Advanced glycation end-products pentosidine and N\^e-carboxymethyllysine are elevated in serum of patients with osteoporosis

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Objective. To investigate serum levels of the advanced glycation end-products (AGEs) pentosidine and N\^e-carboxymethyllysine (CML) in patients classified into different osteoporosis subgroups according to histomorphometric data.

Method. Serum samples were obtained from 116 osteoporotic patients (34 men, 82 women) classified by bone histomorphometry into subgroups with high turnover (HTO, \(n=32\)), low turnover (LTO, \(n=39\)), normal turnover (NTO, \(n=9\)) and cellular uncoupled osteoporosis (CUO, \(n=36\)). Pentosidine was measured by high-performance liquid chromatography, and CML by a competitive enzyme-linked immunoassay.

Results. The entire osteoporosis group had significantly higher pentosidine and CML serum concentrations than healthy subjects. In contrast to healthy subjects, no correlation between levels of AGEs and age could be found. In subgroups characterized by increased bone resorption (HTO, CUO), serum pentosidine correlated significantly with the histomorphometric marker reflecting osteoclast activity/bone resorption (eroded surface as a percentage of trabecular surface). Moreover, in CUO a strong correlation between pentosidine and the mineral apposition rate was found. Surprisingly, in HTO the levels of CML and percentage of eroded surface were significantly negatively correlated.

Conclusion. AGE-modified proteins may be a cause of disturbed bone remodelling in osteoporosis. Our findings do not support the alternative hypothesis that increased AGEs in serum indicate only a more intensive releasing of AGEs in circumstances of increased bone resorption.

KEY WORDS: Osteoporosis, Advanced glycation end-product, Pentosidine, N\^e-Carboxymethyllysine, Bone formation, Bone resorption, Bone histomorphometry.
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rheumatoid arthritis (RA) [9], presumably causing the perpetuation of inflammation in the joint. Keeping that in mind it can be hypothesized that an increase of AGE modification of bone proteins such as collagens is one of the reasons for the activation of NF-κB in different cell types participating in bone turnover, induced by the binding of these AGES on RAGE.

To test the validity of this hypothesis, the study aimed to measure serum levels of the AGES pentosidine and CML in patients with different forms of osteoporosis, and to compare the results with those obtained from age- and sex-matched healthy controls.

In particular we intended to examine the relationship of AGE serum levels and cellular parameters of bone turnover, as investigated by bone histomorphometry.

Patients and methods

Patients

The study included 116 non-diabetic patients with osteoporosis diagnosed by bone densitometry and characterized by bone biopsy (34 men and 82 women, mean age 55 ± 10 yr, all with normal renal function), examined regularly at the Department of Internal Medicine IV, Section Rheumatology and Osteology (University of Jena), and 44 age-matched healthy subjects (18 men and 26 women, mean age 55 ± 8 yr).

The patient group comprised patients with high-turnover (HTO, n = 32), low-turnover (LTO, n = 39), normal-turnover (NTO, n = 9) and cellular uncoupled osteoporosis (CUO, n = 36).

Blood samples were collected on the day before biopsy, centrifuged at 3000 r.p.m. for 10 min and stored at −80°C until testing.

Pentosidine

Pentosidine measurement was performed with some specification [9] using the high-pressure liquid chromatography (HPLC) assay described by Miyata et al. [10]. Synthetic pentosidine was used to obtain a standard curve (kindly provided by Dr T. Miyata, Tokai University, Japan). The intra- and interassay coefficients of variation were <3 and <6%, respectively.

N²-Carboxymethyllysine

CML serum concentrations were determined by a competitive enzyme-linked immunoassay (ELISA) (kindly provided by Roche Diagnostics Gmbh, Penzberg, Germany) as described in [11]. The intra- and interassay coefficients of variation were <5 and <7%, respectively.

Bone histology and histomorphometry

Tetracycline-labelled bone biopsies were obtained from the anterior, superior iliac crest using the Straumann-Burkhart drill. Specimens were fixed in Carnoy’s solution and embedded into methylmethacrylate as undecalcified samples. Specimens of each biopsy, 4 μm thick, were stained after Masson/Goldner, Gomori and Giemsa to identify bone cells, marrow or endosteal fibrosis and to differentiate mineralized bone and osteoid.

For fluorescence examination, 7-μm thick sections were kept unstained. Bone structures and bone cells were measured using the Merz ocular grid and the Osteoplan II system (KONTRON, Munich, Germany). Bone histomorphometry was performed according to the publication of the American Society of Bone and Mineral Research [12].

Dynamic parameters of bone formation were estimated by fluorescence microscopy, for example the mineral apposition rate (MAR; mean distance in micrometres between the two tetracycline labels) and the mineralizing surface (MS; double-labelled surface plus half of single-labelled surfaces, expressed as a percentage of trabecular bone surface, BS). Additionally, the osteoblast-covered surface as a percentage of trabecular bone surface (ObS/BS) was determined.

Parameters of bone resorption were only static ones. They included the eroded surface as a percentage of trabecular bone surface (ES/BS) and the osteoclast-covered surface as a percentage of the trabecular surface containing resorptive cavities (OcS/BS).

After Marie et al. [13], bone formation was classified according to mineralizing surface (<6% = low, 6–10% = normal, >10% = high). To classify the activity of bone resorption we used an analogous subdivision according to osteoclast-covered surface: <1% = low, 1–3% = normal, >3% = high.

The following terms for description of osteoporosis subgroups were used: (i) with high resorption but normal (or high) formation—HTO; (ii) in cases of low formation but normal (or low) resorption—LTO; (iii) with normal ranges for both, formation and resorption—NTO; and (iv) in cases of low formation but high resorption—CUO.

Statistical methods

The results are given as means with standard deviations. ANOVA and the unpaired t-test were used for statistical evaluation; the Pearson correlation test and multiple regression analysis for estimating relationships between variables; P values less than 0.05 were considered to be significant.

In the group of healthy subjects as well as in osteoporotic patients the pentosidine and CML values were normally distributed (Kolmogorov–Smirnov test).

Results

Compared with healthy subjects, patients with osteoporosis showed significantly elevated pentosidine (185 ± 115 vs 133 ± 37 pmol/ml; P < 0.0001) and CML (2590 ± 970 vs 2251 ± 415 pmol/ml; P = 0.002) serum concentrations.

In the patient groups, neither CML nor pentosidine correlated with age. In the healthy subjects, pentosidine and CML were significantly age-related (pentosidine: r = 0.327, P = 0.030; CML: r = 0.333, P = 0.027).

All osteoporosis subgroups had higher pentosidine and CML mean levels than healthy subjects, with significant increases in the HTO and CUO groups for pentosidine and in the HTO and LTO groups for CML (Figs 1 and 2). The mean pentosidine concentration in patients with CUO was found to be significantly higher than that in patients with NTO or LTO. In contrast with the healthy subjects (r = 0.430, P = 0.004), the serum pentosidine and CML levels in the osteoporosis patients did not correlate with each other.

In the whole osteoporosis group significant correlations existed between the serum pentosidine levels...
and those of the bone histomorphometric parameters ES/BS, MS/BS or MAR (ES/BS: \( r = 0.329, P < 0.0001 \); MS/BS: \( r = 0.243, P = 0.015 \); MAR: \( r = 0.334, P = 0.001 \)). In the osteoporosis subgroups, pentosidine correlated significantly with \( \text{OcS/BS} \) in LTO \( (r = 0.328, P = 0.042) \), with ES/BS in HTO \( (r = 0.426, P = 0.015) \) and with ES/BS or MAR in CUO \( (ES/BS: r = 0.377, P = 0.023; \text{MAR}: r = 0.638, P < 0.0001) \). On the other hand, the CML levels in the HTO group correlated negatively with ES/BS \( (r = -0.414, P = 0.019) \).

On multiple regression analysis of the whole osteoporosis group, the most important parameters associated with an increase in ES/BS \( (r^2 = 0.368, P < 0.0001) \) were \( \text{OcS/BS} (\beta = 0.429, P < 0.0001) \), pentosidine \( (\beta = 0.271, P = 0.006) \) and CML \( (\beta = -0.203, P = 0.024) \). All other parameters (sex, age, \( \text{ObS/BS}, \text{MAR}, \text{MS/BS} \)) investigated in the model revealed no association.

**Discussion**

The aim of our study was to look for different AGE serum levels in patients with osteoporosis in relation to histomorphometric findings and compared with healthy subjects. Higher serum levels of AGEs (e.g., pentosidine or CML) in osteoporosis (presuming that the measured levels indicate bone condition) may provide evidence for the hypothesis that this alteration of bone proteins may be one of the initializing factors for osteoclast activation or for braking osteoblasts.

It is known that AGE modifications alter the structure and properties of proteins and are able to activate NF-kB or other transcription factors in different cell types by the binding of AGEs on specific receptors (e.g., RAGE). In the healthy subjects we found a significantly positive correlation between serum levels of pentosidine as well as of CML with age; both AGEs correlated significantly with each other. At similar age distribution, the osteoporosis group was characterized by significantly higher levels of pentosidine and CML compared with the healthy subjects. In the osteoporosis group we could not find any correlation of patient age with AGE levels in the serum nor a correlation of both AGEs to each other. Thus there seems to be no doubt that osteoporosis is connected with more intensive generation of AGEs, yet these results do not clearly indicate whether the increased AGE generation is a causal phenomenon of osteoporosis or an epiphenomenon.

To obtain more detailed information, we investigated whether the measured AGE levels are related to the histomorphometrically characterized osteoporosis subgroups. Although all subgroups of osteoporosis showed higher mean levels of the measured AGEs pentosidine and CML compared with the healthy subjects, significant differences were found for pentosidine only in subgroups with increased bone resorption (HTO or CUO). The highest pentosidine levels were found in the group with histomorphometric findings of increased bone resorption \( (\text{OcS} > 3\%) \) and lowered bone formation \( (\text{MS} < 6\%) \). CML showed significantly higher mean levels in the HTO and the LTO groups.

Furthermore, we investigated correlations between AGE levels and selected histomorphometric parameters of bone turnover. For the whole osteoporosis group we found a positive correlation between serum pentosidine and ES/BS, MS/BS as well as the mineral apposition rate. This may provide evidence that, initially, pentosidine contributes to increased osteoclast formation and/or activity and, possibly in a following step, the osteoblast recruitment or activity is intensified. This is supported by the finding of significant
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relationships between serum pentosidine and the percentage of ES/BS, especially in subgroups with increased bone resorption (HTO, CUO), as well as of a significant correlation between the pentosidine levels and OcS/BS in LTO. Surprisingly, in the HTO subgroup, CML had a significant negative correlation with ES/BS. Perhaps this advanced glycation end-product might be able to brake osteoclast activity or its generation.

In cultured mouse bone cells and in rats implanted subcutaneously with devitalized bone particles, Miyata et al. [14] showed, in 1997, that AGEs were able to enhance osteoclast-induced bone resorption, although the mechanism remained unclear. Calcitonin and ipriflavone, both inhibitors of bone resorption, neutralized the effects of AGEs [14]. Furthermore, AGE-modified collagen was able to regulate the proliferation and differentiation of osteoblastic cells depending on the stage of osteoblastic development [15, 16]. The addition of AGE-bovine serum albumin to cultures of human osteoblast-like cells resulted in a significantly reduced synthesis of collagen I and osteocalcin in these cells [17]. In osteoblast-like cells, RAGE could be identified [18].

When we looked in our study for significant relationships between important variables using multiple regression analysis, for the AGE pentosidine we were able to confirm not only an independent influence on the marker of bone resorption ES/BS, the percentage of eroded bone surface, but also on the bone formation marker MAR. This is a further hint that pentosidine (and possibly other AGEs too) participate in recruitment and/or activation of osteoclasts and—possibly in a second step or caused by mechanisms of coupling—in the bone formation processes, thereby potentially having a role in the entire process of bone remodelling.

In the light of experimental data of other groups, our investigations, especially the results of multiple regression analysis, do not support the alternative hypothesis that the increased pentosidine levels in the serum of osteoporosis patients in circumstances of high turnover or high osteoclast activity only discloses an epiphenomenon, indicating merely a more intensive releasing of AGEs from bone tissue proteins by the activated osteoclasts. However, further investigations concerning the quantification of AGEs in bone tissue and their functional effects on cells contributing to bone remodelling are necessary.

Conflict of interest

The authors have declared no conflicts of interest.

References
