Osseous wound repair under inhibition of the axis of advanced glycation end-products and the advanced glycation end-products receptor

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Background/Purpose: Blockade of advanced glycation end-products (AGE) is able to reduce diabetic complications and control periodontitis. This study aimed to determine whether the application of aminoguanidine (AG), an AGE inhibitor, or N-phenacylthiazolium bromide (PTB), an AGE breaker, facilitates the healing of an osseous wound in non-diabetic animals.

Methods: 2.6 mm diameter full-thickness osseous wounds were created bilaterally in 54 healthy Sprague-Dawley rats. Rats received daily normal saline, AG, or PTB injections respectively and were euthanized after 7 days, 14 days, or 28 days (n = 6). The wound healing pattern was assessed by micro-computed tomography, histology, histochemistry for the fiber arrangement, and the gene expression levels of AGE receptor, tumor necrosis factor-α, type I collagen, and fibronectin.

Results: Under the AG and PTB administration, osteogenesis was apparently promoted in the early stages of healing, but the union of the osseous wound and the fibril re-arrangement...
was apparently retarded. No significant difference was found in any of the micro-computed tomography parameters as compared to the control in the first 14 days, whereas the relative bone volume was significantly higher in the control at Day 28. AGE receptor and tumor necrosis factor-α were depressed in the PTB group, but only temporarily at Day 14 in the AG group. Therefore, at Day 14, type I collagen was significantly upregulated in the PTB group, and fibronectin was significantly increased in the AG group.

Conclusion: Anti-AGE agents reduced inflammation but did not apparently facilitate osteogenesis during the osseous wound repair.

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Introduction

The healing of the osseous wound generated by periodontal inflammation, osseous surgery, or the enucleation of cysts or tumors involves complex molecular signaling. The harmonization of destructive and reconstructive phases is one of the prerequisites for achieving bone regeneration. Inflammation, an immediate immunological response after injury, is a determining factor for tissue destruction. However, the inhibition of inflammation was also reported to damage the bone tissue. Modulation of inflammation to facilitate the healing process has not been confirmed.

Advanced glycation end-products (AGE), the irreversible adducts from the nonenzymatic glycosylation of proteins or lipids, have been found to accumulate in patients with severe metabolic or immunological disturbances or with extensive inflammation or degeneration. AGE have been reported to interfere with cell-matrix interactions by the alteration of cross-linking of the extracellular matrix. The engagement of the cellular receptor for AGE (RAGE) may induce an increase in oxygen tension and lead to the production of inflammatory cytokines. Our recent investigations demonstrated that AGE–RAGE axis activation occurs in parallel with experimental periodontitis progression and was evident in the early stage of dento-alveolar osseous wound healing of nondiabetic animals. Anti-AGE agents, including aminoguanidine (AG), an AGE inhibitor, and N-phenacylthiazolium bromide (PTB), an AGE breaker, have been reported to inhibit the induction of experimental periodontitis of nondiabetic animals as well as to promote cellular viability in the nondiabetic and noninflammatory condition in vitro. The potential modulatory mechanisms are the reduction of inflammation and the increase of matrix synthesis. However, inflammatory destruction recovery is only facilitated with PTB administration. This outcome is presumably related to the limited effect of AG on the highly concentrated pre-existing AGE in the sites of periodontitis.

In this study, we examined the therapeutic potential of anti-AGE agents in the facilitation of osseous wound repair in rats. The efficacy of AG and PTB was investigated respectively by micro-computed tomography (micro-CT), histology, and expression profiles of RAGE, tumor necrosis factor-α (TNF-α), type I collagen, and fibronectin.

Figure 1  (A) The study design. A total of 54 animals (6/group/time point) and 108 sites (2/animal) were evaluated. (B) Illustration of the osseous wound in the mandible. The standardized defect was created using a 2.6 mm diameter customized drill, and the dashline indicates the path of the mandibular canal. AG = aminoguanidine; micro-CT = micro-computed tomography; NS = normal saline; PTB = N-phenacylthiazolium bromide.
Materials and methods

Animal models

All of the animal procedures performed followed the approved protocol number 20130054 from the Institutional Animal Care and Use Committee of the National Taiwan University, Taipei, Taiwan. Fifty-four male Sprague–Dawley rats (250–300 g) were utilized. The study design is illustrated in Fig. 1A, and the sample size was determined by power analysis based on the results from our previous study. We assumed at least 15% difference in relative bone volume between the control and treatment groups, 80% power, $\alpha = 0.05$, and normal distribution and equivalent variance of the samples. Thus, at least six animals/treatment group/time point were used in this study. A 2.6 mm diameter full-thickness osseous defect was created using a customized dental drill on both sides of the mandible of each rat (Fig. 1B). Animals received daily injections of normal saline (control), 100 mg/kg AG (Sigma-Aldrich, St Louis, MO, USA), or 5 mg/kg PTB (Ariel Chemical Co. Ltd., Wuhan, China) and were euthanized at 7 days, 14 days, or 28 days after the creation of the defects ($n = 6$/treatment/time point). From the harvested mandible, tissues within the osseous wound in one randomly selected side were excised for the evaluation of gene expression levels, and the other side of the mandible was fixed in 10% formaldehyde for microcomputed tomography micro-CT imaging and histological examinations.

Micro-CT

The harvested mandibles were examined by Siemens Inveon CT System (Siemens Healthcare, Erlangen, Germany) with an 80-W tungsten anode, a 30–80-kVp variable focus X-ray source, and a 165-mm detector to achieve an effective pixel size of $19 \mu m \times 19 \mu m \times 19 \mu m$ (or $0.019 mm \times 0.019 mm \times 0.019 mm$). The entire osteotomy site was selected as the region of interest, and the micromorphometric bone parameters, including relative bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N), were separately calculated for each region of interest using CTAn software (Skyscan, Kontich, Belgium).

Histology

After fixation, the mandibles were decalcified with 12.5% ethylenediaminetetraacetic acid and embedded in paraffin. Two 5 $\mu m$ sections from the horizontal plane of the center of the osseous wound were selected. One section was stained with hematoxylin and eosin for histological assessment, and the other with Masson’s Trichrome to evaluate the collagen configurations. All of the images were acquired utilizing

Figure 2 Osseous wound repair pattern from a slice of micro-computed tomography imaging from day 7 to 28. The region bounded by the dashline indicates the wound edges of the control specimens at Day 28. AG = aminoguanidine; PTB = N-phe-nacylthiazolium bromide.
AxioCam ICc5 system (Carl Zeiss Microscopy GmbH, Munich, Germany), and closure of the osseous defect was evaluated by the measurement of the distance between the two ends of the osseous edges as measured under 40× magnification.

Real-time polymerase chain reaction analysis

The RNA harvested from the tissue was transferred to cDNA utilizing iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), and the mRNA levels of genes were quantitatively analyzed by StepOnePlus real-time polymerase chain reaction system (Applied Biosystems and Life Technologies, Grand Island, NY, USA) and sequence-specific TaqMan gene expression assays (Applied Biosystems and Life Technologies, Grand Island, NY, USA) for GAPDH (housekeeping gene), RAGE, TNF-α, type I collagen (major extracellular matrix protein in the periodontium), and fibronectin (extracellular matrix protein for cell attachment). Because the mRNA levels of bone-specific genes, including osteopontin and osteocalcin, were not detectable in most harvested specimens of our preliminary trials, the assessment of bone-specific genes was excluded in the present study. The gene expression levels were normalized to GAPDH, and data were evaluated compared to the relative expression of the averaged levels of the control group at the same time point. All experiments were performed in triplicate.

Statistical analysis

One-way ANOVA followed by Tukey’s post hoc test were used to compare the differences between the control and AG or PTB-treated groups at each time point. The data are presented as the mean ± standard deviation of measurements, with p < 0.05 considered to be statistically significant.

Results

Gross observations

All animals recovered uneventfully from the anesthesia and interventions and survived until the date of euthanasia. However, swelling in the submandibular area was noted in all animals during the first 3 days, which was completely resolved at Day 7, without observable differences among groups (data not shown).

Osseous wound repair from the micro-CT imaging

Mineralization initiated at the wound edges in all groups at Day 7 (Fig. 2, upper panel). At Day 14, the wound was apparently completely filled with bone spongiosa in the control group, whereas partially wound fill and the union of the newly-formed bone from the wound edges was noted in the AG and PTB groups (Fig. 2, middle panel). At Day 28, the border of the osseous wound was generally indistinguishable in the control group (Fig. 2, lower panel). Greater wound fill was noted in the AG and PTB groups relative to Day 14, with thicker bone spongiosa noted in the PTB group. However, mineralization of the AG and PTB groups was obviously inferior relative to the control group.
Quantitative micro-CT assessments

Generally, all of the micro-CT parameters, except Tb.Sp, gradually increased and appeared to reach a steady status at Day 14 in the AG and PTB groups, whereas the persistent increase of mineralization was noted in the control group until Day 28 (Fig. 3A–C). BV/TV was slightly higher in the AG and PTB groups relative to the control at Day 7, and no obvious difference was noted among all three groups at Day 14 (Fig. 3A). However, BV/TV was significantly higher in the control relative to the AG and PTB groups at Day 28. No obvious difference was noted among all three groups at Day 7 and Day 14 (Fig. 3B and C), and the control group demonstrated significantly greater Tb.Th than that of the AG and PTB groups at Day 28 ($p < 0.05$, Fig. 3B). Tb.Sp was slightly and insignificantly greater in the PTB group relative to the AG and control groups at Day 14, whereas no obvious difference in Tb.Sp was noted among the three groups at Day 7 and Day 28 (Fig. 3D).

Descriptive histology

At Day 7, the osseous wound was mainly occupied with fibrous tissue. The osteogenesis was immature and restricted at the edge of the wound in the control group (Fig. 4A). In the AG and PTB groups, the newly-formed bone appeared to be evenly distributed within the wound (Fig. 4B). At Day 14, the union of the osseous wound by the thin lamellar bone was noted. The edge of the osseous wound was still distinguishable in the control group (Fig. 4C). The newly-formed bone was apparently thicker and evidently deposited in the inner native bone surface of the AG and PTB groups, whereas the union of the osseous wound was not observed (Fig. 4D). Therefore, at Day 28, the osseous wounds were invisible in four specimens of the control group, two specimens of the AG group, and two specimens of the PTB group (data not shown). Histologically, the newly-formed bone was corticalized and without distinguishable junction to the native bone in the control group (Fig. 4E). Although the bone trabeculae were thicker and better organized relative to those at Day 14, corticalization was still not achieved in most specimens of the AG and PTB groups at Day 28 (Fig. 4F).

Observations under Masson’s trichrome staining

At Day 7, the wound edge was occupied with loose fibrous matrix and thin spongiosa in the control group (Fig. 5A). In
the AG and PTB groups, a fibril matrix was apparently densely aligned in the neogenic bone, and the bone surface was relatively flat compared to the control group. Higher cellularity with fewer interposed collagen fibrils was noted in the remainder of the wound area (Fig. 5B). The bone trabeculae appeared to be thicker at Day 14 and Day 28 in all three groups (Fig. 5C–F). However, in the AG and PTB groups, the fibril arrangement was apparently different in the native and newly-formed bone at Day 14 (Fig. 5D), and the border between the native and newly-formed bone was clearly distinguishable at Day 28 (Fig. 5F).

**Gene expression profiles**

RAGE was apparently downregulated in PTB-treated animals from Day 7 to Day 28 and significantly downregulated in both AG and PTB groups ($p < 0.05$) at Day 14 (Fig. 6A). TNF-$\alpha$ was significantly downregulated in the PTB group at Day 7 ($p < 0.05$) and Day 14 ($p < 0.01$) and in the AG group at Day 14 ($p < 0.05$, Fig. 6B). The type I collagen level was significantly higher in the PTB group relative to the control group at Day 14 ($p < 0.05$), whereas a difference was not obvious at Day 28 (Fig. 6C). Fibronectin was apparently higher in the AG group relative to that of all other groups (Fig. 6D), and a significant difference compared to the control was noted at Day 14 ($p < 0.05$).

**Discussion**

The accumulation of AGE and the activation of the AGE–RAGE axis have been shown to affect wound healing dynamics in diabetics. Blockade of this signaling pathway facilitated the recovery phase. The existence of the AGE–RAGE axis could also directly influence the activities
of osteoblastic/osteoclastic activities and lead to the impaired formation of the osseous tissue in the diabetes.\textsuperscript{22} Given that the activation of the AGE–RAGE axis is prominent in the early reparative stage of the periodontal osseous defect in systemically healthy rats and that the level was parallel to the degree of inflammation,\textsuperscript{15} the present study might be the first report of the antiglycation treatment in osseous wound repair. The results demonstrate that neo-osteogenesis was apparently accelerated with the treatment of anti-AGE agents (i.e., AG or PTB) at Day 7 (Figs. 4B and 5B). This effect might be related to the inhibition of inflammation via the downregulation of the AGE–RAGE signaling pathway (Fig. 6A and B). However, no significant difference was noted in any of the micro-CT parameters at this stage (Fig. 3). As Kim et al\textsuperscript{23} demonstrated, the differentiation process of the neuron precursor cells was unlikely triggered but potentially promoted by RAGE signaling. It is inferred that the differentiation potential of osteoprogenitor cells was also not directly triggered in the present study, and thus the promotion of osteogenesis was limited.

Following the recession of inflammation, the levels of RAGE and TNF-\(\alpha\) in the AG and PTB groups were not significantly different from those of the control group at Day 28 (Fig. 6A and B). However, osteogenesis as well as the union of the osseous wound was apparently retarded in the AG and PTB groups (Figs. 3 and 4). Unlike the control, newly-formed bone was deposited in the inner surface of the mandible with a distinctly different fibril arrangement than the native bone (Fig. 5D and F), presumably due to the resultant hypoglycosylation caused by the persistent antiglycation treatment. Hypoglycosylation had been reported to affect the structural integrity and force transmission between the cytoskeleton and the extracellular matrix and to lead to peripheral tissue dystrophy in extreme cases,\textsuperscript{24,25} and a similar situation might apply to the healing osseous wound. Thus, the upregulation of structural matrix expression in the AG and PTB groups at Day 14 (Fig. 6C and D) might not necessarily lead to the facilitation of the osseous wound repair in the later stage. By contrast, in order to confirm the capability of osteogenesis, examinations on the expression levels of bone-specific markers would be needed.

The inhibition of RAGE-regulated signaling was apparently different in the AG and PTB groups. RAGE and TNF-\(\alpha\) were downregulated at Day 7 and sustained until Day 28 in the PTB group, whereas the downregulation of RAGE and TNF-\(\alpha\) was only prominent at Day 14 in the AG group (Fig. 6A and B). This difference may be due to the short plasma half-life (\(<1\) hour) of AG, whereby the high AG concentration required to trap dicarbonyl intermediates to inhibit AGE formation and reduce inflammation as well.\textsuperscript{26} Therefore, as inflammation in the current model was relatively low and was only elicited immediately after the creation of the defect, the resultant promotive effect in the osseous wound repair was consequently similar in both the AG and PTB groups.

The study has limitations. Firstly, the pattern and etiology of the osseous defect are different from those in human jaw bones, and the size of the defect was relatively small.
due to the limited size of rat jaw bone and the proximity of the mandibular canal to the mandibular border. The pattern and dynamics of wound healing observed in this study might differ from the clinical conditions in humans. Secondly, AG and PTB were systemically administered without targeting to the specific tissue, and the dose utilized was a single effective dose in the modulation of experimental periodontitis. The concentration might be suboptimal and uncontrollable in the osseous wound. Thirdly, as the tissue agents precisely are still needed.

Development of vehicles to control the release of anti-AGE in the current mode of anti-AGE treatment. Further mechanistic investigation in more clinically relevant models and the development of vehicles to control the release of anti-AGE agents precisely are still needed.

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