Advanced glycation end products affect growth and function of osteoblasts

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Abstract

Objective

Advanced glycation end products (AGEs) have been implicated in the pathogenesis of bone-destructive disorders. Yet reports on the influence of AGEs on human osteoblasts remain lacking. The aim of the study is to investigate the influence of AGE-modified bovine serum albumin (AGE-BSA) on cell growth and expression of osteoblastic markers associated with osteogenesis and osteoclastogenesis.

Methods

Human osteoblasts established from bone tissue specimens were stimulated with AGE-BSA and investigated in vitro. Expression of mRNA for the receptor for AGEs (RAGE), nuclear factor kappa B subunit p65 (NFκB p65), tumour necrosis factor alpha (TNF-α), matrix metallo proteinase-1 (MMP-1), receptor activator of NFκB ligand (RANKL), osteoprotegerin, collagen type I (Col1), osteocalcin (OC) and alkaline phosphatase (ALP) were measured using real-time polymerase chain reaction (PCR). Respective protein expressions were evaluated by western blot analysis or ELISA. NFκB activation was investigated by luciferase assay and electrophoretic mobility shift assay (EMSA). Cell cycle analysis, cell proliferation and markers of necrosis and early apoptosis were assessed.

Results

AGE-BSA was actively taken up into osteoblasts and induced cell cycle arrest and an increase in necrotic, but not apoptotic cells. The increased expression of RAGE and TNF-α together with NFκB activation indicates an AGE-mediated inflammatory response. The decreased expression of Col1, OC and ALP presumably reflects a diminished osteogenic potential, whereas upregulation of RANKL and TNF-α enhances osteoclastogenesis.

Conclusion

The present study demonstrates that AGE-BSA affects the growth and function of osteoblasts. Modulation of the expression of various target genes involved in bone metabolism provides evidence that AGEs accumulated in the bone matrix have the potential to suppress osteogenic and to promote osteoclastogenic properties of osteoblasts in vivo, thereby leading to functional and structural impairment of bone.

Key words

Osteoblasts, Advanced Glycation End Products, RAGE (receptor for advanced glycation end products), NFκB, bone formation and resorption

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Received on August 10, 2010; accepted in revised form on April 7, 2011.

Competing interests: none declared

Introduction

Disturbed bone remodelling contributes to the development of age-related bone diseases such as osteoporosis, a major public health problem associated with aging (1, 2). It is known that with increasing age advanced glycation end products (AGEs) accumulate in bone tissue. In this physiological process AGEs, chemical modifications of proteins by carbohydrates (including metabolic intermediates generated during the Maillard reaction), are formed (3). Bone tissue is susceptible to AGE accumulation due to its low turnover and over the length of a lifespan biomechanical, biochemical, and cellular characteristics of bone tissue are affected. The binding of AGEs to their specific receptor (RAGE) leads to activation of the nuclear factor kappa B (NFκB), which is then followed by an increased expression of proinflammatory cytokines and the receptor itself (4, 5).

During bone remodelling, osteoblasts and osteoclasts arranged within the basic multicellular units are in close proximity to AGE-modified bone proteins such as collagen type I (Col1) and their degradation products. These are RAGE-expressing cells and interactions with AGEs may influence cell differentiation, maturation and function (6-9). Thus, protein glycation is clearly implicated as a strong contributor to the development of age-related bone disorders.

Several important roles in bone remodelling have been attributed to osteoblasts, including production of bone matrix and bone mineralisation (by expression of osteogenic markers), expression of osteoclastogenic cytokines (*e.g.* receptor activator of NFκB ligand, RANKL; tumour necrosis factor alpha, TNF-α) as well as secretion of matrix metalloproteinases (*e.g.* MMP-1) (10). Most published studies have used murine or rat osteoblast-like cell lines to investigate interactions with AGEs (11-21). Human osteoblasts isolated from bone tissue have been evaluated in only six studies (6, 9, 22-25). No data yet exists on the impact of AGEs on the osteoblastic expression of RANKL and osteoprotegerin.

The aim of this study is to elucidate AGE-mediated changes in gene expression and function in an *in vitro* model using human osteoblasts rather than animal cell lines, offering the advantage of a better simulation of the *in vivo* situation. In contrast to numerous studies in the literature, we used AGE-modified bovine serum albumin (AGE-BSA) prepared by incubation of BSA with glucose, in the physiological setting one of the more important saccharides involved in glycation in mammalian metabolism (26). Similar to *in vivo* glycated proteins, modification of our AGE-BSA was done using the naturally occurring structures N^{ε}-carboxymethyllysine (CML), pentosidine and 3-desoxyglucosone-derived imidazolone (3).

Our results demonstrate that AGE-BSA induces cell cycle arrest and cell death, upregulation of RAGE together with activation of NFκB, suppression of osteogenesis (downregulation of Col1, osteocalcin, OC; and alkaline phosphatase, ALP), and promotion of osteoclastogenesis (upregulation of RANKL, TNF-α, and MMP-1). Thus, AGEs seem to lower the capacity of osteoblasts to form normal bone and increases osteoclastogenic potential.

Materials and methods

Specimen selection and osteoblast cultures

Bone tissues were obtained at the time of knee replacements from 18 patients with osteoarthritis (7 women, 11 men; 64±9 years). Informed consent for the study was given by all patients and the study was approved by the local ethics committee. Subchondral bone was harvested from femoral and tibial joint surfaces by means of a sharp curette. Human osteoblast cell cultures were prepared from these bone specimens as previously described (25). Briefly, minced trabecular bone sections were suspended in Dulbecco's Modified Eagle Medium: F12 Nutrient Mixture (D-MEM/F12) supplemented with 10% foetal calf serum (FCS) and gentamicin (100 µg/ml) (Gibco, Karlsruhe, Germany) and maintained at 37°C in a 5% CO_2-humidified atmosphere. The medium was changed twice weekly. Two weeks later, the cells were passaged and expanded near confluence. To avoid any additional influence on

cell differentiation no osteogenic supplements were added. Only the third to seventh passage cells were used for the experiments after the medium was replaced by RPMI 1640 (with 10% FCS and 100 µg/ml gentamicin).

Preparation of AGE-BSA

AGE-BSA and control-BSA (Co-BSA) were prepared as previously described (27). A fatty acid-poor and endotoxin-free type of BSA (Calbiochem, La Jolla, CA, USA) was incubated under sterile conditions at 37°C for 50 days in phosphate buffered saline (PBS) with and without the addition of glucose (90 mg/ml), then filtrated to remove unbound glucose and glucose degradation products (Millipore Labscale TFF System, Bedford, MA, USA), and lyophilised. After glycation, the AGE-BSA had a 90-fold higher content of CML than control-BSA (12.47 versus 0.14 nmol/mg protein in Co-BSA) and a 10-fold higher pentosidine concentration (22.8 versus 2.3 pmol/mg protein in Co-BSA). CML was measured by ELISA (MicroCoat Biotechnologie GmbH, Bernried, Germany) and pentosidine by HPLC (Merck-Hitachi, Darmstadt, Germany) (28).

In addition, as demonstrated by western blot analysis, AGE-BSA but not Co-BSA showed strong staining for imidazolone (data not shown). The accumulation of CML, pentosidine and imidazolone indicates that the AGE-BSA used in the study is modified with a variety of AGE structures which may conform with *in vivo* generated forms.

Experimental conditions

After optimising the dose and time course of AGE-BSA treatment all experiments were conducted in RPMI 1640 containing 0.1% FCS supplemented with 5 mg/ml AGE-BSA or 5 mg/ml Co-BSA (corresponding to 75 µmol/l). Cells were incubated for a period of up to seven days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For histochemical studies, cells were seeded in chamber slides (Nunc, Rochester, NY, USA) and treated as described before. AGE uptake of osteoblasts was confirmed by immunohistochemical staining and western blot analysis for the detection of AGE-modified albumin.

Immunohistochemical staining

Immunohistochemical staining was performed using the Vectastain® Elite ABC Kits (Vector Laboratories, Burlingame, CA, USA) as specified (27). The following primary antibodies were used: anti-OC, anti-ALP (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-CML (Roche Diagnostics, Penzberg, Germany), and anti-imidazolone (kindly provided by Toshumitsu Niwa, Japan). Secondary antibodies were conjugated either with fluorescein (for OC and ALP staining) or with horse radish peroxidase (HRP) using aminoethylcarbazole as substrate (for CML and imidazolone staining). Counterstaining was performed with DAPI or Mayer's haematoxylin. For the negative controls, primary antibodies were replaced by rabbit or mouse immunoglobulin in the same concentration as the primary antibody.

Cell proliferation and viability tests

To evaluate the influence of AGE-BSA on the number of cultured cells, osteoblasts seeded in six-well plates were incubated with either AGE- or Co-BSA for a period up to seven days. On days two, five and seven, cells were detached and counted (CASY Cell Counter, Innovatis, Reutlingen, Germany). To assess proliferation, osteoblasts were transferred into 96-well plates (3000 cells/well) and incubated for 24 h. Bromodeoxyuridine (BrdU) incorporation as a parameter for DNA synthesis was measured by the Cell Proliferation ELISA and cell viability using the MTT Cell Proliferation Kit (Roche Diagnostics, Mannheim, Germany). Each measurement was performed on three different osteoblast cell lines with n=6 per treatment group.

Cell cycle analysis and evaluation of cell death

Osteoblasts were harvested after two days of Co-BSA or AGE-BSA treatment. For cell cycle analysis cells were stained with propidiumiodide and analysed by a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lake, NJ, USA).

To investigate whether AGE-BSA induces early apoptosis and necrosis, osteoblasts were stained with annexin-V-fluorescein and propidiumiodide simultaneously (Annexin-V-FLUOS Staining Kit, Roche Diagnostics, Mannheim, Germany).

Reverse transcriptase and real-time PCR

Total cellular RNA was extracted from the treated osteoblasts using a RNA isolation kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesised from 3 µg of total RNA with the Reverse Transcription System (Promega, Madison, WI, USA).

Real-time PCR was performed with the Realplex Mastercycler instrument (Eppendorf AG, Hamburg, Germany) using the LightCycler®480 SYBR Green I Master-Kit (Roche Diagnostics, Mannheim, Germany). The cDNA samples were amplified according to the manufacturer's instructions. The sequences of the chosen primers and the cycler conditions are given in Table I.

The quantity of mRNA was calculated using the threshold cycle (Ct) value for amplification of each target gene and GAPDH as a reference gene. To compare results between AGE-BSA and Co-BSA treatments, the $2^{\Delta\Delta Ct}$ formula was used for relative quantification (29).

Western blot analysis

Western blot analysis was performed as described (27). Osteoblasts stimulated with Co-BSA or AGE-BSA were lysed in complete Lysis-M buffer (Roche Diagnostics, Mannheim, Germany) and protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Aliquots of 20 µg protein/lane were electrophoresed in a 12% SDS-polyacrylamide gel, then transferred to PVDF membranes and immunohistochemically stained.

Primary antibodies to the following antigens were used: ALP, BSA, Col1, NFκB p65, OC, osteoprotegerin, RANKL (Santa Cruz Biotech, Santa Cruz, CA, USA), MMP-1 (R&D Systems, Minneapolis, MN, USA), RAGE (Acris Antibodies, Hiddenhausen, Germany), and vinculin (Sigma, St. Louis, MO, USA). Antigen-antibody complexes were detected with

Table I. DNA sequences of the sense and antisense primers for real-time PCR analysis and cycler conditions.

Gene	Accession number	Primer sequences	Annealing temperature (°C)	Number of cycles	Product size (bp)
Alkaline phosphatase	[GenBank:NM_000478]	5‘-CCACGTCTTCACATTTGGTG -3’ (sense) 5’-AGACTGCGCCTGGTAGTTGT-3’ (antisense)	58	30	196
COL1A1	[Genbank:NM_000088]	5‘-CCCTGGAAAGAATGGAGATG-3’ (sense) 5’-CCATCCAAACCACTGAAACC-3’ (antisense)	53	30	150
GAPDH	[GenBank:J02642]	5’-CAATGACCCCTTCATTGACC-3’ (sense) 5’-TGGACTCCACGACGTACTCA-3’ (antisense)	59	30	197
MMP-1	[GenBank:NM_002421]	5‘-CTGGGAGCAAACACATCTGA-3’ (sense) 5’-CTGGTTGAAAAGCATGAGCA-3’ (antisense)	60	30	239
NFκB p65	[GenBank:NM_021975]	5'-AGTACCTGCCAGATACAGACGAT-3’ (sense) 5’-GATGGTGCTCAGGGATGACGTA-3’ (antisense)	62	30	215
Osteocalcin	[GenBank:NM_199173]	5’-GTGCAGAGTCCAGCAAAGGT-3‘ (sense) 5‘-TCAGCCAACTCGTCACAGTC-3‘ (antisense)	58	30	175
Osteoprotegerin	[GenBank:U94332]	5‘-TGCAGTACGTCAAGCAGGAG-3’ (sense) 5’-CCCATCTGGACATCTTTTGC-3’ (antisense)	53	30	175
RAGE	[GenBank:AB036432]	5’-GGAAAGGAGACCAAGTCCAA-3’ (sense) 5’-CATCCAAGTGCCAGCTAAGA-3’ (antisense)	59	30	166
RANKL	[GenBank:AF019047]	5’-GCTTGAAGCTCAGCCTTTTG-3’ (sense) 5’-CGAAAGCAAATGTTGGCATA-3’ (antisense)	59	40	192
TNF-α	[GenBank:NM_000594]	5'-GGCAGTCAGATCATCTTCTCGAA-3’ (sense) 5’-AAGAGGACCTGGGAGTAGATGA-3’ (antisense)	62	40	195

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; COL1A1: collagen type I alpha 1; MMP-1: matrix metalloproteinase-1; NFκB p65: nuclear factor kappa B p65 subunit; RAGE: receptor for advanced glycation end products; RANKL: receptor activator of NFκB ligand; TNF-α: tumour necrosis factor alpha.

HRP-labelled secondary antibodies (DakoCytomation, Glostrup, Denmark) and visualised using enhanced chemiluminescent reagents (Perkin Elmer LAS, Boston, MA, USA). Quantification was done by measuring relative density normalised for vinculin.

Measurement of TNF-α concentrations

TNF-α concentrations in the osteoblast culture supernatants were determined by using an enzyme immunoassay (R&D Systems, Minneapolis, MN, USA). Cells were stimulated in six-well plates with either Co-BSA or AGE-BSA for 48 hours. The media were harvested and stored at -80°C until the measurements were performed. Cells were detached and counted. The results were corrected by the numbers of osteoblasts in the wells.

Electrophoretic mobility shift assay (EMSA) for NFκB

Osteoblasts isolated from three different patients were grown in RPMI with 10%FCS. Cells were then treated for 24 hours with either Co-BSA, AGE-BSA, or RPMI/0.1%FCS. TNF-α-stimulated cells (10 ng/ml TNF-α for two hours) were used as a positive control for NFκB activation. EMSA of nuclear extracts was performed as previously described (27). In competition assays, the 100-fold molar excess of unlabelled oligonucleotides (NFκB consensus and mutant oligonucleotides, Santa Cruz Biotech) were added 30 min prior to the addition of a labelled probe.

The following sequences were used:

NFκB consensus 5'-AGTTGAGGGG-ACTTTCCCAGGC-3',

NFκB mutant 5'-AGTTGAGGCG-ACTTTCCCAGGC-3',

For the supershift an antibody against NFκB p65 (Santa Cruz Biotech) was added to the reaction 30 min before the addition of the labelled probe. The protein-DNA complexes were resolved on 6% polyacrylamide gel in TBE-buffer.

NFκB transactivation assay

To test whether AGE-mediated NFκB activation leads to target gene binding and activation in living cells, osteoblasts were transfected with the pNFκB-Luc reporter plasmid (Clontech, Mountain View, CA, USA) together with the pSV-β-galactosidase plasmid (Promega, Madison, WI, USA). The pNFκB plasmid contains multiple copies of the κ enhancer fused to the herpes simplex virus thymidine kinase promoter. Binding of NFκB to the κ enhancer results in activation of the luciferase reporter gene. Transfection was performed using the Neon™ transfection system consisting of the transfection kit and the Neon device (Invitrogen, Carlsbad, CA, USA) according the manufacturer’s guidelines. The transfected cells were suspended into RPMI/10%FCS, plated on six-well plates and incubated overnight without antibiotics. The following day, cells were stimulated with Co-BSA or AGE-BSA for 24 hours. Using the Luciferase reporter assay system (Promega) and the Luminescent β-gal Reporter System 3 & Detection Kit II (Clontech), luciferase and β-galac-

tosidase activities were measured with a luminometer (Infinite M200, Tecan, Männedorf, Switzerland). Luciferase activities were normalised to the corresponding β-galactosidase levels.

Statistical analysis

All data are reported as means ± SEM. Statistical analysis was performed using SPSS 15 for Windows (SPSS, Chicago, IL, USA). Results were analysed with the Kruskal-Wallis test followed by the Mann-Whitney U-test. P-values <0.05 were considered significant.

Results

Characterisation of osteoblasts and AGE uptake

The presence of marker proteins for osteocalcin and alkaline phosphatase was determined by immunohistochemical staining (Fig. 1A). The AGE-BSA treated cells showed intensive intracellular staining for CML and imidazolone, two representative members of the AGE family, indicating AGE-BSA uptake into the cytoplasm of the osteoblasts (Fig. 1B). This observation was confirmed by western blotting. AGE-BSA treated osteoblasts showed a strong accumulation of intracellular CML as well as imidazolone compared to the cells treated with Co-BSA. The intensively stained bands corresponded to either monomeric (~70 kDa) or oligomeric forms of AGE-BSA (>200 kDa), a result of crosslink formation during the glycation process (Fig. 1C).

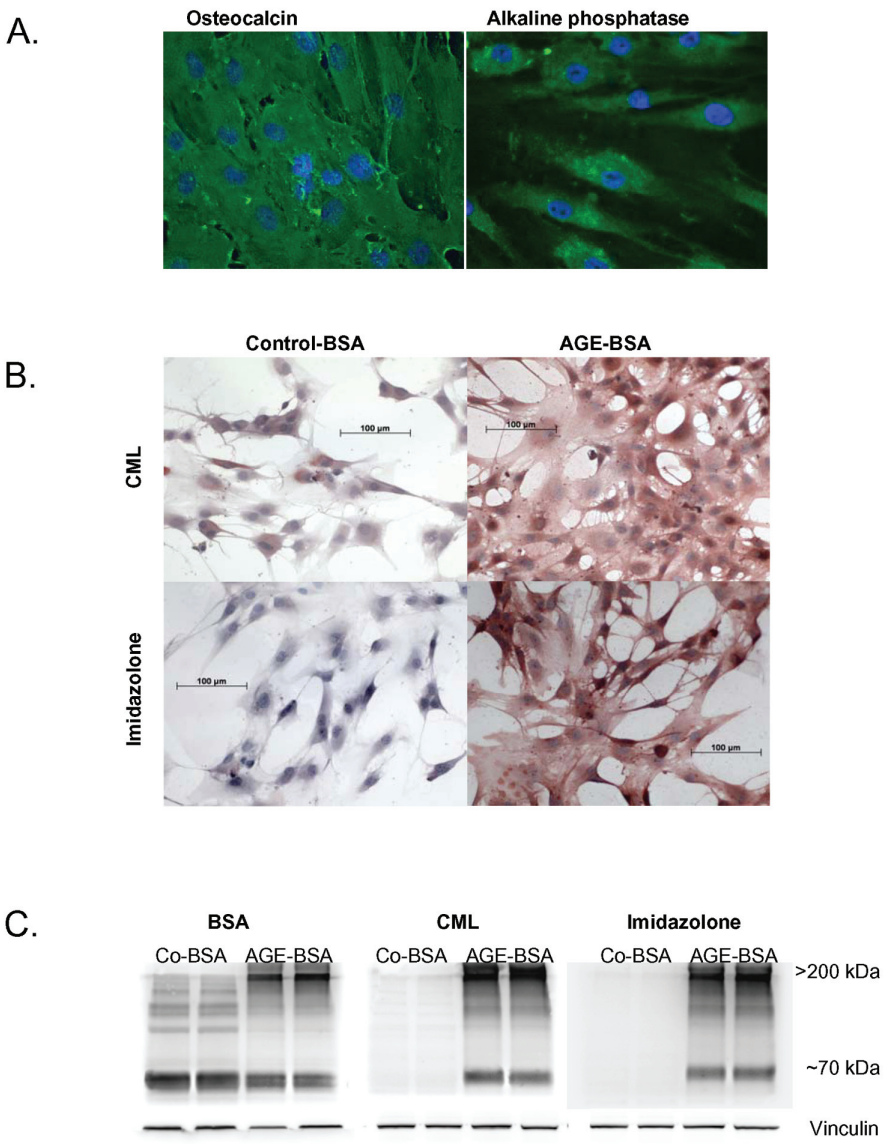

Fig. 1. A. Immunohistochemical staining of human osteoblasts cultured from bone tissues. Cultured cells stained positive for their characteristic proteins osteocalcin and alkaline phosphatase. **B**. Osteoblasts were stimulated with Co-BSA or AGE-BSA (5 mg/ml) for 24 hours. The intensive intracellular staining for CML and imidazolone in AGE-BSA treated cells in comparison to Co-BSA suggests active uptake of AGEs. **C.** Western blot for BSA, CML and imidazolone. Osteoblasts treated with AGE-BSA had substantially more CML and imidazolone-modified protein than cells incubated with Co-BSA. The intensively stained bands corresponds to either monomeric (~70 kDa) or oligomeric forms of AGE-BSA (>200 kDa), a result of crosslink formation during the glycation process.

Cell proliferation, cell viability, cell cycle and evaluation of cell death

Survival of the osteoblasts was affected by AGE-BSA. As shown in Figure 2A, the total number of osteoblasts was significantly reduced from day two to day seven after AGE-BSA treatment. Moreover, AGE-BSA significantly suppressed DNA synthesis as well as the metabolic activity, reflecting decreased proliferation and viability (Fig. 2B).

Cell cycle analysis revealed that the total number of cells in the subG_1+G_1 phase was significantly higher after AGE-BSA stimulation than after Co-BSA treatment. In contrast, the number of cells grown in the presence of AGE-BSA in the S+G_2 phase was significantly lower compared to Co-BSA treated osteoblasts (Fig. 2C).

After two days of AGE-BSA incubation, a significant decrease of vital cells was accompanied by a significant increase of necrotic and late apoptotic cells. An increase in AGE-induced early apoptotic cells could not be detected (Fig. 2D).

RAGE and NFκB p65 expression

As shown in Figures 3A and B, in comparison to Co-BSA, RAGE mRNA and protein expression in the AGE-BSA-stimulated cells were significantly upregulated after two days. mRNA expression of NFκB p65 was also significantly increased (Fig. 3C), resulting in a substantially higher protein expression (Fig. 3D).

NFκB p65 activation

To investigate NFκB activation in the Co-BSA and AGE-BSA-treated osteoblasts, nuclear extracts were analysed by EMSA. Nuclear extracts of TNF-α-

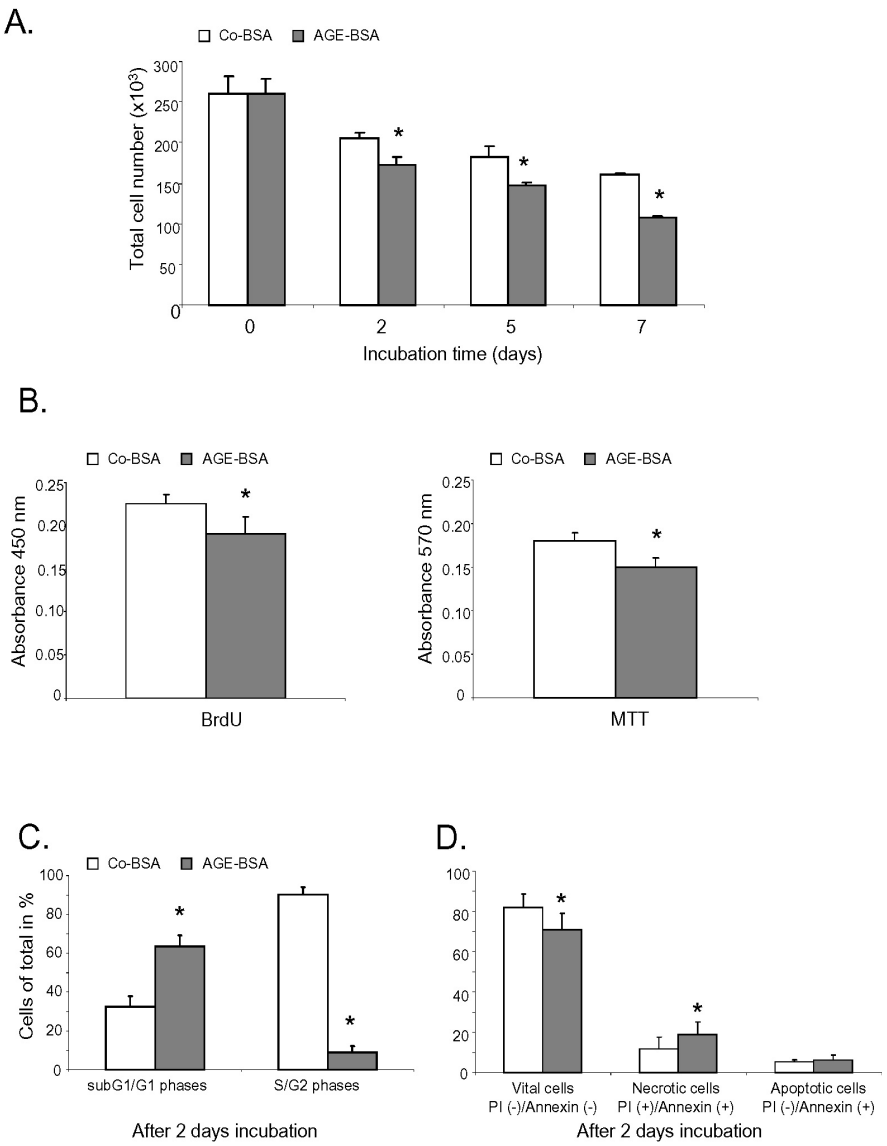

Fig. 2. A-C: Cell proliferation, cell viability, cell cycle analysis, and evaluation of cell death.
A. Total cell number. Treatment by AGE-BSA significantly reduced total cell number after two to seven days in comparison with Co-BSA (*$p \leq 0.01$, n=6). **B**. Cell proliferation and viability. Incubation of osteoblasts for 24 hours with AGE-BSA significantly reduced cell proliferation as measured by incorporation of BrdU ($p<0.01$, n=6), as well as metabolic activity determined by the MTT assay (*$p<0.001$, n=6). **C**. Cell cycle analysis. Compared to Co-BSA, two-days treatment with AGE-BSA significantly increased the percentage of cells in the subG1 and G1 phases, whereas the respective levels in the S and G2 phases decreased (*$p<0.01$, n=9). **D**. Quantification of cell death. Two-day incubation of cells with AGE-BSA significantly reduced the percentage of vital cells (annexin-V and propidiumiodide negative) and increased the number of necrotic cells (annexin-V and propidiumiodide positive) (*$p<0.05$, n=9). The percentage of apoptotic cells (annexin-V positive and propidiumiodide negative) was not significantly influenced by AGE-BSA treatment.

activated cells served as a positive control. To demonstrate the specificity of the assay, aliquots of the control nuclear extracts were incubated with unlabelled oligonucleotides as competitors (NFκB cold, NFκB mutant cold). As shown in Figure 3E, DNA-binding was reduced in the presence of the cold NFκB probe, but not with NFκB mutant oligonucleotides. AGE-BSA, but not Co-BSA treatment or stimulation with 0.1% FCS resulted in NFκB activation and the formation of NFκB-DNA complexes. The specificity of the NFκB binding was confirmed by supershifts using the anti-NFκB p65 antibody.

To confirm the AGE-BSA-mediated NFκB activation in the living cells, a reporter plasmid containing multiple copies of the κ enhancer was transfected into two osteoblast cell lines. After transfection, cells were incubated for 24 hours with either Co-BSA or AGE-BSA. As shown in Figure 3F, the luciferase activity normalised to β-galactose activity was significantly higher in both investigated cell lines in the AGE-BSA-treated cells compared to the Co-BSA treated cells.

Expression of TNF-α and MMP-1

As NFκB activates transcription of proinflammatory cytokines such as TNF-α, and this cytokine increases the expression of matrix metalloproteinase 1 (MMP-1) (30), we next tested for expression of TNF-α as well as MMP-1.

As shown in Figure 4A, the mRNA expression of TNF-α was significantly upregulated on days one and two after AGE-BSA treatment compared to Co-BSA. This was confirmed by TNF-α protein release into the culture supernatants as determined by ELISA assay. On day two, significantly increased TNF-α levels were found in the media of AGE-BSA-stimulated osteoblasts compared to Co-BSA (AGE-BSA *versus* Co-BSA stimulation: 11.1±1.0 *versus* 6.3±1.1 pg/10^5 cells). The relative increase of TNF-α release in the media of the AGE-BSA treated cells in comparison to Co-BSA is shown in Figure 4B.

The observed AGE-induced upregulation of TNF-α was accompanied by dramatically increased levels of MMP-1 (Fig. 4C and D). In comparison to Co-BSA, the MMP-1 mRNA as well as protein expression was significantly higher during the seven-day treatment period with AGE-BSA.

Expression of Col1, OC and ALP

To investigate whether AGEs affect the osteogenic potential of osteoblasts, the mRNA and protein expression of the bone formation markers Col1, OC and ALP were determined. As shown in Figure 5A, seven-day treatment with AGE-BSA compared to Co-BSA downregulated the mRNA expression of all measured osteogenic genes with significant decreases at day two and seven for Col1, and from day one to seven for OC and ALP. Western blot analysis confirmed the real-time PCR results demonstrating significantly re-

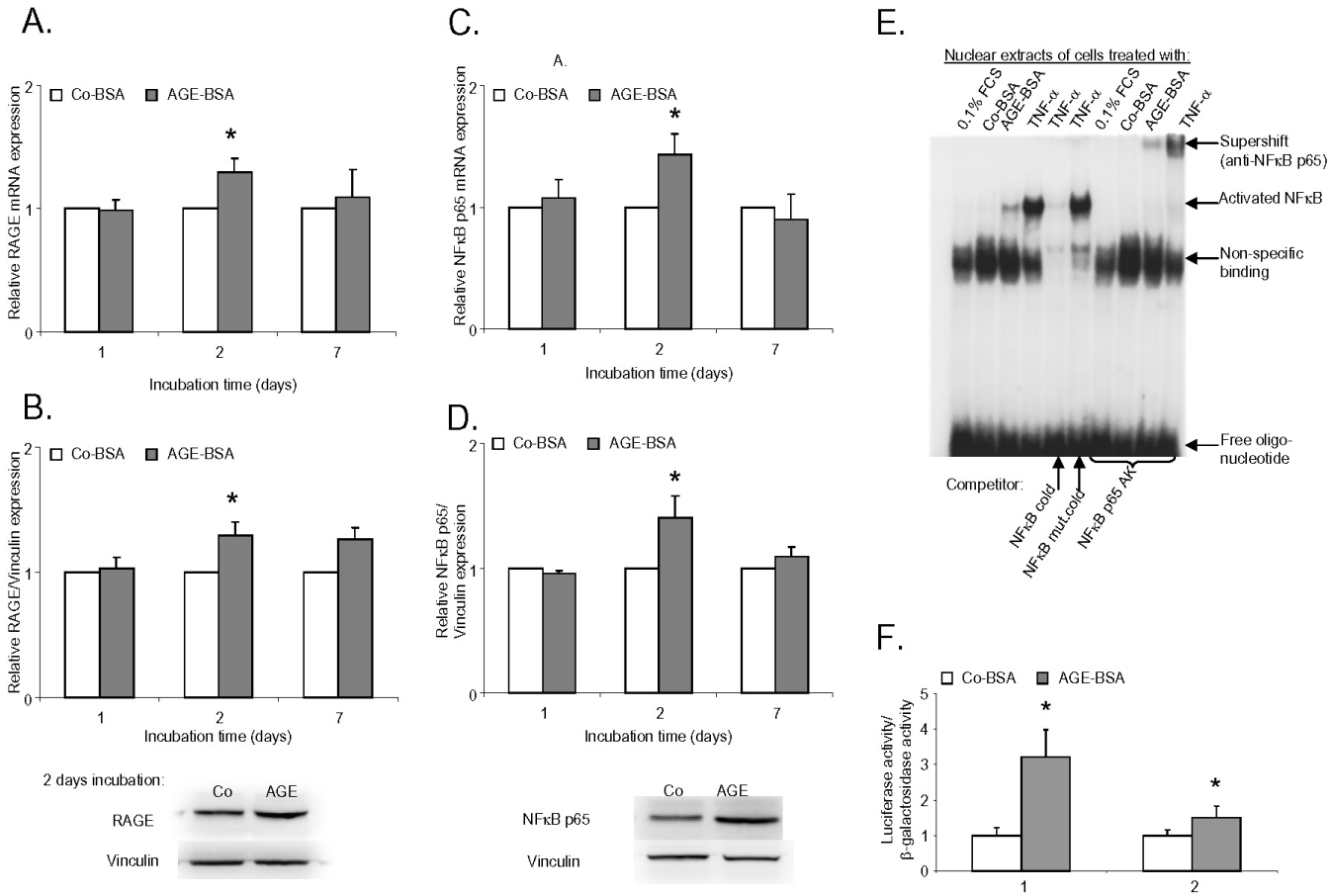

Fig. 3. A-D: RAGE and NFκB expression, NFκB activation.
A. RAGE mRNA expression. RAGE mRNA expression was significantly higher after two-day treatment with AGE-BSA (*p=0.02, n=18). **B**. RAGE protein expression. As determined by western blot, AGE-BSA significantly induced RAGE protein expression after two days (*p<0.05, n=6). As an example, a representative western blot is shown. **C**. NFκB p65 mRNA expression. mRNA expression of NFκB p65 subunit was also significantly increased after two days of AGE-BSA incubation (*p<0.05, n=10). **D**. NFκB p65 protein expression. AGE-BSA in comparison with Co-BSA significantly stimulated p65 protein expression two days after stimulation (*p<0.05, n=6). A representative western blot is given. **E**. EMSA for NFκB. Nuclear extracts of osteoblasts stimulated either with 0.1% FCS, Co-BSA or AGE-BSA for 24 hours were investigated. Nuclear extracts of TNF-α-activated cells served as positive control. To demonstrate the specificity of the assay, aliquots of the control nuclear extracts were incubated with the indicated unlabelled oligonucleotides as competitors. The DNA-binding was reduced in the presence of cold NFκB probe, but not with NFκB mutant oligonucleotide. AGE-BSA, but not Co-BSA treatment or stimulation with 0.1% FCS resulted in NFκB activation and the formation of NFκB-DNA complexes. The specificity of NFκB binding was confirmed by supershifts using the NFκB p65 antibody. The shown EMSA is representative of three independent experiments with similar results. **F**. Transactivation of NFκB. A luciferase reporter plasmid containing consensus binding sites for NFκB was transfected in two different osteoblast lines. AGE-BSA significantly enhanced NFκB transactivation compared with Co-BSA (*p<0.01, n=6).

duced protein levels for Col1, OC and ALP, respectively (Fig. 5B).

Expression of RANKL and osteoprotegerin

Osteoblasts regulate bone resorption by producing the osteoclastogenic cytokines RANKL and osteoprotegerin (31). Consequently, we also investigated whether expression of these cytokines were influenced by AGE-BSA. Relative to Co-BSA, AGE-BSA treatment significantly increased RANKL mRNA and protein expression on days one and two (Fig. 6A and B). The osteoprotegerin mRNA expression as well as the respective protein expression was found to not be significantly influenced by AGE-BSA treatment (Fig. 6C and D).

Discussion

Osteogenesis may be impaired in the elderly by the accumulation of advanced glycation end products in bone tissue and their effects on osteoblasts. Molecular mechanisms of AGEs are difficult to investigate *in vivo*. Here we rely on cell cultures of human osteoblasts and not on animal cell lines in an *in vitro* model. As AGEs accumulate in the bone tissue, they are in close proximity to osteoblast cells. To mimic *in vivo*, we used AGE-BSA which is described to have a high affinity to RAGE (32). As glycated BSA is modified by a variety of AGE structures and its complexity resembles *in vivo* generated forms, a physiologically plausible model is approximated.

We investigated the effect of AGE-BSA on cell proliferation and viability, on the expression of a complex set of bone formation parameters (ALP, OC, Col1) and bone resorption parameters (RANKL/osteoprotegerin, TNF-α, MMP-1) as well as the activation of the RAGE-NFκB pathway. To the best of our knowledge, up to now very few studies are available that have addressed this issue.

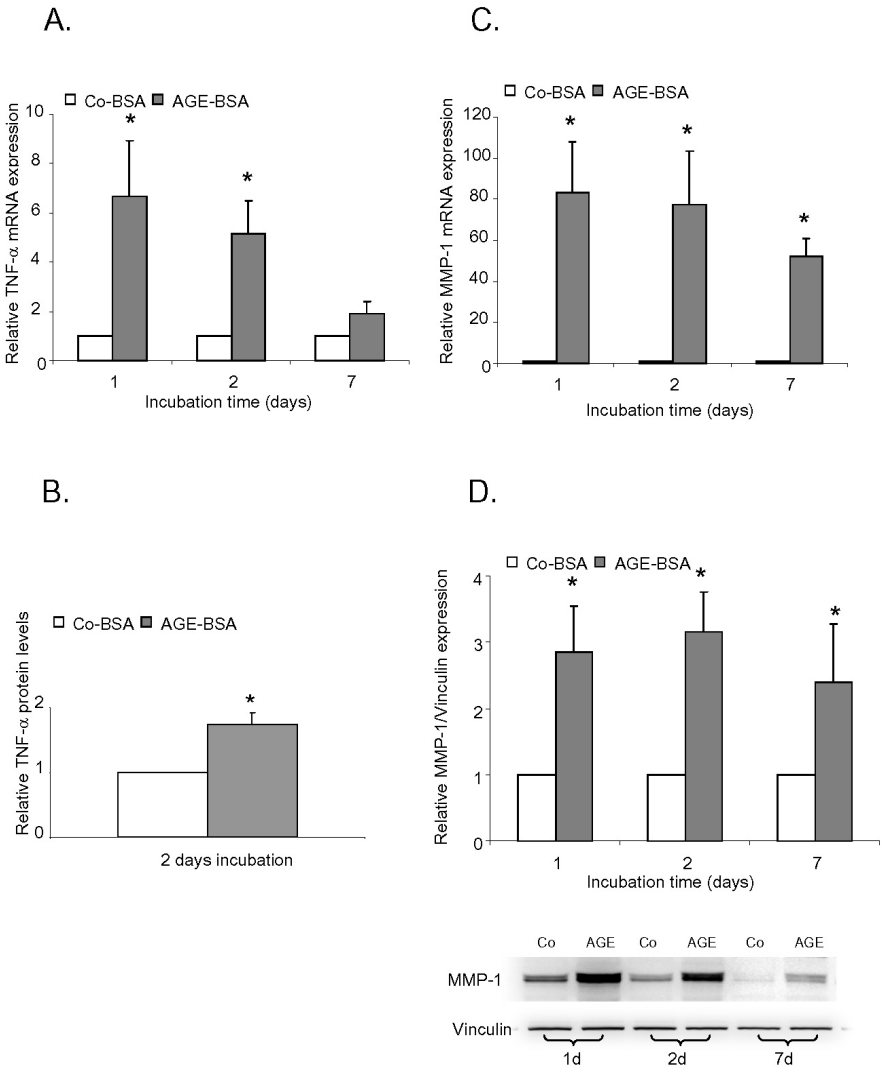

Fig. 4. Stimulation of TNF-α and MMP-1 by AGE-BSA.
A. TNF-α mRNA expression. Transcripts for TNF-α were significantly increased after one and two days (*p<0.01, n=16). **B.** TNF-α protein release. Stimulation of osteoblasts with AGE-BSA released significantly more TNF-α into the culture supernatants than treatment with Co-BSA. Concentrations of TNF-α in the supernatant were normalised to cell number (*p<0.01, n=6). **C.** MMP-1 mRNA expression. As compared to Co-BSA, the MMP-1 mRNA expression of AGE-BSA stimulated cells was significantly higher during the seven-day treatment period (*p≤0.01, n=11). **D**. MMP-1 protein expression. The MMP-1 protein expression was significantly elevated as demonstrated by western blot (*p<0.02, n=6). A representative blot is shown.

Our study demonstrates that AGE-BSA is actively incorporated by osteoblasts. As described recently, AGE-BSA uptake into the cytoplasm occurs via internalisation of RAGE after ligand binding. Moreover, the internalisation of the AGE-RAGE complex was shown to be essential for mediating cellular activation in response to AGEs (33).

Our findings that AGEs induced a decrease in osteoblast proliferation and viability support the recent similar results which have been reported for a variety of cells including mouse and rat osteoblast-like cells, as well as human synovial fibroblasts (12, 13, 19, 27). Our study also showed that AGE-BSA-mediated cell cycle arrest resulted in necrotic cell death which was accompanied by a loss of osteoblasts. This observation agrees with our previous investigations of synovial fibroblasts and podocytes (27, 34). Cell cycle arrest in these cells was combined with AGE-induced upregulation of the cell cycle inhibitor protein $p27^{Kip1}$. A similar association has also been reported for human osteoblasts (35).

In contrast to our study, AGE-mediated apoptosis has been recently described for human osteoblasts (24). In that study, long-term low dose exposure to CML-modified collagen (7 days) stimulated markedly higher increases in the number of apoptotic cells than short-term stimulation with higher doses (24 hours). The increase in apoptosis and not necrosis, as found in our experiments, may be caused by the use of CML collagen, which is exclusively modified by CML and not by a variety of AGE structures. However both studies found that the AGE-induced reduction in the number of osteoblasts contributes in general to reduced bone formation in elderly individuals.

As of today only little is known about the effect of AGEs on the expression of RAGE and the activation of NFκB in osteoblasts. AGE-BSA-induced upregulation of RAGE has been documented for animal osteoblastic linage cells (15, 18, 20, 21), and for human osteoblasts in our preliminary investigation (25). A decrease in RAGE gene expression was observed when osteoblasts were stimulated with pentosidine which was, in contrast to AGE-BSA, not bound to a protein (9). AGE-BSA induced NFκB activation has been shown for mouse osteoblastic cells by Takagi *et al*. (6). Activation of NFκB caused by stimulation of osteoblasts with CML-modified collagen has been reported by Alikhani and colleagues (24).

In light of the complex role of osteoblasts in bone remodelling, the present study also examined the impact of AGEs on the RAGE-NFκB axis as well as on the expression of osteogenic and osteoclastogenic markers. In line with the above mentioned literature (6, 15, 18, 20, 21, 24, 25), upregulation of RAGE together with NFκB p65 activation could be demonstrated. To evaluate the functional impact of this interaction on the transcription of proinflammatory cytokines, the expression of TNF-α as a representative downstream mediator was determined. Significantly increased mRNA and protein levels were found, indicating an inflammatory response of osteoblasts due to stimulation with AGEs. Moreover, TNF-α, described as an osteoclastogenic cytokine, stimulates osteoclast formation as well as the expression of matrix met-

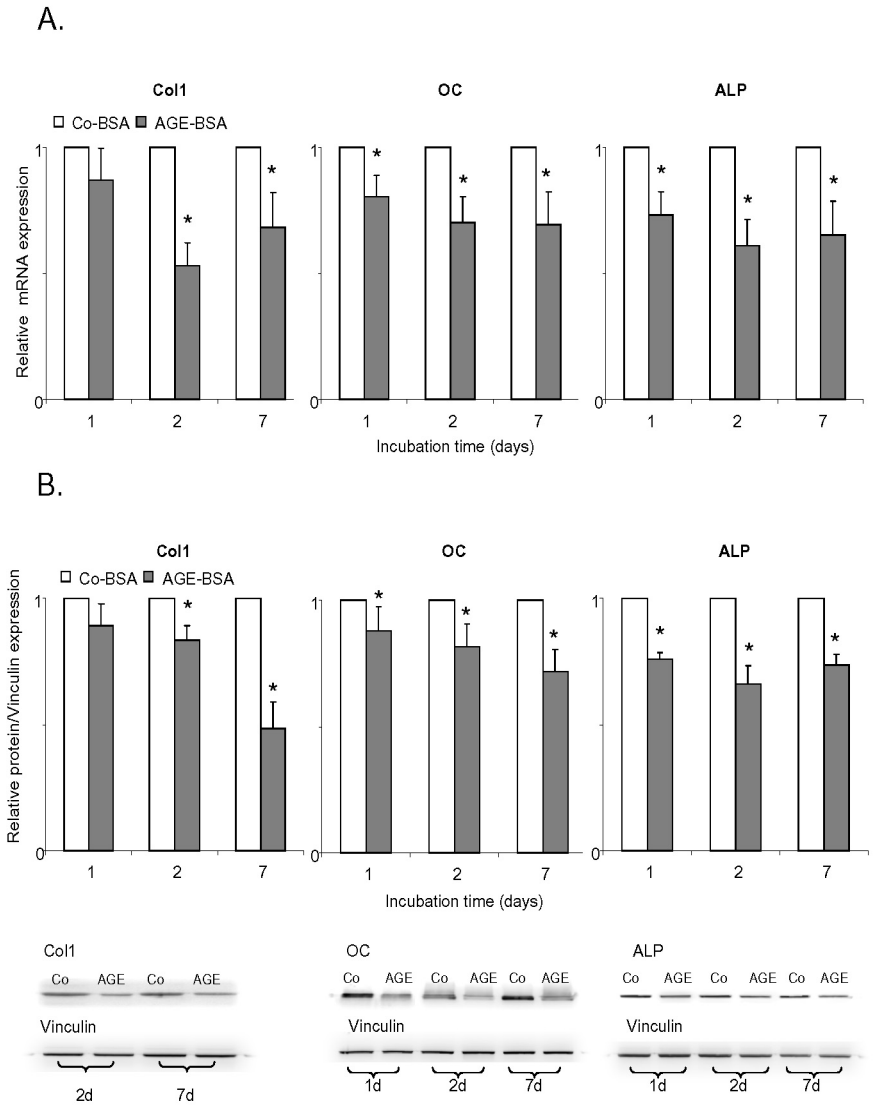

Fig. 5. Inhibition of osteogenic genes and protein expression.
A. mRNA expression. Incubation of osteoblasts with AGE-BSA downregulated the mRNA expression of the osteogenesis-related genes collagen type I (Col1), osteocalcin (OC) and alkaline phosphatase (ALP). The measured decreases reached significance at days two and seven for Col1 (*p<0.02, n=15), and from day one to seven of AGE-BSA treatment for OC (*p<0.04, n=15) and ALP (*p<0.02, n=15).
B. The protein expression of Col1, OC and ALP was lower in AGE-BSA treated cells than in Co-BSA stimulated osteoblasts (*p<0.03, n=6). Examples of representative blots are given.

alloproteinases such as MMP-1 (30, 36). We could clearly show that the observed AGE-induced upregulation of TNF-α in our study was accompanied by substantially increased expression levels of MMP-1. MMPs secreted from osteoblasts degrade the unmineralised osteoid that lines the bone surface and expose RGD adhesion sites within the mineralised bone that are necessary to facilitate osteoclast attachement (10).
RANKL and osteoprotegerin are the master regulator cytokines in osteoclastogenesis. Whereas RANKL is the essential factor for osteoclast formation, activation and survival, osteoprotegerin is its soluble decoy receptor and counteracts these effects (31). In the present investigation we showed for the first time that AGE-BSA treatment of osteoblasts significantly increased the RANKL mRNA and protein expression, but affected osteoprotegerin only marginally, which may possibly reflecting an influence in favour of osteoclastogenesis. TNF-α and IL-1 are among the factors that can stimulate RANKL expression (37). Whether AGE-induced upregulation of RANKL in our study reflects a primary effect, or is a secondary effect due to the elevated expression of proinflammatory cytokines such as TNF-α, needs further investigation.
It is worth noting however, that as the RAGE gene promoter region contains NFκB binding sites, the activation of NFκB upregulates RAGE gene expression (38); as the binding of AGEs to RAGE activates NFκB, TNF-α has the ability to induce RAGE expression through activation of NFκB (39), and this suggests a self-perpetuating cycle among AGEs, RAGE, NFκB signalling, and cytokines.
In contrast to an upregulation of osteoclastogenic factors, the present study clearly shows that AGE-BSA treatment of osteoblasts downregulates the expression of the bone formation markers Col1, OC and ALP. These findings concur with previous data (9, 11, 22) and indicate that AGEs contribute to impaired bone formation. Similar effects have been shown for TNF-α, which is generally recognised as contributing to decreased bone mineral density by inhibiting osteoblast differentiation and bone formation. Inhibitory effects of TNF-α on the synthesis of Col1 and OC have also been reported (40, 41). We have shown that the AGE-RAGE interaction in osteoblasts leads to NFκB activation and on the other hand increases the expression of TNF-α. Thus, the AGE-RAGE interaction itself, the upregulation of TNF-α, and both combined in a complex network with NFκB as a key regulator (*e.g.* by sharing NFκB downstream signalling pathway), may induce diminished expression of matrix proteins and disturbed mineralisation.
In summary, human osteoblasts *in vitro* are affected by AGEs in a very complex way. The present investigations demonstrate that AGEs modulate cell proliferation and expression of genes involved in bone formation as well as bone resorption processes. Thus, the study provides evidence that AGEs accumulated in the bone matrix have the potential to inhibit osteogenic and to promote osteoclastogenic properties of osteoblasts *in vivo*. This may reflect a molecular mechanism by which AGEs trigger disturbed remodelling leading to functional and structural impairment of bone.

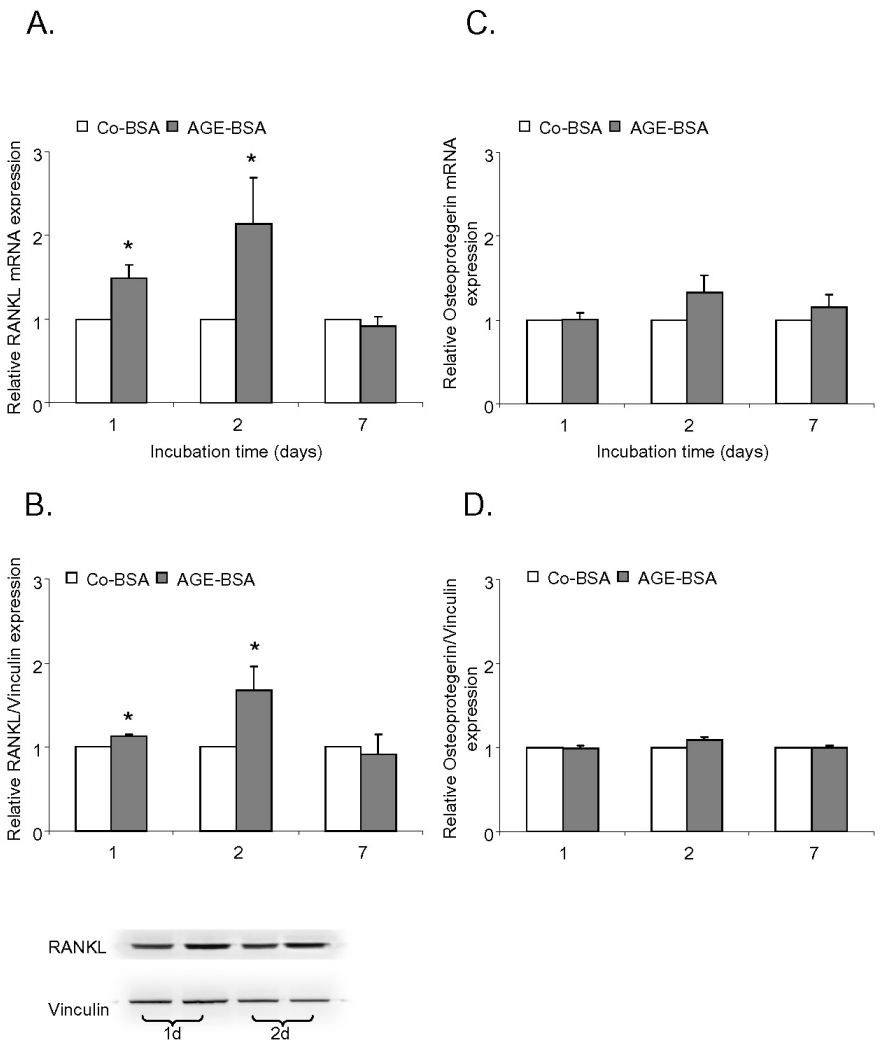

Fig. 6. Stimulation of RANKL and osteoprotegerin expression in osteoblasts by AGE-BSA.
A. RANKL mRNA expression. In comparison to Co-BSA, AGE-BSA significantly induced RANKL mRNA expression at days one and two of treatment (*p<0.05, n=18). **B.** RANKL protein expression. Quantification of western blots demonstrated a significant increase of RANKL protein expression (*p<0.05, n=8). As an example, a representative western blot is shown. **C.** Osteoprotegerin mRNA expression. The osteoprotegerin mRNA expression as well as the respective protein expression (**D.**) was found to not be significantly influenced by AGE-BSA treatment.

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