. Since ADM2 has been shown to activate protein kinase B (AKT) signaling in various cells[43–45], and activated AKT could preserve β -catenin through phosphorylation and inactivation of glycogen synthase kinase-3 β (GSK-3 β)[46], we assume that the osteogenesis-protecting effect of ADM2 on BMSCs may contribute to the activation of the AKT/GSK-3 β / β -catenin pathway. Although this study does not include an exploration of the mechanisms by which ADM2 directly improves osteogenic potential of BMSCs, further studies are required to help develop a comprehensive and in-depth understanding of the relevance of ADM2 with bone regeneration under diabetic conditions.

The present study has several limitations. First, the detailed mechanisms underlying the ability of ADM2 to activate PPAR γ remain to be fully elucidated. Second, the feasible mechanisms contributing to the rescue effect of ADM2 on AGE-impaired osteogenic potential have not been verified. Lastly, although ADM2 could rescue the osteogenic potential of BMSCs impaired by AGEs, this comprehensive effect fails to prove that the pathways regulated by ADM2 are all beneficial to osteogenic differentiation. Since PPAR γ is a vital factor for adipogenic differentiation of BMSCs[47], ADM2 may potentially possess the ability to inhibit osteogenesis, contributing to its PPAR γ -activating effect, which may lead to ADM2 inhibition of bone regeneration in non-diabetic individuals, thus affecting the indications for the application of ADM2 in clinical practice. Consequently, the effects and mechanisms of ADM2 on the osteogenic differentiation of BMSCs under normal conditions remain to be further investigated.

Conclusions

This study demonstrates that ADM2 reverses AGE-induced M1/M2 imbalance partly through the PPAR γ /I κ B α /NF- κ B signaling pathway and restores AGE-impaired osteogenic potential of BMSCs simultaneously, revealing ADM2 as a novel factor to accelerate bone regeneration under diabetic

conditions during DO. Moreover, our study also provides a novel therapeutic strategy for diabetic patients undergoing DO, which is managing both inflammation and osteogenesis parallelly.

Abbreviations

ADM2, adrenomedullin 2; AGE, advanced glycation end product; AKT, activate protein kinase B; ALP, alkaline phosphatase; Arg-1, arginase1; ARS, alizarin red S; BMD, bone mineral density; BMDM, bone marrow-derived macrophage; BMP2, bone morphogenetic protein 2; BMSC, bone marrow mesenchymal stem cell; BSA, bovine serum albumin; BV/TV, bone volume/tissue volume; CT, computed tomography; DAPI, 4',6-diamidino-2-phenylindole; DO, distraction osteogenesis; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; E-modulus, modulus of elasticity; FBS, fetal bovine serum; GSK-3 β , glycogen synthase kinase-3 β ; H&E, hematoxylin-eosin; hematoxylin-eosin; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; I κ B α , nuclear factor kappa-light-chain-enhancer of activated B cells inhibitor alpha; MRC1, macrophage mannose receptor 1; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; OCN, osteocalcin; OIM, osteogenic induction medium; OSX, osterix; P/S, penicillin-streptomycin; PBS, phosphate buffer saline; PFA, paraformaldehyde; PGL, plasma glucose level; PPAR γ , peroxisome proliferator-activated receptor γ ; qRT-PCR, quantitative real-time polymerase chain reaction; RAGE, receptor of advanced glycation end product; SD, sprague dawley; SO-FG, safranin O-fast green; STZ, streptozotocin; T1DM, type 1 diabetes mellitus; TGF- β , transforming growth factor β ; TNF- α , tumor necrosis factor α ; Wnt, wingless/integrated.

Declarations

Ethics approval and consent to participate

All experimental procedures on Sprague-Dawley rats were performed in accordance with the Animal Research Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and in compliance with National Institutes of Health Guide for the care and use of laboratory animals.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

QLK and JX conceived and designed the experiments. FW performed the experiments. QLK, JX, and FW wrote the manuscript. FW, JX, and QLK analyzed the data and prepared all the figures. LCK, WBW, LS, MWW provided technical support. YMC provided financial support. All authors reviewed and agreed upon the manuscript.

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Tables

Table 1 Primers for quantitative real-time polymerase chain reaction (qRT-PCR)

Gene	Forward (5'–3')	Reverse (5'–3')
ALP	CCGCAGGATGTGAACTACT	GGTACTGACGGAAGAAGGG
Arg-1	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC
GAPDH (Mouse)	AAATGGTGAAGGTCGGTGTG	AGGTCAATGAAGGGGTCGTT
GAPDH (Rat)	ATGGCTACAGCAACAGGGT	TTATGGGGTCTGGGATGG
IL-6	AGCCAGAGTCCTTCAGAGAGAT	GCACTAGGTTTGCCGAGTAGAT
iNOS	CGAGACGGATAGGCAGAGATTG	CTCTTCAAGCACCTCCAGGAA
MRC1	CCTATGAAAATTGGGCTTACGG	CTGACAAATCCAGTTGTTGAGG
OCN	CAGACAAGTCCCACACAGCA	CCAGCAGAGTGAGCAGAGAGA
OPN	GGCCGAGGTGATAGCTT	CTCTTCATGCGGGAGGT
OSX	GGAAAAGGAGGCACAAAGAA	CAGGGGAGAGGAGTCCATT
TGF- β	CGGAGAGCCCTGGATACCA	GCCGCACACAGCAGTTCTT
TNF- α	GCTGAGCTCAAACCCTGGTA	CGGACTCCGCAAAGTCTAAG

Figures

Figure 1

ADM2 reversed AGE-induced M1 macrophage polarization to M2 phenotype. (A, B) Expression of M1-specific genes (*iNOS*, *IL-6*, and *TNF-α*) (A) and M2-specific genes (*Arg-1*, *MRC1*, and *TGF-β*) (B) of BMDMs treated with vehicle, AGEs, AGEs+ADM2, and AGEs+ADM2+GW9662 was assessed by qRT-PCR. (C) The expression of CD86 (M1 surface marker) and CD206 (M2 surface marker) on BMDMs in each group was examined by flow cytometry. (D) Quantification of mean fluorescence intensity (MFI) of the surface markers. (E) ELISA for production of pro-inflammatory cytokine (*TNF-α*) and anti-inflammatory cytokine (*TGF-β*) in the supernatant of BMDMs in each group. * $P < 0.05$; ** $P < 0.01$.

Figure 2

ADM2 reversed the imbalance of M1/M2 polarization induced by AGEs via PPAR γ /I κ B α /NF- κ B pathway. (A) Western blot of PPAR γ , I κ B α , p65, and p-p65 in BMDMs treated with vehicle, AGEs, AGEs+ADM2, and AGEs+ADM2+GW9662. (B) Quantitative analysis of the protein levels of PPAR γ and I κ B α relative to GAPDH and the phosphorylated level of p65. (C) Representative immunocytochemistry images showing the expression and distribution of p65 in BMDMs in each group. Scale bar: 50 μ m. (D) Quantitative analysis of the nuclear-translocation of p65. *P < 0.05; **P < 0.01

Figure 3

ADM2 improved the AGE-impaired osteogenic potential of BMSCs. (A-D) Osteogenesis of BMSCs treated with OIM, OIM+AGEs, and OIM+AGEs+ADM2 was determined by ALP staining (A), ALP activity assays (B) and alizarin red staining (C). Calcium deposition was assessed by measuring the optical density (D). Scale bar: 200 μ m. (E, F) Expression of osteogenic-specific genes and proteins of BMSCs in different groups were assessed by qRT-PCR (E) and Western blot (F). (G) Quantitative analysis of the protein levels of BMP2, OCN, and OSX relative to GAPDH. * $P < 0.05$; ** $P < 0.01$

Figure 4

ADM2 accelerated bone consolidation during distraction osteogenesis in diabetic rats. (A) Mechanical tests (E-modulus, ultimate load, and energy to failure) of the distracted tibias. The values were normalized to the corresponding contralateral normal tibias. (B) Representative X-rays of distraction regenerate at various time points of consolidation phase in the four groups. (C) Representative 3-D and longitudinal images of the tibial distraction zone after 2 and 4 weeks of consolidation and treatment. (D) Quantitative analysis of bone mineral density (BMD) and bone volume/tissue volume (BV/TV) of the regenerated bone. * $P < 0.05$; ** $P < 0.01$

Figure 5

ADM2 accelerated mature callus formation during distraction osteogenesis in diabetic rats. Representative images of H&E, Masson, and Safranin O-Fast Green staining from the middle of the distracted tibias showed various amounts of trabecular bones, fibrous tissue and cartilaginous tissue in all four groups. Compared to the control group, the distraction gaps showed increased fibrous and cartilaginous tissues and less trabecular bone in the center zone in the DM group. Upon treatment of ADM2, the distraction gaps showed more mature trabecular bone and less fibrous and cartilaginous tissues. The bone-regenerating effect of ADM2 was partially reversed by GW9662 administration. Immunohistochemical analysis for osteocalcin (OCN) showed a similar tendency with stronger staining in the control and ADM2 groups than that in the DM and GW9662 groups. Scale bar: 200 μ m.

Figure 6

ADM2 reversed the imbalance of M1/M2 polarization during distraction osteogenesis in diabetic rats. (A) Immunofluorescence staining images of CD68 and CD86 for the distraction area sections from the four groups. Scale bar: 100 μ m. (B) Quantitative analysis of CD68+CD86+ / CD68+ M1 macrophage ratio for the distraction area sections from the four groups. (C) Immunofluorescence staining images of CD68 and CD206 for the distraction area sections from the four groups. Scale bar: 100 μ m. (D) Quantitative analysis of CD68+CD206+ / CD68+ M2 macrophage ratio for the distraction area sections from the four groups. *P < 0.05; **P < 0.01

Figure 7

Working model of ADM2 restoration of AGE-induced imbalanced macrophage polarization and impaired osteogenesis. Under pathological diabetic conditions, AGEs interact with RAGE in macrophages and BMSCs, activating AGE-RAGE signaling. In macrophages, PPAR γ is downregulated subsequently, leading to the inhibition of I κ B α and the activation and nuclear translocation of p65, which plays a vital role in the M1 polarization of macrophages. The inflammatory cytokines secreted by M1 macrophages would lead to an inflammatory microenvironment, thus indirectly impairing the osteogenic differentiation of BMSCs. Besides, the activation of AGE-RAGE signaling in BMSCs could directly impair the osteogenic potential of BMSCs. Finally, reduced osteogenesis of BMSCs causes impaired bone regeneration in T1DM. ADM2 reverses AGE-induced M1 polarization of macrophages to M2 phenotype, which contributes to the regenerative microenvironment, by activating PPAR γ and attenuates AGE-impaired osteogenic potential of BMSCs simultaneously, thus accelerating diabetic bone regeneration during DO.