Non-enzymatic Glycation of Bone Collagen Modifies Osteoclastic Activity and Differentiation*§

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Type I collagen, the major organic component of bone matrix, undergoes a series of post-translational modifications that occur with aging, such as the non-enzymatic glycation. This spontaneous reaction leads to the formation of advanced glycation end products (AGEs), which accumulate in bone tissue and affect its structural and mechanical properties. We have investigated the role of matrix AGEs on bone resorption mediated by mature osteoclasts and the effects of exogenous AGEs on osteoclastogenesis. Using in vitro resorption assays performed on control- and AGE-modified bone and ivory slices, we showed that the resorption process was markedly inhibited when mature osteoclasts were seeded on slices containing matrix pentosidine, a well characterized AGE. More specifically, the total area resorbed per slice, and the area degraded per resorption lacuna created by osteoclasts, were significantly decreased in AGE-containing slices. This inhibition of bone resorption was confirmed by a marked reduction of the release of type I collagen fragments generated by the collagenolytic enzymes secreted by osteoclasts in the culture medium of AGE-modified mineralized matrices. This effect is likely to result from decreased solubility of collagen molecules in the presence of AGEs, as documented by the reduction of pepsin-mediated digestion of AGE-containing collagen. We found that AGE-modified BSA totally inhibited osteoclastogenesis in vitro, most likely by impairing the commitment of osteoclast progenitors into pre-osteoclastic cells. Although the mechanisms remain unknown, AGEs might interfere with osteoclastic differentiation and activity through their interaction with specific cell-surface receptors, because we showed that both osteoclast progenitors and mature osteoclasts expressed different AGEs receptors, including receptor for AGEs (RAGEs). These results suggest that AGEs decreased osteoclast-induced bone resorption, by altering not only the structural integrity of bone matrix proteins but also the osteoclastic differentiation process. We suggest that AGEs may play a role in the alterations of bone remodeling associated with aging and diabetes.

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Non-enzymatic glycation is a common post-translational modification of proteins induced by the spontaneous condensation of reducing sugars (e.g. glucose) and metabolic intermediates (e.g. triose phosphates, glyoxal, and methylglyoxal) with free amino groups in lysine or arginine residues. The early step of the so-called Maillard reaction is the formation of a Schiff base adduct to protein. This early glycation product undergoes a reversible rearrangement to form an Amadori product adduct to protein (i.e. an intermediate glycation product). Schiff bases and Amadori products then undergo a complex series of rearrangements, oxidations, and/or dehydrations along different chemical pathways to produce a class of irreversible adducts to proteins, the advanced glycation end products (AGEs)3 (reviewed in Refs. 1 and 2). A direct consequence of these highly diverse reaction pathways leading to AGE formation is that a wide variety of AGEs with different chemical structures is formed. Some AGEs are adducts to the protein (e.g. carboxymethyllysine and carboxyethyllysine), whereas others present protein-protein cross-links (e.g. pentosidine, glyoxal-derived lysine dimer, and methylglyoxal-derived lysine dimer). Since the initial characterization of the pentosidine cross-link by Sell and Monnier (3, 4), many AGEs (and more recently advanced lipoxidation end products) have been identified. However, it is unclear which, if any, of these AGEs can be designated as the most functionally relevant, and new AGEs are continuously being discovered. Once formed, AGEs are removed from the tissue only when the protein involved is degraded. Consequently, the most extensive accumulation of AGEs will occur in tissues characterized by low turnover and containing consequently long-lived proteins, such as collagen in the extracellular matrix of connective tissues (e.g. cartilage, bone, tendon, and skin) (5).

Formation and accumulation of AGEs are characteristic features of tissues of aged people, but they also occur in patients with diabetes mellitus, mainly those with poorly controlled hyperglycemia. AGEs have also been strongly implicated in the pathogenesis of AGE-related and diabetic complications, such as retinopathy, glomerulopathy, neuropathy, and diabetic atherosclerosis (6, 7). It has recently been reported that AGEs are

3 The abbreviations used are: AGE, advanced glycation end product; RAGE, receptor for AGEs; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; FCS, fetal calf serum; MEM, α-minimal essential medium; RT, reverse transcription; TRAP, tartrate-resistant acid phosphatase; RANK-L, receptor of activator of nuclear factor κB-ligand; ELISA, enzyme-linked immunosorbent assay; PYD, pyridinoline; DPD, deoxypyridinoline; AMG, aminoguanidine; ICTX, β-isomerized and cross-linked C-telopeptide fragment of type I collagen.
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involved in skeletal diseases such as osteoarthritis, which is a chronic disabling disorder of aged people. Accumulation of AGES in articular cartilage affects cellular characteristics and increases stiffness and brittleness of the tissue, thereby rendering it more prone to mechanical damage (8, 9). AGES modify the structure and the subsequent functions of proteins by creating intra- or intermolecular cross-links (10); these could partially explain the deleterious effects of AGES on the biomechanical properties of connective tissues. AGES also interfere with the susceptibility of matrix proteins toward proteolytic degradation. Degradation of AGE-modified cartilage collagen by matrix metalloproteinases is impaired compared with unmodified collagen (11), and pepsin mediated-solubilization of collagen is decreased in AGE-modified collagen (10, 12). AGES have also been found in bone tissue (4, 13); in particular, pentosidine has been shown to accumulate with age in cortical bone of human femur (14). Accumulation of AGES in bone collagen matrix has been shown to alter the mechanical properties of bone, decreasing its toughness, and could therefore contribute to skeletal fragility (15–17). Indeed, decreased bone strength and osteopenia have been shown in rodent models of diabetes (18, 19). Osteopenia and osteoporosis are often observed in patients with type 1 diabetes, and recent cohort studies indicate that diabetes itself is associated with increased risk of fractures (20). However, the role of AGES in decreased bone strength in diabetes and subsequent contribution to fracture risk remain unclear.

Eventually, AGES modify cellular behavior by interacting with specific cellular receptors, such as RAGE (receptor for AGES). In a craniotomy model in type I diabetic mice, the degree of bone healing was reported to be 40% of that of non-diabetic animals, and RAGE is expressed at higher levels in the healing bone tissue of diabetic animals compared with control diabetic animals (21). RAGE has been shown to be expressed in osteoblasts (21, 27) and may modulate AGE-dependent signaling in osteoblasts (27). This observation could explain in part the decreased expression of transcription factors responsible for osteoblastic differentiation in type I diabetic mice model (28).

Taken together, these findings suggest that AGES may play a role in the pathogenesis of diabetic osteopenia as well as that of age-related decreased bone strength and increased risk of fracture; however, the details of such deleterious mechanisms remain to be clarified. Bone mass is tightly regulated by the differentiation and activity of bone-resorbing cells (osteoclasts) and bone-forming cells (osteoblasts) through a process called bone remodeling. The contribution of non-enzymatic glycation to bone remodeling has been poorly investigated in vivo, mainly due to a lack of suitable animal models; for instance, insulin deficiency or resistance in diabetic animal models might interfere with cell differentiation or function processes. Although the role of AGES in osteoblasts differentiation and function has been well characterized in vitro (23, 26, 27, 29), their contribution to osteoclast activity and differentiation is unknown. In the present study, we first investigated the effects of matrix AGES on bone resorption by osteoclasts, using an in vitro resorption assay on AGE-modified mineralized matrices. We then extended our analysis to the contribution of exogenous AGES to osteoclastogenesis.

EXPERIMENTAL PROCEDURES

In Vitro AGE-Matrix and AGE-BSA Formation—Elephant ivory slices (6-mm diameter, kindly donated by the Centre de conservation et d’étude des collections, Lyon, France) and bone cortices (3-mm diameter) from 3-month-old calves were used in these experiments. Human cortical bone slices (3-mm diameter) were prepared from a 40-year-old male femur obtained from necropsy (Faculté de Médecine Laennec, Lyon, France). Slices (0.3-mm thick) were cut with an Isomet low speed saw (Buehler, Lake Bluff, IL), cleaned by sonication, sterilized for 2 min in 70% ethanol, and stored dry at −20 °C until AGE-matrix formation or in vitro resorption assay. In vitro AGE formation in mineralized tissues was performed according to adapted protocols of those previously described for tendon or cartilage collagens (30, 31). Ivory and bone slices were incubated with 0.2 M D-ribose in 10 ml of phosphate-buffered saline (PBS) solution, containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.5 mM phenylmethylsulfonyl fluoride, for 45 and 60 days at 37 °C, respectively. Alternatively, the slices were incubated with 0.05–0.6 M D-ribose in 10 ml of Tris-buffered saline solution (TBS, pH 7.4), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.5 mM phenylmethylsulfonyl fluoride, for 2–7 days at 37 °C, to modulate the level of AGES formed. Control incubations were also performed with PBS or TBS. Incubation solutions were replaced every day, and the pH was controlled routinely to ensure optimal buffering activity during the glycation process. AGE-BSA was prepared according to a modified version of methods previously described (32, 33). Briefly, 50 mg/ml BSA (Fraction V, sterile filtered, Sigma-Aldrich) was incubated at 37 °C, under sterile conditions, with 0.6 M D-ribose for 1 week or with 0.5 M glucose and 0.3 M lysine for 4 weeks in PBS (pH 7.4) containing 100 units/ml penicillin and 100 μg/ml streptomycin. The unincorporated sugars were removed by dialysis against PBS. These products were designated as AGE-1 and AGE-2, respectively. Non-AGE-modified BSA was incubated in the same conditions except for the absence of reducing sugars. Glycated BSA (1–5 mol of fructose per mol of albumin, Sigma-Aldrich) was used as a negative control. BSA derivatives were quantified using the Biorad protein assay system, and the concentrations of pentosidine were determined as described hereafter (Table 3).

Rabbit Osteoclast Isolation and Resorption Assay—Osteoclasts were isolated from the long bones of 4-day-old New Zealand rabbits. Bones were removed, cleaned of soft tissues and bone marrow, split, and scrapped into M199 medium supplemented with 10% fetal calf serum (FCS, Invitrogen) and 20 μM HEPES (Invitrogen). Cells were centrifuged at low speed, resuspended in αMEM containing 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2% FCS (all products from Invitrogen), and seeded on ivory or bone slices in...
48- or 96-well culture plates, respectively, for 3 h at 37 °C and 5% CO₂. After this setting period, non-adherent cells were removed, slices were washed in PBS, and the remaining cells were incubated for 3–8 days in complete αMEM containing 2% FCS and at pH ~7.0 (34). At the end of culture, cells were removed in 1 mM NaOH for 20 min, and the slices were rinsed three times in water and stained with acid hematoxylin for 30 s and then with 1% (w/v) toluidine blue in 1% (w/v) sodium borate for 30 s. The number and the area of resorption lacunae were then measured by light microscopy using LUCIA® Software (Nikon, France), as previously described (35). Results are expressed as the number of lacunae and total area resorbed per slice, as well as the area resorbed per lacuna per slice.

**Biochemical Assessment of Bone Collagen Degradation in Culture Media**—The release of type I collagen fragments, subsequent to resorption of bone and dentin slices, was also quantified in the culture supernatants using two immunological methods: (i) the concentrations of cross-linked and isomerized C-terminal fragments of type I collagen (βCTX) were determined using the CrossLaps for Culture ELISA kit (Nordic Bioscience Diagnostics, Herlev, Denmark) as recommended by the manufacturer; and (ii) the concentrations of the helical peptide corresponding to the 620–633 fragment of human type I collagen were measured using the METRA® Helical Peptide EIA kit (Quidel Corp., San Diego, CA). Collagen fragments released into the culture media were also analyzed by measuring the hydroxyproline concentration using the Hydroxyproline by high-performance liquid chromatography reagent from Bio-Rad, after acid hydrolysis of the conditioned culture media.

**Murine Osteoclast Differentiation**—Bone marrow cells of 6- to 8-week-old male NMRI mice were seeded at 5000 cells/mm² and cultured for 8 days in differentiation medium: αMEM containing 10% FCS (HyClone Laboratories, Perbio Science, Cheshire, UK), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 30 ng/ml recombinant mouse macrophage colony-stimulating factor, and 30 ng/ml soluble recombinant RANK-L, mixed with 2.5 μg/ml mouse anti-polystyidine antibody. Culture media were replaced every second day with growth and differentiation factors up to 15 days. For the bone resorption assay, differentiating cells (at day 10) were detached and seeded on control- and ribose-incubated bovine bone slices and cultured for 8 days in the presence of growth and differentiation factors.

**Tartrate-resistant Acid Phosphatase Activity Assay and Cytochemistry**—TRAP activity in the culture media was measured using a colorimetric assay (38, 39). The reaction buffer (412 mM acetic acid, 0.209% Triton X-100, 412 mM NaCl, 4.12 mM EDTA, 10.6 mM ascorbic acid, 10.2 mM 4-nitrophenylphosphate, 41.6 mM Na₂ tartrate at pH 5.5) was added to conditioned media, incubated for 1 h at 37 °C in the dark, and then stopped with 100 μl of 300 mM NaOH. Colorimetric changes were measured at 405 nm with 650 nm as the reference using an ELISA reader. Cellular TRAP activity was measured using the Leukocyte Acid Phosphatase kit (Sigma-Aldrich). Briefly, the cells were washed twice with PBS, fixed for 5 min with 2% glutaraldehyde in PBS, and stained according to the manufacturer’s instructions.

**Biochemical Analyses of Bone Collagen**—The amount of pyridinoline (PYD), deoxypyridinoline (DPD), and pentosidine was measured after hydrolysis of the bone or ivory slices. Briefly, slices (~7 mg wet weight) were hydrolyzed by HCl at 110 °C during 20 h. Collagen cross-links were extracted from hydrolysates using solid phase extraction cross-links Chromabond column (Macherey-Nagel, France). Separation of the different cross-links was performed by high-performance liquid chromatography on an Alliance 2695 separation module using an Atlantis dC18 column (3 μm, 4.6 × 100 mm) protected by an Atlantis dC18 guard cartridge (3 μm, 4.6 × 20 mm, Waters Corp., Milford, MA). Molecules were separated by using a gradient solution. Solvent A consisted of 0.06% of heptfluorobutyric acid, and solvent B was 50% of solvent A and 50% of acetonitrile. The flow rate was 1.2 ml/min, and the column temperature was 40 °C. The separation of PYD and DPD was performed during the first 12 min of an isocratic step at 14% of solvent B, and pentosidine was eluted during the following 24 min of gradient from 14 to 31% solvent B. PYD and DPD were monitored for fluorescence at an emission of 395 nm and an

**Human CD-14-positive Monocytes Isolation and Osteoclast Differentiation**—Human monocytes were isolated from peripheral blood of healthy donors (Etablissement Français du Sang, Lyon). Blood, diluted 1:1 with 4 °C PBS, was carefully layered on Ficoll-Paque™ Plus (GE Healthcare, Sweden) and centrifuged at 700 × g for 20 min. The lymphocytes interface was collected, washed twice with 4 °C PBS followed by centrifugation at 700 × g for 12 min, and resuspended in cold PBS containing 2% fetal bovine serum. CD-14-positive cells were isolated using magnetic beads coated with a mouse monoclonal anti-CD14 antibody according to the recommendation of the manufacturer (Dynabeads CD14 monocytes/macrophages, Dynal Biotech, Invitrogen). CD-14-positive cells were resuspended in αMEM containing 10% of fetal calf serum (HyClone Laboratories), 25 ng/ml recombinant human macrophage-colony stimulating factor, and 25 ng/ml soluble recombinant RANK-L, mixed with 2.5 μg/ml mouse anti-polysynthetic antibody. Culture media were replaced every second day with growth and differentiation factors up to 15 days. For the bone resorption assay, differentiating cells (at day 10) were detached and seeded on control- and ribose-incubated bovine bone slices and cultured for 8 days in the presence of growth and differentiation factors.
**TABLE 1**

| Oligonucleotide primers used for conventional RT-PCR and quantitative real-time RT-PCR |  |
|---|---|
| Gene | Primer sequence (strand) | Product size (bp) | Temperature (°C) | PCR cycles | Ref. or accession no. |
| **Primers used for conventional RT-PCR** |  |  |  |
| RAGE | 5'–AGGCCGGTGGATGAAACTGACACA–3' (+) | 702 | 58 | 37 | AB061668 |
| AGE-R1 | 5'–GGAGCCGAGAAGAAAGCTGAC–3' (−) | 440 | 60 | 32 | NM_007838 |
| AGE-R2 | 5'–AGCTGTCAGTACGCAAC–3' (+) | 331 | 60 | 32 | NM_008925 |
| AGE-R3 | 5'–GGATCCAGGAAGGAGCTGAC–3' (−) | 263 | 60 | 26 | X16834 |
| CTR | 5'–ACCCAGACCTTGAGATGTC–3' (−) | 255 | 54 | 40 | (37) |
| TRAP | 5'–GGCTTCCAGAAGGAGCTGAC–3' (−) | 292 | 55 | 27 | (37) |
| Gapdh | 5'–ATCTGTCAGACCCAGAAGAC–3' (+) | 443 | 57 | 27 | (65) |
|  | 5'–ACCCAGACCTTGAGATGTC–3' (−) | 443 | 57 | 27 | (65) |
| **Primers used for quantitative RT-PCR** |  |  |  |
| RAGE | 5'–GTGGGACTGCTGGGCTGAG–3' (+) | 162 | 58 | 40 | NM_007425 |
| Gapdh | 5'–GGCTTCCAGAAGGAGCTGAC–3' (−) | 76 | 58 | 40 | (66) |

excitation of 297 nm, and then wavelengths were shifted to 385 nm and 353 nm, respectively, for determination of pentosidine (multi-λ fluorescence detector, Waters 2475). Pyridinium cross-links were quantified against a calibrator supplied by Metra Biosystems Ltd. Pentosidine standard was synthesized and calibrated with a standard of pentosidine generously given by Dr. M. Takahashi (University of Saitama, Japan). Hydroxyproline content was measured by high-performance liquid chromatography using the hydroxyproline by high-performance liquid chromatography reagent from Bio-Rad.

Collagen Solubility—After demineralization in 0.5 M EDTA, 0.05 M Tris, pH 7.5, at 4 °C for 72 h, bone slices were rinsed and submitted to limited pepsin digestion in 0.5 mg/ml pepsin (Sigma-Aldrich) in 0.5 M acetic acid for 4 h at 37 °C with constant shaking. The reaction (1 ml) was stopped by the addition of 2 μl of 1 mm pepstatin and centrifuged for 10 min at 12,000 rpm. Collagen concentration was determined in the supernatant and pellet by measuring the amount of hydroxyproline (as described above) after acid hydrolysis in 6 N HCl for 20 h at 110 °C. Collagen solubility was expressed as the collagen concentration in the supernatant after pepsin digestion as a percentage of total recoverable collagen in pellet and supernatant.

RT-PCR—Total RNA was extracted from RAW264.7 cells using the RNeasy kit (Qiagen), and digested with RNase-free DNase (Promega, Madison, WI) to remove any contaminating genomic DNA. Reverse transcriptions were performed as described previously (40), using 1 μg of RNA and 12.5 ng of anchored oligo-dT23 primers (5'-d123VVV-3', where V represents A, C, or G nucleotides) per μl, in the presence of 200 units of SuperScript II-RNase H− (Invitrogen). Polymerase chain reactions were carried out as described (40), using specific primers designed according to sequences available in the data-banks or published by other authors (Table 1). Primers for mouse glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were used to ascertain that an equivalent amount of cDNA was synthesized. Controls where reverse transcriptase was omitted and where cDNAs were replaced with water were also performed to demonstrate the specificity of the reactions (data not shown). Quantitative real-time PCR reactions were performed as described previously (40) using an iCycler (Bio-Rad). Gene expression levels were determined using the comparative Ct method using Gapdh as reference. The ground condition was set to 1, and expression data are presented as bar graphs of mean values ± S.D.

Indirect Immunofluorescence and Direct Fluorescence—For actin cytoskeleton direct fluorescence microscopy, cells seeded on glass coverslips were fixed, permeabilized, and stained with Cy3-labeled phalloidin (Molecular Probes, Eugene, OR). For immunofluorescence microscopy, fixed cell preparations were processed as described (41). Rabbit polyclonal anti-RAGE (H-300) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The fluorescein isothiocyanate-conjugated rabbit anti-rabbit-IgG antibody was from DAKO (Carpinteria, CA), and Cy3-conjugated goat anti-rabbit-IgG antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). All photomicrographs were obtained by a Leica DMRB microscope equipped with a Nikon DXM1200 digital camera, using a Leica PL Fluotar 20×/0.50 objective lens and photographing at ambient temperature in the absence of immersion oil. Image contrast was adjusted using Adobe Photoshop after image acquisition with the camera’s LUCIA® software (Nikon, France). Control experiments for which the primary antibody was omitted were also performed (data not shown).

Statistical Analyses—Data are presented as means ± S.D., unless otherwise noted. Comparison between experimental conditions for bone-resorption activity and osteoclastic differentiation assays were assessed using the non-parametric Mann-Whitney U test.

RESULTS

In Vitro AGE-Matrix Formation—To analyze the effects of collagen AGEs on bone resorption we used cortical bone slices from 3-month-old calves, in which the formation of AGEs was induced in vitro, by incubating the slices with r-ribose in PBS at 37 °C for various times. After 60 days of incubation in the presence of 0.2 m ribose, there was a browning of the bone matrix, which characterizes the Maillard reaction (Fig. 1A). The forma-
tion of AGEs was confirmed by the marked induction of pentosidine concentration in the 3-month-old cortical bone slices (Fig. 1B). In contrast, there was no change in the content of the mature enzymatic collagen cross-links PYD (Fig. 1C) and DPD (Fig. 1D) in slices incubated at 37 °C either with or without ribose.

**Effects of AGEs on Osteoclastic Bone Resorption**—We assessed the ability of mature osteoclasts to degrade bone tissue by performing an *in vitro* bone resorption assay using unfractionated bone cells from rabbit long bones cultured for 3 or 8 days on control and AGE-containing slices. This cell preparation is enriched in mature TRAP-positive osteoclasts (Fig. 2A), which are able to degrade a mineralized matrix, as documented by the formation of resorption lacunae (or pits) (Fig. 2B). After 3 days in culture, the total area resorbed per slice did not change significantly (Fig. 2C), but we observed a significant increase in the number of resorption pits (Fig. 2D) in ribose-incubated slices compared with untreated ones. Consequently, the mean area resorbed per pit per slice markedly decreased (Fig. 2E). We next extended the resorption period to up to 8 days. After this longer culture period, the area resorbed per slice (86%) (Fig. 3A), the number of lacunae (51%) (Fig. 3B), and the area degraded per lacuna (72%) (Fig. 3C) were markedly decreased in ribose-incubated slices compared with control bones.

To ascertain that the effects of matrix AGEs on bone resorption that we observed for rabbit osteoclasts were not species-specific features but representative of a more general phenomenon, we performed similar experiments using *in vitro* differentiated mouse osteoclasts. Fully differentiated osteoclasts were detached from plastic dishes and seeded on control- and ribose-incubated ivory slices and incubated for 8 days. As expected, incubation of ivory slices with ribose increased markedly their pentosidine content (up to 240 mmol/mol collagen, Fig. 4A), whereas the amount of PYD and DPD cross-links did not change significantly (data not shown). Consistent

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**FIGURE 1. In vitro matrix AGEs formation in cortical bone slices.** 3-month-old bovine bone slices (3 mm-width, 300 μm-thick) (A) were incubated for 60 days in PBS buffer alone (Control) or with 0.2 M D-ribose at 37 °C. AGEs formation in the presence of ribose is observed by the browning of the bone matrix. Pentosidine (B), pyridinoline (C), and deoxypyridinoline (D) concentrations were determined in control- and ribose-incubated bone slices. Pentosidine formation is induced in the presence of ribose, whereas the concentrations of enzymatic cross-links, pyridinoline and deoxypyridinoline, did not change during the incubation. Statistical non significance (n.s.) for the differences between control- and ribose-incubated slices using the nonparametric Mann-Whitney U test are indicated (n = three slices). n.d., not detected.

**FIGURE 2. Assessment of bone resorption after 3 days of culture with rabbit unfractionated bone cells.** A, staining for the TRAP activity of unfractionated bone cells seeded on glass coverslips and cultured for 3 days. Bar, 10 μm. B, lacunae of resorption created by unfractionated bone cells cultured for 3 days on ivory slices and stained with toluidine blue and hematoxilin. Bar, 80 μm. Unfractionated bone cells, enriched in mature osteoclasts, were seeded on control- or ribose-incubated bone slices and cultured for 3 days. The area resorbed (C) and the number of resorption lacunae (D) per slice as well as the area resorbed per lacuna per slice (E) were determined microscopically using the LUCIA® software. The results are shown as box plots showing the median, and inferior and superior quartiles (n = 10 slices). F, unfractionated bone cells were seeded on control- or ribose-incubated ivory slices (6 mm-width) and cultured for 3 days. Bone resorption was assessed biochemically using the Crosslaps Culture ELISA determining the concentration of βCTX released in the culture media. The results are expressed as mean ± S.D. for 5 slices. A representative experiment from at least three independent experiments is shown in C–F. n.s., not significant. ***, p < 0.01.
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with the findings obtained with rabbit osteoclasts, there was a marked reduction of the total area resorbed per slice and the area degraded per lacuna (−44% and −42%, respectively, Fig. 4, B and D) when the cells were seeded on ribose-incubated slices. However, the number of resorption lacunae was not altered (Fig. 4C).

With both rabbit and mouse osteoclast experiments, there was no significant difference of TRAP activity in the culture medium between control-incubated and ribose-incubated slices, indicating that the effects observed on bone and ivory matrix resorption were not due to differences in osteoclast seeding (data not shown).

The effects of AGEs on bone resorption were also investigated directly by measuring the release of collagen fragments in the conditioned media of rabbit and mouse osteoclast cultures. We used two immunological assays, the Crosslaps for Culture ELISA kit and the Helical Peptide EIA kit, which, respectively, assess the cross-linked and isomerized C-telopeptide fragment (βCTX) and the helical region of the α1 chain of type I collagen (42, 43). There was a marked reduction in the concentration of βCTX released into the culture media of osteoclasts seeded on ribose-incubated slices, not only after 8 days of culture (Figs. 3D and 4E), but also after 3 days of resorption (Fig. 2F). In a similar manner, the concentration of helical peptide detected in the culture media from AGE-modified slices diminished markedly compared with those from control slices (Figs. 3E and 4F). The reduction of bone (and ivory) matrix degradation was also confirmed by the decrease of the hydroxyproline concentration (Fig. 3F and data not shown), which provides a global read-out of the collagen release in the conditioned media.

Dose-dependent Inhibition of Bone Resorption by Matrix AGEs—In human bone, pentosidine has been shown to accumulate in an exponential manner with aging (14). The maximal range of pentosidine found in bone tissues varies from 50 mmol/mol of collagen in femora (14) to 140 mmol/mol of collagen in vertebrae (44). To analyze the dose-dependent effects of matrix AGEs on bone resorption mediated by osteoclasts using concentrations of collagen AGEs closer to the physiological ranges, protocols for in vitro matrix AGEs formation were modified. In a first attempt, slices were incubated in Tris-buffered saline buffer in the presence of increasing concentrations of D-ribose (0.2 M, 0.4 M, and 0.6 M) for a shorter time (up to 6 days). By this method, we could not obtain a dose-dependent formation of pentosidine, as a plateau was rapidly reached even when lower molarities (down to 0.05 M) of D-ribose were used (data not shown). However, when slices were incubated with the same molarities of D-ribose (0.2 M), but during various times (2, 4, or 6 days), we could observe a slight dose-dependent effect for the formation of pentosidine for 4 and 6 days of incubation, although not significant (Fig. 5A). In contrast, the content of the PYD cross-link did not change (Fig. 5B). The dose-dependent increase of pentosidine was associated with a dose-dependent reduction in the concentration of the collagen products helical peptide (Fig. 5C) and hydroxyproline (Fig. 5D) released in the conditioned media of osteoclasts.

To further modulate the concentrations of matrix AGEs, bone slices were also incubated with 0.3 M aminoguanidine (AMG), an inhibitor of AGE formation (45), in the presence or absence of D-ribose (0.6 M) for 6 days. AMG alone did not affect the concentration of PYD (Fig. 5F) and had no influence on the release of helical peptide in the culture media (Fig. 5G). However, the presence of AMG partially inhibited the browning of the bone matrix induced by the incubation with D-ribose (data not shown). In the presence of ribose, AMG markedly inhibited (>7-fold) the formation of pentosidine molecules in bone slices (Fig. 5E), and the release of helical peptide (Fig. 5G) and hydroxyproline (Fig. 5H) after 8 days of resorption was significantly higher than in slices incubated with ribose alone. When
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PBS was instead used of TBS during incubation, we found very similar results both for the formation of pentosidine in bone slices (supplemental Fig. S1A) and the inhibition of osteoclast-mediated bone resorption in AGE-containing matrices (supplemental Fig. S1B).

Because sugars are present in low amounts in connective tissues (46), we also tested whether we could induce in vitro the formation of AGES using lower concentration of reducing sugars. We found that incubating bone slices with only 0.05 M D-ribose at 37 °C. The results are expressed as mean ± S.D. for three slices. Microscopic (B–D) and biochemical (E–F) assessments of resorption mediated by mature mouse osteoclasts cultured for 8 days on collagen- or ribose-incubated slices. The area resorbed (B) and the number of resorption lacunae (C) per slice as well as the area resorbed per lacuna per slice (D) were determined using the LUCIA® software. The degradation of type I collagen and its release in the osteoclast culture media was determined using Crosslaps Culture Elisa (E) and Helical Peptide Elisa (F) assays. The results are expressed as mean ± S.D. for five slices. A representative experiment from at least three independent experiments is shown in B–F. n.s., not significant; **, p < 0.02; ***, p < 0.01.

Inhibition of Bone Resorption by Matrix AGEs in Human Cortical Bone—To confirm that the accumulation of pentosidine in human bone matrix also interferes with bone resorption mediated by osteoclasts, rabbit unfractonated bone cells were seeded on control- and ribose-incubated bone slices and cultured for 8 days. The increase in pentosidine content (80 mmol/mol of collagen, Fig. 6A), comparable to the physiological levels of pentosidine observed in vivo, was associated with a significant reduction of the area resorbed per slice (Fig. 6C), the area degraded per lacuna (Fig. 6E), and the concentration of the helical peptide product (Fig. 6F) released in the osteoclast culture media.

Dose-dependent Decrease of Collagen Solubility by Matrix AGEs—AGEs may alter the function of proteins by creating intramolecular and/or intermolecular cross-links. We investigated whether the inhibition of collagen release in the culture media of osteoclasts seeded on AGE-modified mineralized slices could be due to an increase in matrix insolubility by AGEs. We assessed the ability of collagen molecules in bone slices incubated or not with D-ribose to be digested by pepsin. Pepsin digestion is commonly used to determine in vitro the solubility of collagen molecules (47). The presence of AGES in bone slices diminished the solubility of collagen molecules in a dose-dependent manner (Table 2).

AGEs Receptors Are Expressed by Osteoclasts—We first analyzed by RT-PCR the expression of the different receptors of AGES that are expressed by undifferentiated (osteoclast progenitors) and fully differentiated osteoclasts. We used monocyte/macrophage RAW264.7 cells that have the potential to differentiate into osteoclast-like cells in vitro in the presence of recombinant RANK-L. We showed that undifferentiated RAW264.7 cells expressed genes encoding several AGES receptors, including AGE-R1, AGE-R2, AGE-R3 (also called Galectin-3), and RAGE (Fig. 7A). RAW264.7 cells differentiated in the presence of RANK-L expressed not only the calcitonin receptor and the TRAP encoding genes, two established markers of osteoclasts, but also the genes coding for the different AGE receptors (Fig. 7A). However, we could not see any difference in the level of expression of these receptors between undifferentiated and differentiated osteoclasts, except for AGE-R2 encoding gene whose expression slightly increased in mature osteoclasts (Fig. 7A). We then investigated the expression and localization of RAGE in human and murine osteoclasts by indirect immunofluorescence (Fig. 7B and supplemental Fig. S3, C and D). RAGE was expressed in multinucleated and TRAP-positive cells that exhibited actin podosomes in vitro (Fig. 7B and supplemental Fig. S3, C and D), three characteristic features of mature osteoclasts (36, 48). In non-permeabilized cells, RAGE was localized all over the cell surface (Fig. 7B), whereas it localized mainly to punctuate structures in permeabilized cells (supplemental Fig. S3, C and D), most likely corresponding to the Golgi apparatus and the secretory vesicles.
AGEs Inhibit Osteoclastic Differentiation in a Dose-dependent Manner—We then investigated whether exogenous AGEs could interfere with osteoclastogenesis. We performed in vitro differentiation assays using mouse primary and immortalized osteoclast precursor cells in the presence of AGE-containing BSA (Fig. 8). BSA was used as a carrier protein for the in vitro formation of AGEs, performed by its incubation either with \(\alpha\)-ribose (AGE-1), or with \(\alpha\)-glucose and lysine (AGE-2). We also used a commercial glycated BSA as a negative control, because this modified BSA contains only immature glycation products. The content of pentosidine in the different preparations of modified BSA is shown in Table 3. We first analyzed the ability of AGE-modified BSA to stimulate RAGE expression. We treated undifferentiated RAW264.7 cells with 50 \(\mu\)g/ml of AGE-1 for 48 h and showed that RAGE gene expression was stimulated (2.5-fold), compared with BSA alone (Fig. 7, C and D), whereas the level of AGE-R3 expression level remained unchanged (Fig. 7C). We then analyzed the differentiation process from primary osteoclast progenitors by assessing the TRAP activity in the different conditions as well as the number of TRAP-positive cells with more than three nuclei (Fig. 8, A and B). The presence of two increasing concentrations of control BSA, as well as of glycated BSA, did not interfere with the differentiation process. However, the two AGE-modified BSA inhibited in a dose-dependent manner the osteoclastic differentiation process, because the TRAP activity and the number of multinucleated cells markedly decreased in the presence of AGE-1 and AGE-2 (Fig. 8, A and B). Similar results were obtained in the monocytic cell line RAW264.7 (Fig. 8C) and in human primary monocytes (data not shown). AGE-1 seemed to have a more potent inhibitory effect than AGE-2. We then determined the kinetic of the effects of AGEs along the differentiation process. We used the RAW264.7 cells whose differentiation process is well documented. The cells were stimulated with RANK-L (at day 0), then AGE-1 or BSA was added at different days (from day 0 to day 3), and the cell phenotype was analyzed at the end of the differentiation (day 4). We determined the TRAP activity (Fig. 8D) as well as the fusion process, by counting the cells with more than three nuclei (Fig. 8E). AGE-1 inhibited totally the differentiation process when added at day 0 and partially at day 1 but not signifi-
cantly thereafter. These results showed that the first step of osteoclastogenesis was inhibited, mainly the commitment of osteoclasts progenitors into pre-osteoclastic cells.

DISCUSSION

Despite the growing identification of new AGEs molecules in vitro, few have been characterized in vivo, and very few have been identified in bone. Pentosidine, the best characterized AGE, has been shown to accumulate with aging in human femora (14) and vertebrae (44). Recently, imidazolone and N\textsubscript{ε}-carboxymethyllysine have been detected in human bone by immunohistochemistry, and the intensity of the staining has been shown to correlate with the age of the patients (13). AGEs accumulation may have deleterious effects in bone tissue as they modulate the functional properties of target tissue. Indeed, in

TABLE 2

Bone matrix AGEs decreased pepsin-mediated collagen solubilization in a dose-dependent manner

| Experimental condition | TBS | d-Ribose (0.2 M) |
|-----------------------|-----|-----------------|
| Incubation time       | 6 days | 2 days | 4 days | 6 days |
| Pentosidine (mmol/mol collagen) | ND* | 7 ± 2b | 168 ± 44b | 209 ± 42b |
| Collagen solubility (%) | 21.6 ± 1.3 | 18.5 ± 2.7 | 11.7 ± 2.3b | 12.5 ± 0.5b |

*ND, not detected. 
\(^b\)p < 0.05 between control- and ribose-incubated slices.
different ex vivo models, using either untreated bone samples (16, 17) or bone specimens where matrix AGEs formation was induced in vitro (15, 49), collagen AGEs have been demonstrated to have deleterious effects on bone mechanical properties. Here, we investigated the role of collagen AGEs on resorption by osteoclasts and demonstrated that matrix AGEs inhibited the resorption of bone and ivory by mature osteoclasts of different species (Figs. 2–6), including human (supplemental Fig. S3B).

Different methods were used to induce the formation of matrix AGEs. In particular, we compared the effect of incubation of D-ribose either in phosphate- and Tris-buffered saline solutions and found that the concentration of pentosidine obtained was similar in the two conditions (supplemental Fig. S1A). These results differ from those of Acharya and colleagues (50) who found that Tris inhibited the glycation process of the globular RNase protein, most likely by trapping the reducing sugar aldotriose. Although the reasons for these discrepant results are unclear, it may be possible that differences of reducing sugars and/or the substrate used in the two studies can be involved. Indeed, collagen is organized into fibrils that are embedded in a mineralized matrix in bone tissue. The use of either type of buffers also did not influence the degradation of bone collagen in vitro (supplemental Fig. S1B), suggesting that in our experimental settings data obtained with TBS solution are valid.

To our knowledge, only one study has reported the effects of AGEs on bone resorption. Miyata et al. (51) showed that bone resorption was enhanced using mouse unfractionated cell containing osteoclasts when cultured on AGE-modified dentin slices. However, this conclusion was mainly drawn by counting the number of resorption lacunae formed by osteoclasts on the slices after 4 days in culture. In our study, we also observed an increase in the number of resorption lacunae when rabbit unfractionated bone cells were cultured for 3 days on bone and dentin slices (Fig. 2 and data not shown). However, when bone resorption was assessed using specific biochemical markers of collagen break-
down, we observed a decrease of dentin collagen fragments released in the culture media (Fig. 2F). The mechanism responsible for the increase in the number of resorption lacunae obtained at 3 days of culture is presently unknown. It is possible that the modification of bone matrix with AGEs could create a microenvironment favorable for the adherence of osteoclasts in the early step of bone resorption. However, our experiments clearly indicate that AGEs have an overall inhibitory effect on bone matrix degradation as we observed a decrease of type I collagen fragments released in the conditioned media after 3 (Fig. 2F) and 8 days (Figs. 3 and 4) of culture. AGEs may interfere with the collagenolytic activity of proteases secreted by osteoclasts, either by masking the cleavage sites or by rendering them less accessible to proteases. Alternatively, AGEs could trap collagen fragments into the cross-linked bone matrix, by creating intermolecular cross-links between matrix proteins. This hypothesis seems to be supported by our findings showing that the presence of AGEs into the bone matrix decreased the solubility of bone collagen, as documented by the pepsin digestion experiment (Table 2). Importantly, when the culture period was extended for up to 8 days, there was a consistent decrease in both the number and the area of resorption lacunae formed by osteoclasts seeded on AGE-modified slices. When seeded on mineralized slices, osteoclasts alternate between resorption and migration phases (36). However, the cellular and molecular mechanisms regulating or triggering either of those two processes are still unknown. It is possible that AGEs contained in collagen molecules modulate the switch between resorption and migration states, by inhibiting the migratory process or by impairing the completion of the resorption phase. Eventually, it is also tempting to speculate that AGEs could accelerate the rate of apoptosis in mature osteoclasts, because it has been shown that exogenous AGEs or matrix AGEs could increase cell death in fibroblasts (52, 53). These mechanisms would explain the decrease of the number of resorption pits and/or in the area resorbed per lacuna for rabbit cells in AGE-containing slices compared with control slices. For the experiments where mouse mature osteoclasts were used, the resorption assay was performed in the presence of RANK-L, a factor known to prolong osteoclast survival by inhibiting apoptosis (54). In this context, we did not observe a difference in the number of lacunae (Fig. 4C) but did find a decrease of the area resorbed per slice (Fig. 4B) and of the area degraded per lacuna (Fig. 4D), supporting a cellular effect of AGEs on osteoclast survival.

Miyata and colleagues (51) also observed an increase in dentin resorption, i.e. an increase of the number of resorption lacunae, when the cells were cultured for 4 days in the presence of AGE-modified BSA or AGE-containing β2-microglobulin. We performed similar experiments using AGE-1 and AGE-2 and rabbit unfractionated bone cells, and bone resorption was assessed after 8 days using biochemical assays. In these conditions, we were not able to detect any significant differences on collagen release when osteoclasts were cultured in the presence of BSA or AGE-modified BSA (data not shown), indicating that exogenous AGEs did not interfere directly with the resorption activity of osteoclasts per se, and more specifically with the degradation of type I collagen occurring in the sub-osteoclastic compartment. The same authors showed that AGEs enhanced demineralized bone resorption in vivo, when AGE-modified bone particles were implanted subcutaneously in rat (51). However, we previously demonstrated in our laboratory that this subcutaneous degradation process involves macrophages but not osteoclasts (55). Altogether, our findings demonstrate that matrix AGEs inhibited osteoclast-mediated bone resorption in vitro.

We demonstrated for the first time that osteoclastogenesis was totally inhibited in vitro in the presence of the AGES, AGE-1 and AGE-2, but not the intermediate glycation products, such as glycated BSA (Fig. 8). The inhibitory effect involves not only the pentosidine molecule (present in AGE-1), but also uncharacterized AGEs, because pentosidine was not formed in AGE-2 (Table 3), as expected from the source of reducing sugars used (32). This finding indicates that osteoclast progenitors express specific receptors for AGEs. There are several molecules reported to interact with AGEs, among those the AGE-R1–AGE-R2–AGE-R3 complex of receptors (56), CD36 (57), scavenger receptors class AI/AII (SR-A) (58), scavenger receptor class B type I (SR-BI) (59), and RAGE (60). Using RT-PCR, we found that undifferentiated RAW264.7 cells expressed AGE-R1–, AGE-R2–, AGE-R3–, and RAGE-encoding genes, and that the level of expression remained unchanged with the osteoclastic differentiation (except for AGE-R2). We also found that AGE-1 up-regulated RAGE expression (Fig. 7). It has been shown that AGEs themselves have the ability to stimulate RAGE expression in osteoblastic cells (27), as well as AGE-R3 (Galectin-3) expression (61). However, we could not see any change in the level of expression of AGE-R3 in RAW264.7 cells, probably due to cell-type specificity. This suggests that AGEs may inhibit osteoclastogenesis mainly by a RAGE-dependent manner, although RAGE-independent pathways cannot be excluded. Further studies are needed to determine the involvement of the different AGES receptors and their downstream effectors in the inhibition of osteoclastogenesis induced by AGEs.

We showed that AGEs affect osteoclastogenesis by targeting directly the osteoclast precursor cells in vitro. This finding should be confirmed in vivo, where the osteoclast differentiation takes place in a microenvironment involving the presence of different cellular actors, including stromal cells/osteoblasts, and the regulatory molecules, including RANK-L and osteoprotegerin. Collagen AGEs have been shown to impair osteoblast proliferation and to inhibit osteoblastic differentiation in a cell-stage-dependent manner (22–25). Knowing that osteoclastogenesis is intimately linked to osteoblast differentiation and function in vivo (62), it is tempting to speculate that AGEs could also inhibit osteoclastic differentiation in an indirect manner, by inhibiting the differentiation of osteoblasts and the subsequent expression of RANK-L by those cells in vivo. This could explain the discrepancy in the number of pits observed using rabbit unfractionated bone cells and mouse mature osteoclast cells. The former is a heterogenous cell population also containing osteoclast precursors and stromal/osteoblastic cells able to perform in vitro osteoclastogenesis when seeded on slices, whereas the latter contains mainly fully differentiated osteoclasts. Consequently, the decrease in the number of lacu-
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markers of bone resorption, no correlation was observed with the histomorphometrical

genesis, we showed that exogenous AGEs inhibited osteoclasto-

nymely by decreasing the solubility of collagen. In addi-

tion, the increase of fracture risk that is observed in aging people (64)

components AGE may (i) impair the biomechanical properties

affected the biomechanical properties of bone collagen and thus increase the brittleness of the tissue, as it has been demonstrated for articular cartilage (8, 63), and (ii) decrease the solubility of bone collagen and impair the normal bone turnover, thus leading to a decline in AGEs removal in bone tissue. Concomitantly, through the interaction with cellular membrane receptors, AGEs could modulate not only the osteoblastic differentiation and function but also osteoclastogenesis, and consequently decrease bone remodeling and collagen turnover, enhancing the accumulation of more AGEs in bone matrix. Consequence of such effects would be deleterious for the overall bone strength and could partially contribute to the increase of fracture risk that is observed in aging people (64) and patients suffering from diabetes (20).

In conclusion, we demonstrated for the first time that bone resorption by osteoclasts is impaired by bone and dentin matrix AGEs, likely by decreasing the solubility of collagen. In addition, we showed that exogenous AGEs inhibited osteoclastogenesis in vitro, most likely by interacting with the cell-surface-specific receptor RAGE. All together these results indicated that AGEs could contribute to some of the physiopathological mechanisms of altered bone remodeling observed with aging and diabetes.

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