Advanced glycation end product modification of bone proteins and bone remodelling: hypothesis and preliminary immunohistochemical findings

G Hein, C Weiss, G Lehmann, T Niwa, G Stein, S Franke

Background: The process of bone remodelling is disturbed in the development of osteoporosis.

Objective: To investigate if proteins in osteoporotic bone are modified by advanced glycation end products (AGEs), and whether these alterations are related to measures of bone remodelling based on histomorphometric findings.

Methods: Bone specimens taken from the iliac crest by bone biopsy of eight osteoporotic patients were investigated by histomorphometry and by immunohistochemical staining with the AGEs imidazolone and N'-carboxymethyllysylsine.

Results: Both AGEs were found in all bone specimens. The intensity of staining correlated with patient age. The percentage of bone surface covered with osteoblasts showed a significantly negative correlation with the staining intensity of both AGEs.

Conclusions: It is known that AGEs can regulate proliferation and differentiation of osteoblastic cells and that AGE-specific binding sites are present in cultured osteoblast-like cells. Moreover, AGE induced biological effects in these cells might be mediated by RAGE (receptor of AGE) or by other AGE receptors in different stages of osteoblast development. The inverse relation between AGE staining intensity and the percentage of bone surface covered with osteoblasts in the trabecular bone may provide evidence that AGE modification of bone proteins disturbs bone remodelling.

It is currently known that bone is a permanently regenerating organ with a high number of removal sites at any time—the basic multicellular units. Physiological remodelling seems to be essential for preservation of bone quality.1,2

In this context questions arise as to what are the mechanisms behind the initiation of the remodelling in an encosed bone site? What form of alterations intensifies the attraction of osteoclast precursors and triggers their maturation or disturbs osteoblast recruitment and/or function in the remodelling process?

Advanced glycation end products (AGEs) are chemical modifications of proteins by carbohydrates, including metabolic intermediates formed during the Maillard reaction. The generation of AGEs is an inevitable process in vivo.3

AGEs constitute a heterogeneous class of structures. They are recognised by specific receptors on different cell types. The most well known receptor of AGEs is the RAGE. A remarkable feature of AGEs is their ability to mediate an additional cross linking of altered protein subunits, resulting in a high resistance to proteolytic digestion. The binding of an AGE on its cellular receptor (for example RAGE) activates the nuclear factor κB (NF-κB) in these cells, followed by an increased expression of, for example, cytokines such as interleukin 6, interleukin 1, and tumour necrosis factor α, as well as growth factors and adhesion molecules.4,5

Among the well described and chemically identified AGEs are N'-carboxymethyllysylsine (CML), pentosidine, and imidazolone. Long living proteins such as collagen molecules in bone are particularly predestined to undergo such chemical modification.6

It seems probable that AGE modification takes place in bone tissue and that this results in disturbed proliferation and function of different cell types participating in bone turnover.

We hypothesise that embedded in the process of post-translational protein modification, AGE modification of bone proteins may be one of the reasons for the initiation of bone remodelling, and for a disturbance of this process as well.

PATIENTS AND METHODS

Tetracycline labelled bone biopsy specimens were used from eight patients with osteoporosis as diagnosed by bone densitometry (T score <−2.5 SD, DXA, Hologic QDR 4500A). Table 1 gives details of the patients. The specimens were taken from the iliac crest using the Straumann-Burkhardt drill (at days 16, 15, 5, and 4 before each patient received 250 mg of tetracycline).

Informed consent for the study was given by all patients and the local ethics committee.

Bone histomorphometry was performed according to the publication of American Society of Bone and Mineral Research7 and as already described by us.8 The following variables were determined:

- Mineral apposition rate (MAR; μm/day)
- Mineralising surface/bone surface (MS/BS; %)
- Osteoid surface/bone surface (OS/BS; %)
- Osteoblast covered surface/bone surface (ObS/BS; %)
- Erodable surface/bone surface (ES/BS; %)
- Osteoclast covered surface/bone surface (OcS/BS; %).

Immunohistochemistry

Sections (~4 mm x 10 mm), 4 μm thick, were stretched with 80% ethanol on Superfrost Plus slides (before use coated with a solution containing 0.003% poly-L-lysine and 1.3% Ponnal express) and dried overnight at 60°C. (poly-L-lysine, Sigma Diagnostics Inc, St Louis, USA; Ponnal express, Henkel KGaA, Düsseldorf). Sections were deplasticised with 2-methoxy-ethylacetate and rehydrated with distilled water through a decreasing ethanol series and then decalcified with 3% acetic acid for 3 minutes. After washing with a rinsing buffer

Abbreviations: AGE, advanced glycation end product; CML, N'-carboxymethyllysylsine
(phosphate buffered saline), the sections were encircled with a water repellent wax pen (Dako, Glostrup, Denmark) and placed in a humidified chamber. The procedure was carried out at room temperature, unless incubation with primary antibodies was done at 4°C overnight. Polyclonal rabbit anti-CML antibody (supplied by Roche Diagnostics, Penzberg, Germany) and monoclonal anti-imidazolone antibody were used as primary antibodies. The imidazolone antibody was prepared and characterised as previously described. Staining was performed using Vectastain Elite ABC Kits (Vector Laboratories, Burlingame, USA). Aminoethylcarbazole was used as a chromogen. The sections were counterstained with Mayer’s haematoxylin. For the negative control, the primary antibody was replaced by phosphate buffered saline.

For imaging and documentation a computer assisted Axioplan microscope with an Axiocam HRc digital camera and AxioVision 4.1 software was used (Carl Zeiss AG, Germany).

For quantitative assessment of CML and imidazolone staining intensities of bone areas all selected samples were stained in one batch without counterstaining. Thirty fields (magnification ×200) of each sample were scanned in the monochrome mode of the camera. After highlighting the bone area in the fields, the mean densitometric grey levels of these selected areas could be calculated. Finally, the average of all grey levels obtained by measuring 30 fields for each sample was used as an equivalent for the AGE staining intensity in bone and expressed as arbitrary units. Spearman’s correlation test was used for statistical analysis.

RESULTS
The mean (SD) histomorphometric data were: OS/BS (%) = 2.51 (2.47), ObS/BS (%) = 0.93 (1.39), MS/BS

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<th>Table 1 Characteristics of patients</th>
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Figure 1 Immunohistochemical detection of CML (A) and imidazolone (B) in osteoporotic bone (magnification ×100). CML showed a stronger immunoreactivity of the bone marrow than the trabecular bone. Imidazolone staining was much more intensive in the bone tissue than in the marrow cells. Control staining for CML (C) and imidazolone (D). Negative controls included the omission of the respective primary antibody. Immunohistochemical detection of CML (E) and imidazolone (F) positive osteoclasts (arrows) in osteoporotic bone (magnification ×400).
Figure 2  The staining levels of CML (A) and imidazolone (B) correlated negatively with the percentage of osteoblast covered bone surface (CML staining intensity—Obs/BS: $r_s = -0.781$, $p = 0.022$; imidazolone staining intensity—Obs/BS: $r_s = -0.805$, $p = 0.016$).

Correlation with histomorphometric findings
The presence of the AGEs CML and imidazolone was shown in all bone specimens investigated. No significant correlation was found between the intensity of AGE staining and the cellular markers of bone resorption (ES/BS, OcS/BS) and also no correlation with the mineral apposition rate MAR, the mineralising surface MS/BS, and the osteoid surface OS/BS. Figure 2 shows a significant negative correlation between the intensity of AGE staining and the bone surface covered with osteoblasts (CML staining intensity—Obs/BS: $r_s = -0.833$, $p = 0.010$; imidazolone staining intensity: $r_s = 0.857$, $p = 0.007$) as well as with each other ($r_s = 0.905$, $p = 0.002$).

DISCUSSION
Already in 1997 Miyata et al reported that enhanced bone resorption was seen when mouse unfractionated bone cells containing osteoclasts were cultured on AGE modified dentin slides.11

As described by McCarthy et al the accumulation of AGEs in the bone extracellular matrix regulated the proliferation and differentiation of osteoblastic cells. These effects appear to depend on the stage of osteoblastic development.12 Moreover, they could show that AGE-specific binding sites are present in cultured osteblast-like cells and that these AGE receptors are regulated by the stage of osteoblastic differentiation.13 As reported recently, AGE-induced biological effects in osteoblasts could be mediated by RAGE in the later stages of development, and mediated by other AGE receptors in the earlier pre-osteoblastic stage.14

Also, our own previous investigations provide evidence that AGE modified proteins may be a cause of altered bone remodelling in osteoporosis.9 The group with osteoporosis had significantly higher pentosidine and CML serum levels than the non-osteoporotic age matched controls.

subgroups with signs of high osteoclast activity we found that serum pentosidine correlated significantly with markers reflecting osteoclast activity/bone resorption (for example, eroded surface as a percentage of trabecular surface), suggesting that pentosidine influences the activity of osteoclasts in vivo. A strong correlation between pentosidine and the mineral apposition rate (MAR) was also found.

In this study we aimed at determining if advanced alterations of bone protein by glycation are clearly detectable in osteoporotic bone, and if a relationship between AGE accumulation in bone tissue and any histomorphometric variables could be demonstrated. The results show that AGE modification is present in all the osteoporotic bone taken from the eight patients; each, however, with a different intensity.

Whereas Miyata et al using in vitro experiments with osteoclasts could show enhanced bone resorption by the addition of AGE modified albumin, we showed a significantly negative relationship between the staining intensity of both AGEs CML and imidazolone in osteoporotic bone, on the one hand, and the percentage of trabecular bone surface covered with osteoclasts on the other hand. The higher the staining intensity, the lower the portion of trabecular surface with formation activity (covered with osteoclasts).

Our results are in agreement with those of Katayama et al.15 They reported that AGEs in collagen inhibit phenotypic expression of osteoclasts.

Thus it seems that the glycation of bone proteins influences processes both of remodelling: osteoclastic bone resorption and osteoblastic bone formation.

Whether the result will be an accelerated bone resorption (with possible development of “high turnover” osteoporosis) or a decreased bone formation (resulting possibly in a “low turnover” osteoporosis), may depend on the specificity of generated glycation end products.

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