Advanced Glycation End Products Enhance Osteoclast-Induced Bone Resorption in Cultured Mouse Unfractionated Bone Cells and in Rats Implanted Subcutaneously with Devitalized Bone Particles

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Abstract. Advanced glycation end products (AGE) are formed in long-lived matrix proteins by a nonenzymatic reaction with sugar. The presence of AGE in β₂-microglobulin-amyloid fibrils of dialysis-related amyloidosis, one of the characteristic features of which is an accelerated bone resorption around amyloid deposits, was recently demonstrated. This suggested a potential link of AGE in bone resorption and initiated this investigation of whether AGE enhance bone resorption. When mouse unfractionated bone cells containing osteoclasts were cultured on dentin slices, both AGE-modified β₂-microglobulin and BSA increased the number of resorption pits formed by osteoclasts, whereas their normal counterparts or those modified with the early glycation products did not. AGE proteins, however, did not increase the number of newly formed osteoclasts, even in the coculture of mouse bone marrow cells with osteoblastic cells isolated from mouse calvaria. Enhanced bone resorption was also observed when unfractionated bone cells were cultured on AGE-modified dentin slices. AGE-enhanced bone resorption was effectively inhibited by calcitonin and ipriflavone, both of which are inhibitors of bone resorption. AGE-enhanced bone resorption was further supported by in vivo evidence that rat bone particles—upon incubation with glucose for 60 days (AGE-bone particles)—when implanted subcutaneously in rats, were resorbed to a much greater extent than control bone particles upon parallel incubation without glucose. These findings suggest that AGE enhance osteoclast-induced bone resorption. Although the mechanism remains unknown, AGE are unlikely to promote differentiation of osteoclast progenitors into osteoclasts, suggesting that AGE activate osteoclasts or alter microenvironments favorable for bone resorption by osteoclasts. The modification of bone matrices with AGE might play a role in the remodeling of senescent bone matrix tissues, further implicating a pathological significance of AGE in dialysis-related amyloidosis or osteoporosis associated with diabetes and aging. (J Am Soc Nephrol 8: 260–270, 1997)

Advanced glycation end products (AGE) are the pigmented and fluorescent adducts that are formed, over the months, by a non-enzymatic reaction between sugar and long-lived proteins such as matrix collagens, called the Maillard reaction (1). Several lines of evidence have suggested that AGE proteins play a role in normal tissue remodeling, i.e., the removal and replacement of senescent extracellular matrix components (2). However, under pathological conditions such as diabetes, renal failure, and aging, the accumulation of AGE proteins might lead to tissue damage through a variety of mechanisms: through an alteration of the structure and function of tissue proteins (2), by the stimulation of cellular responses (3–8) via receptors specific for AGE proteins (9,10), or by the generation of reactive oxygen intermediates (11,12). AGE have thus been implicated in the pathogenesis of atherosclerosis (3,4,13), diabetic nephropathy (5,6), dialysis-related amyloidosis (7,8,14–17), and Alzheimer’s disease (18–20).

Recently, we demonstrated that AGE are present in amyloid fibrils of dialysis-related amyloidosis (14,15). This is a serious osteoarticular complication of long-term hemodialysis patients, in which amyloid fibrils consisting predominantly of β₂-microglobulin (β₂m) deposit particularly in periarticular bones (21,22). One of the characteristic features of this complication is an accelerated bone resorption around amyloid deposits, which leads to subchondral erosion and bone cysts—detected by radiological examination—in most patients undergoing hemodialysis for more than 10 yr (21,22). Further studies in our group demonstrated that the β₂m modified with AGE (AGE-β₂m), but not intact β₂m, induces chemotaxis of monocytes and secretion of bone-resorbing cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 from mono-
cyte-derived macrophages (7,8,17). These findings suggest a potential link of AGE to bone resorption.

The AGE levels of matrix proteins have been shown to be markedly elevated in diabetic patients and elderly subjects when compared with healthy young subjects (23,24). Taking into account the observations that the AGE modification accumulates in long-lived proteins in vivo (1,2) and that among these are bone matrix proteins such as Type I collagen, we assumed that AGE might play a pathophysiological role not only in dialysis-related amyloidosis, but also in the remodeling of senescent bone matrix tissues or osteoporosis associated with diabetes and aging. In the study presented here, we examined the effect of AGE on osteoclast-induced bone resorption in vitro utilizing the pit-formation assay with an unfractionated bone cell culture system containing osteoclasts from newborn mice. Because bone resorption is regulated by two different processes, i.e., differentiation of osteoclast progenitors into osteoclasts and activation of osteoclasts (25), we examined the effect of AGE on osteoclast formation by means of the mouse coculture system of bone marrow cells with primary osteoblastic cells. An inhibitory effect of clinically used anti-bone resorbing agents on bone resorption enhanced by AGE was also evaluated. Furthermore, we investigated the bone-resorbing effect of AGE in vivo in rats that were implanted subcutaneously with bone particles.

Materials and Methods

Both normal and electrophoretically acidic isoforms of βm were purified from the urine of nondiabetic hemodialysis patients who were free of urinary infection as described previously (14). Our previous study demonstrated that the acidic βm, but not normal βm, had the characteristic physicochemical properties of AGE proteins, i.e., brown color, fluorescence, and tendency for polymerization, and exhibited positive immunoreactivity to anti-AGE antibody (14). The levels of pentosidine (26), which is a glycoxidation marker postulated to be AGE (11), were 2.12 mmol/mol in acidic βm and below the detection limit (0.02 mmol/mol) in normal βm, assessed by HPLC assay according to the method by Odetti et al. (27). This also supports that AGE are present in the acidic βm, but not in normal βm.

Proteins modified with the Maillard products such as AGE or Amadori products, the early Maillard products leading to AGE, were also prepared in vitro. Five hundred micrograms of normal human βm or BSA (essentially fatty acid-free grade; Sigma, St. Louis, MO) was incubated with 0.1 M tris-glycine (Wako Pure Chemicals, Osaka, Japan) at 37°C for 60 days or 10 days in 500 μL of 0.1 M phosphate buffer (pH 7.4) containing 1.5 mM phenylmethanesulfonyl fluoride (PMSF; Sigma) under sterile conditions. The presence of AGE in glycated βm after a 60-day incubation and the presence of Amadori products in glycated βm after a 10-day incubation were shown previously (14–17). The pentosidine levels, determined by HPLC assay, were 0.09 mmol/mol and 1.74 mmol/mol in the glycated βm after a 10-day and 60-day incubation, respectively, and below the detection limit (0.02 mmol/mol) and 2.28 mmol/mol in normal BSA and the glycated BSA after a 60-day incubation, respectively. The levels of Amadori product (fructoselysine) were also determined by colorimetric assay using a kit (Fructosamine Test Roche-II; Nihon Roche Ltd., Tokyo, Japan) (17): 0.05 mol/mol in normal βm, and 0.44 mol/mol and 0.70 mol/mol in the glycated βm after a 10-day and 60-day incubation, respectively.

Removal of Endotoxin

To perform the experiments in endotoxin-free materials, we carefully removed endotoxin from the proteins by incubating them with endotoxin-adsorbent (Pyro SepR; Daicel Chemical Industries, Ltd., Tokyo, Japan) for 2 h at 4°C, followed by filtration through a 0.22 μm-pore filter (Ultrafree C3-GV; Nihon Millipore Ltd., Tokyo, Japan) to remove the adsorbent. The endotoxin levels were measured by a kit (Toxicolor® System; Seikagaku Corp., Tokyo, Japan), and all the materials and media used in this study were found to be endotoxin-free (<0.5 U of endotoxin/mL).

Unfractionated Bone Cell Preparation

Unfractionated bone cells were prepared according to the method of Takada et al. (28). In brief, the femur and tibia of 11- to 13-day-old ICR mice (Japan Charles River, Tokyo, Japan) were aseptically isolated and minced with scalpel blades into 20 mL of a culture medium consisting of α-Minimum Essential Medium (α-MEM) containing 5% fetal bovine serum (FBS), 10 mM HEPES (pH 7.0), 100 μg/mL kanamycin, and 80 μg/mL gentamicin. The mixture of cell suspension and bone fragments were gently pipetted for 5 min and allowed to stand for 5 min. The resulting supernatant was used for the experiments.

To determine the number of osteoclasts in the supernatant, an aliquot was smeared on a slide glass and stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme for osteoclasts, according to the method of Burstone (29). TRAP-positive multinucleated cells (MNC) with three or more nuclei (roughly corresponding to 0.05% of the total cells) were counted as osteoclast-like cells.

Pit-Formation Assay

Dentin slices with 0.1 mm-thickness and 6 mm-diameter were prepared by our previous method (30) and placed in a 96-well culture dish (NUNC; Nunc, Roskilide, Denmark). The unfractionated bone cell suspension (4 × 10^5 cells/dentin slice) with a TRAP-positive MNC density of 200 cells/dentin slice was seeded on to dentin slices and allowed to stand for 2 h at 37°C in a CO2 incubator. After removal of nonadherent cells, the cells were further cultured for 4 days in the medium containing several concentrations of either AGE-βm or normal βm, or 10^{-9} M 1α, 25-dihydroxyvitamin D3 (1α, 25(OH)2D3). In some experiments, the cells were cultured on dentin slices with or without 10^{-6} M 20% trypsine-EDTA and 0.2% trypsin-EDTA in vitro-prepared AGE-βm in the presence of 10^{-4} M aldosterone (Peninsula Laboratories Co., Belmont, CA) or 10^{-5} M iriflavin (7-isopropoxyisoflavone, synthesized at Takeda Chemical Industries, Osaka, Japan). Experiments were completed by removing the culture medium and adding 0.1 M cacodylate buffer solution (pH 7.4) containing 2% paraformaldehyde.

Total number of TRAP-positive MNC on each dentin slice was counted after TRAP staining of the cells. Cells were then removed from dentin slices by ultrasonication for 30 s in distilled water, and air-dried. The slices were stained with hematoxylin and the number of densely stained pits was counted under light microscopy.

In some experiments, dentin slices were preincubated with or without 0.5 M glucose in 20 mL of 0.3 M phosphate buffer (pH 7.4) at 37°C for 40 days. The presence of AGE in the glycated dentin slices was confirmed immunohistochemically by using anti-AGE antibody and supported by the observation, as described below, that the pentosidine levels in rat bone particles increased upon incubation with glucose. Glycated dentin slices obtained in this manner were used as AGE-modified dentin slices (AGE-dentin slices).

Osteoclast-Formation Assay

The effect of AGE on osteoclast development was examined in two different culture systems: the mouse unfractionated bone cell culture
Figure 1. Effect of normal β2m and AGE-β2m purified from the urine of long-term hemodialysis patients on osteoclast-induced bone resorption. (A) Microscopy of hematoxylin-stained resorption pits (indicated by arrowheads) on dentin slices cultured for 4 days in the presence of 10^{-7} M normal β2m (left panel) or AGE-β2m (right panel). (Original magnification, ×40.) (B) The numbers of resorption pits and (C) TRAP-positive MNC on dentin slices. Mouse unfractionated bone cells (4 × 10^5 cells/dentin slice) containing TRAP-positive MNC (200
system and the mouse coculture system of bone marrow cells with primary osteoblast-rich cell populations. For the former system, the unfractonated bone cell suspension (2 x 10^6 cells/well) with a TRAP-positive MNC density of 100 cells/well were cultured in a 96-well plate without dentin slices in the absence of 1α, 25(OH)2D3 for 4 days. After the depletion of TRAP-positive MNC was confirmed, the cells were further incubated for 6 days in the culture medium containing either 10^-6 M AGE-β2m or 10^-8 M 1α, 25(OH)2D3. The number of newly formed TRAP-positive MNC in the culture medium was then counted after TRAP staining of the cells. Our previous study demonstrated pre-existing TRAP-positive MNC are depleted by culturing in the absence of 1α, 25(OH)2D3, but new TRAP-positive MNC formation is induced by the addition of 1α, 25(OH)2D3 in culture dishes even after the complete deplet of TRAP-positive MNC (30).

Mouse coculture system of bone marrow cells with osteoblastic cells was performed according to the method established by Taka-hashi et al. (31). In brief, bone marrow cells were isolated from tibia of 7- to 9-wk-old mice (32) and primary osteoblastic cells were isolated from newborn mice calvaria by collagenase digestion (33). The bone marrow cells (3 x 10^5 cells/well) and osteoblastic cells (3 x 10^4 cells/well) were cocultured for 6 days in 0.3 mL of α-MEM containing 10% FBS with several concentrations of either AGE-β2m, AGE-BSA, or their normal counterparts, 10^-8 M 1α, 25(OH)2D3, or 10^-6 M prostaglandin E2 (Wako Pure Chemicals) in a 48-well plate. The number of newly formed TRAP-positive MNC was then counted after TRAP staining of the cells.

In Vivo Resorption Assay

Bone resorption was evaluated in rats that were implanted with devitalized bone particles according to the method of Lian et al. (34). In brief, the femoral bones were removed from 12- to 15-wk-old male Sprague-Dawley rats (Japan Charles River, Tokyo, Japan), stripped of soft tissue, washed in cold ethanol, lyophilized, pulverized in a liquid nitrogen mill, and sieved into 75- to 250-μm particles. These bone particles (1 mg) were then incubated with or without 0.5 M glucose in 5.0 mL of 0.3 M phosphate buffer (pH 7.4) at 37°C for 60 days. Determination of pentosidine levels by HPLC assay confirmed the presence of AGE in the glycated bone particles (990 pmol/mg), but not in nonglycated counterparts (0.97 pmol/mg). Glycated bone particles obtained in this manner and those obtained by parallel incubation without glucose were used as AGE-modified bone particles (AGE-bone particles) and control bone particles, respectively.

AGE-bone particles or control bone particles (50 mg each) were packed into 1-mL plastic syringes to which 40 μL of 10% gelatin was then added, and were compressed and lyophilized. These bone-particle pellets were then implanted subcutaneously into front chests of five 4-wk-old male Sprague-Dawley rats. Two and 4 wk later, bone particles and surrounding tissues were removed and the bone mineral content in these specimens was determined by dual-energy x-ray absorptiometry using a bone densitometer (Model DSC-600; Aloka, Tokyo, Japan). All studies were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the institutional Animal Care and Use Subcommittee.

Statistical Analyses

Data were expressed as means ± SE from quadruplicate cultures or five rats. Intergroup differences in means were statistically assessed using Dunnett’s multiple-comparison method and by t test.

Results

AGE Enhance Osteoclast-Induced Bone Resorption

A 4-day culture of unfractonated bone cells containing TRAP-positive MNC on dentin slices in the presence of AGE-β2m purified from the urine of long-term hemodialysis patients significantly increased the number of resorption pits formed by osteoclasts, compared with control cells (Figures 1, A and B). However, there was no statistically significant difference in the number of resorption pits between control cells and those cultured with normal β2m. An increase in the number of resorption pits indicates an acceleration of bone resorption by osteoclasts. It is notable that AGE-β2m did not affect the number of TRAP-positive MNC during the 4-day culture (Figure 1C), suggesting that pre-existing osteoclasts likely formed most of the resorption pits. 1α, 25(OH)2D3, a well-known bone-resorbing agent that stimulates osteoblasts to release factors that affect osteoclasts (25), increased the number of resorption pits together with the increase of the number of TRAP-positive MNC, in good agreement with our previous results (30).

The effect of AGE on osteoclast-induced bone resorption was also examined using in vitro preparations of AGE-proteins. A 4-day culture of the unfractonated bone cells with the glycated β2m after a 60-day incubation (in vitro-prepared AGE-β2m) increased the number of resorption pits (Figure 2A) without an increase of the number of TRAP-positive MNC (control, 20.8 ± 3.0 cells/dentin slice; in vitro-prepared AGE-β2m, 19.3 ± 2.4 cells/dentin slice). By contrast, there was no statistically significant difference in the number of resorption pits between control cells and those cultured with the glycated β2m after a 10-day incubation, which contained the early Amadori products (fructoselysine) but few AGE (assessed by its fluorescence [17] and pentosidine level). This indicates that, among the glycated β2m, the pigmented and fluorescent AGE-β2m enhanced bone-resorbing activity of osteoclasts, whereas the β2m modified with Amadori products had no such activity. The bone-resorbing effect of AGE-β2m is thought to be the result of not β2m, but of AGE, because in vitro-prepared AGE-BSA also increased the number of resorption pits under the same culture conditions, whereas normal BSA did not (Figure 2B).

With or without 1α, 25(OH)2D3, a 4-day culture of unfractonated bone cells on AGE-dentin slices, which were prepared by incubating dentin slices with glucose for 40 days, significantly increased the number of resorption pits formed by...
Figure 2. Effect of in vitro-prepared AGE-\(\beta_2\)m (A) and AGE-BSA (B) on the number of resorption pits on dentin slices. Mouse unfractionated bone cells (4 \(\times\) 10^5 cells/dentin slice) containing TRAP-positive MNC (200 cells/dentin slice) were cultured on dentin slices for 4 days in a medium containing several concentrations of glycated proteins after a 10-day (in vitro-prepared Amadori-\(\beta_2\)m) or 60-day (in vitro-prepared AGE-\(\beta_2\)m or AGE-BSA) incubation. The number of resorption pits on the dentin slices was then counted. The levels of fructoselysine and pentosidine in the glycated proteins were discussed in the Methods section. Note that the glycated proteins after a 60-day incubation contain both AGE and Amadori products, and that the glycated protein after a 10-day incubation contains Amadori products but few AGE. Data are expressed as means \(\pm\) SE from quadruplicate cultures. * \(P < 0.05\) versus control by Dunnett’s multiple comparison.

osteoclasts, compared with the cells cultured on control dentin slices upon parallel incubation without glucose (Figure 3). The number of TRAP-positive MNC, however, did not increase by being cultured on AGE-dentin slices (20.5 \(\pm\) 2.6 and 22.8 \(\pm\) 2.4 cells/dentin slice in the absence and presence of \(10^{-10}\) M 1\(\alpha\), 25(OH)$_2$D$_3$, respectively), compared with control dentin slices (19.8 \(\pm\) 1.4 and 22.3 \(\pm\) 2.2 cells/dentin slice in the absence and presence of \(10^{-10}\) M 1\(\alpha\), 25(OH)$_2$D$_3$, respectively).

**AGE Have No Effect On New Osteoclast Formation**

Next, we examined whether AGE had an effect on new TRAP-positive MNC formation independently from their effect on bone resorption. As shown in Figure 4, TRAP-positive MNC in the unfractionated bone cells completely disappeared in the culture dishes without dentin slices after a 4-day incubation in the absence of 1\(\alpha\), 25(OH)$_2$D$_3$. When 1\(\alpha\), 25(OH)$_2$D$_3$ was added on the fourth day under such culture conditions, the number of TRAP-positive MNC increased (open squares), indicating the formation of new TRAP-positive MNC during the following 6 days of culture. However, both AGE-\(\beta_2\)m purified from patients in vivo (open circles) and prepared in vitro (open triangles) did not increase the number of TRAP-positive MNC. This suggests that AGE have no effect on the formation of new osteoclasts in the mouse unfractionated bone cell culture system.
Osteoblastic cells are required for the differentiation of osteoclast progenitors into osteoclasts and the efficiency of osteoclast formation is greatly enhanced by the presence of osteoblastic cells (25,31–33). Takahashi et al. (31) demonstrated that various colony-stimulating factors stimulated osteoclast formation in the coculture system of bone marrow cells with osteoblastic cells, whereas these factors did not stimulate in the bone marrow culture system alone, thereby suggesting that the coculture system is suitable for examining the effect on osteoclast formation. We therefore used a coculture system of bone marrow cells with primary osteoblastic cells isolated from mouse calvaria. However, even in the presence of osteoblastic cells, no TRAP-positive MNC were formed after adding AGE-β2m or AGE-BSA into the culture dishes (Figure 5). By contrast, many TRAP-positive MNC appeared in response to 1α, 25(OH)2D3 or prostaglandin E2.

**Calcitonin and Ipriflavone Effectively Inhibit AGE-Enhanced Bone Resorption**

Both calcitonin and ipriflavone are known to prevent bone loss in various types of animal models with experimentally induced osteoporosis, and are also known to be effective for the treatment of osteoporosis in humans (35–37). We examined the effect of calcitonin and ipriflavone on AGE-enhanced bone resorption. Unfractionated bone cells containing TRAP-positive MNC were cultured for 4 days in the medium containing *in vitro*-prepared AGE-β2m in the presence of calcitonin (10−8 M) or ipriflavone (10−5 M). A previous study demonstrated that both agents, at these concentrations, significantly reduced the number of resorption pits induced by 1α, 25(OH)2D3 (30). Both calcitonin and ipriflavone significantly (*P < 0.05*) reduced the number of resorption pits induced by *in vitro*-prepared AGE-β2m (Figure 6A). However, the mechanisms of the action of calcitonin and ipriflavone may be different, because the latter reduced the number of TRAP-positive MNC whereas the former did not (Figure 6B). No cytotoxic effects were observed in calcitonin- or ipriflavone-treated cells on the dentin slices after a 4-day incubation, when assessed by trypan blue staining.

**AGE Enhance Bone Resorption in Rats Implanted with Bone Particles**

The pathophysiological significance of AGE in bone resorption hinges upon the *in vivo* demonstration of enhanced bone resorption by AGE modification. Therefore, AGE-bone particles or control bone particles were implanted subcutaneously into rats and their resorption was examined. Histological examination revealed the presence of MNC in resorptive lacunae of bone particles (data not shown). Bone particles were resorbed with time, and 60.8% of AGE-bone particles and 43.7% of control bone particles were resorbed after 4 wk of implantation (Figure 7). Notably, the degree of resorption of AGE-bone particles was much greater than that of control bone particles: the amount of the remaining AGE-bone particles after 4 wk was 69.8% of that of the control group.

**Discussion**

The study presented here has provided *in vitro* and *in vivo* evidence that AGE enhance osteoclast-induced bone resorption. First, when mouse unfractionated bone cells containing osteoclasts were cultured on dentin slices, AGE-β2m, but not normal β2m, purified from the urine of long-term hemodialysis patients increased the number of resorption pits formed by osteoclasts. Second, AGE-β2m and AGE-BSA prepared *in vitro* by incubating with glucose for 60 days increased the number of resorption pits, whereas their normal counterparts or those modified with the early glycation products did not. Third, the osteoclast-induced bone resorption was enhanced on AGE-dentin slices, compared with unmodified dentin slices. Fourth, the effect of AGE on bone resorption was effectively inhibited by calcitonin and ipriflavone, both of which are inhibitors of bone resorption. The results from these *in vitro* studies were further confirmed by *in vivo* evidence that bone particles upon incubation with glucose for 60 days (AGE-bone particles), when implanted subcutaneously in rats, were resorbed to a much greater extent than control bone particles upon parallel incubation without glucose. These findings suggest that the modification of bone matrix proteins with AGE might, in part, play a role in the remodeling of senescent bone matrix tissues. The AGE-enhanced bone resorption is also supported by our preliminary data that AGE-β2m, whether purified from patients *in vivo* or prepared *in vitro*, induces net calcium efflux from cultured neonatal mouse calvaria to a much greater extent than normal β2m (Sprague S, and Miyata T, unpublished observation).

The finding that AGE did not increase the number of newly formed osteoclasts in the coculture system of bone marrow cells with osteoblastic cells, as well as in the unfractionated bone cell culture system, likely suggests that AGE have no effect on osteoclast formation. We previously demonstrated that AGE-β2m, but not normal β2m, stimulates human mono-
nuclear phagocytes to secrete cytokines such as IL-1β (7,17). Although IL-1β is a potent stimulator of osteoclast formation, the concentration of IL-1β necessary for this process is approximately 1 ng/mL (38), which is much higher than that secreted from human mononuclear phagocytes by the action of AGE-β2m (7,17). However, although concentrations of secreted IL-1β may not have been sufficient to stimulate osteoclast formation in the cell culture system, AGE-induced secretion of IL-1β in vivo may yield high local concentrations of IL-1β, sufficient to act in a paracrine manner to induce differentiation of osteoclast progenitors in tissues. At least, the mechanism by which AGE enhance bone resorption in the present cell culture system is thought not to promote differentiation of osteoclast progenitors into osteoclasts.

At present, this mechanism remains unknown. However, two possible explanations may be considered. First, AGE might stimulate the activity of osteoclasts. It is inconclusive whether or not AGE can activate osteoclasts directly. An alternative possibility is that osteoclasts are stimulated by soluble factor(s) that are released from osteoblasts by AGE, as demonstrated previously as the mechanism of stimulation of osteoclasts by parathyroid hormone (39,40) and 1α, 25(OH)2D3 (41). Second, the modification of bone matrix proteins with AGE might create a microenvironment favorable for bone resorption and facilitate osteoclasts to resorb bone matrices, e.g., by strengthening the attachment of osteoclasts to AGE-modified bone surface or by making AGE-modified bone matrices susceptible to degradation with lysosomal enzymes secreted by osteoclasts. A previous study demonstrated the alteration in the integrity of articular cartilage as a result of the crosslinks of pentosidine (42), thereby implicating possible involvement in matrix degradation of osteoarthritis. Further study will undoubtedly be necessary to elucidate the molecular mechanism of AGE-enhanced bone resorption by osteoclasts.

Dialysis-related amyloidosis is an osteoarticular complication of long-term hemodialysis patients (21,22). Subchondral
erosion and bone cysts as a result of an accelerated bone resorption and replacement of bone by amyloid deposits are becoming a serious problem, and are causing pathological fractures (21,22,43). Although β2m is shown to be a major constituent in amyloid fibrils (44,45), the mechanism of bone resorption has remained unknown. We previously demonstrated that AGE are present in β2m-forming amyloid fibrils of dialysis-related amyloidosis (14,15). The results of the study presented here suggest that AGE-β2m in amyloid fibrils accelerates bone resorption around long-lived amyloid deposits, implicating a role of AGE in the pathogenesis of dialysis-related amyloidosis, especially in bone resorption.

Our preliminary immunohistochemical studies using anti-AGE and anti-pentosidine antibodies revealed that human bone tissues obtained from elderly subjects contain a significant amount of AGE, compared with those from young subjects (Miyata T, and Kawai R, unpublished observation). This is not surprising, because the AGE modification occurs in long-lived matrix proteins such as Type I collagen, a major component of bone matrix (46). Taking into account the observations that the AGE levels in matrix tissues are markedly elevated in diabetic patients and elderly subjects, compared with healthy young subjects (23,24), the study presented here suggests a potential link of the accumulation of AGE in bone matrix tissues to osteoporosis associated with diabetes and aging. However, because bone resorption is a combined result that is determined by many hormonal and local factors, more study will be necessary to clarify the issue as to how much the AGE modification is clinically relevant to bone resorption in these patients.

Finally, this study’s observations that both calcitonin and ipriflavone effectively inhibit AGE-enhanced bone resorption, with the concentrations roughly corresponding to plasma concentrations when administered in vivo at therapeutic doses (47), suggest the possible effectiveness of these agents on AGE-enhanced bone resorption, such as dialysis-related amyloidosis, for which there is no effective method available for prevention and treatment.

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Figure 6. Inhibitory effect of calcitonin and ipriflavone on AGE-enhanced bone resorption. Mouse unfractionated bone cells (4 × 10^5 cells/dentin slice) containing TRAP-positive MNC (200 cells/dentin slice) were cultured for 4 days in the medium with or without 10^{-6} M in vitro-prepared AGE-β2M in the presence or absence of 10^{-8} M chick calcitonin or 10^{-5} M ipriflavone. The numbers of resorption pits (A) and TRAP-positive MNC (B) on the dentin slices were then counted. Data are expressed as means ± SE from quadruplicate cultures. *P < 0.05 and †P < 0.01 control without in vitro--prepared AGE-β2M in the absence of calcitonin and ipriflavone, P < 0.05 and †P < 0.01 versus control with in vitro--prepared AGE-β2M in the absence of calcitonin and ipriflavone, and *P < 0.05 and †P < 0.01 versus the sample without in vitro--prepared AGE-β2M, by t test.


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**Figure 7.** Effect of AGE modification on bone resorption in rats implanted with bone particles. Bone particles were prepared from rat femoral bones, incubated with glucose (AGE-bone particles) or without glucose (control bone particles) for 60 days, and implanted subcutaneously into five rats. Two and 4 wk later, bone particles and surrounding tissues were removed and the bone mineral content was determined by dual energy x-ray absorptiometry. Data are expressed as means ± SE from five rats. *P* < 0.01 versus control bone particles by t test.