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Regulation of advanced glycation end product (AGE) receptors and apoptosis by AGEs in osteoblast-like cells

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Abstract Advanced glycation end products (AGEs) have been proposed as the pathological mechanisms underlying diabetic chronic complications. They may also play a role in the pathogenesis of diabetic osteopenia, although their mechanisms of action remain unclear. We investigated the protein (immunofluorescence) and gene expression (real-time RT-PCR) of two receptors for AGEs, RAGE and galectin-3, as well as their regulation by AGEs, and the apoptotic effect on osteoblast-like cells (UMR106 and MC3T3E1) in culture. AGEs up-regulated the expression of RAGE and galectin-3 in both cells lines. These effects were accompanied by an increase in the corresponding mRNA in the non-tumoral MC3T3E1 but not in the osteosarcoma UMR106 cells. Finally, we demonstrated that a 24 h exposure to AGEs induced apoptosis in both cell lines. Thus, AGEs-receptors may play important roles in the bone alterations described in aging and diabetic patients.

Keywords Advanced glycation end products · RAGE · Galectin-3 · Osteoblasts · Apoptosis · AGE-receptors · Regulation

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Introduction

Advanced glycation end products (AGEs) result from non-enzymatic reactions of carbohydrates and oxidized lipid with proteins [1]. AGEs induce alterations in the structure and function of different proteins. The accumulation of AGEs is a characteristic feature of the tissues in aged people, especially in patients with diabetes mellitus, and these products have been proposed as the pathological mechanisms underlying diabetic chronic complications. Some biological effects of AGEs may result from the cross-linking of matrix and other proteins [2]. AGEs can also act through the activation of several surface receptors/binding proteins, expressed in a wide range of cells including smooth muscle cells, monocytes, macrophages, endothelial cells, astrocytes, microglia, fibroblasts, and osteoblasts [3]. RAGE is the best characterized AGE receptor [4] and responsible for most of the deleterious effects of AGEs, whereas the scavenger receptor and galectin-3 (AGE-R3) are probably implicated in the removal of AGEs [4]. In addition, galectin-3 has been involved in the regulation of apoptosis since it behaves as an anti-apoptotic agent in several systems, although its precise mechanism of action is unknown [5].

Accumulation of AGEs increase stiffness of the collagen network in bone, which may explain some of the reported age-related increase in skeletal fragility and fracture risk [6]. AGEs may also play a role in the pathogenesis of diabetic osteopenia, although their mechanisms of action remain unclear. One mechanism through which AGEs may contribute to pathological processes is by enhanced apoptosis, as supported by in vitro and in vivo studies [7–9]. It has recently been reported that carboxymethyl lysine modified collagen injected in vivo, stimulated apoptosis in calvarial periosteal cells compared to unmodified collagen

[9]. CML-collagen also induced apoptosis in osteoblasts in culture. AGEs may enhance apoptosis indirectly through increasing oxidative stress, or via increased expression of pro-apoptotic cytokines [10, 11].

We previously showed that soluble and matrix-associated AGEs can modulate osteoblast growth and differentiation [12, 13]. These effects are probably mediated by an increase in intracellular oxidative stress and/or the regulation of insulin-like growth factor I/binding proteins production [14, 15]. We have also reported that osteoblastic cell lines express detectable levels of RAGE [16]. Treatment of osteosarcoma-derived UMR106 cells for 24 h leads to a dose-dependent increase in RAGE expression, as determined by Western blot. On the contrary, in proliferating MC3T3E1 pre-osteoblasts, 24–72 h exposure to AGEs does not modify the expression of this AGE receptor [16]. MC3T3E1 and UMR106 cells also express AGE-R3/galectin-3, although its expression is about 15-fold lower in the osteosarcoma UMR106 line. Western blot analysis in both cell lines shows that galectin-3 is mainly present in the cytoplasm and in minor amounts in the microsomal fraction, and that considerable proportion is secreted into the culture medium. After incubation with AGEs for 48 h, cells show increased intracellular levels of galectin-3 while its release to the culture media is decrease [17].

Since osteoblasts play important roles in the maintenance of skeletal mass, the accumulation of AGEs in the bone may have detrimental effects, in part by increasing the expression of their specific receptors and by inducing osteoblast apoptosis. To address this issue, we investigated the protein and gene expression of RAGE and galectin-3, as well as their regulation by AGEs, and the apoptotic effect on osteoblast-like cells in culture.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, and fetal bovine serum (FBS) were from Gibco, (Life Technology, Buenos Aires, Argentina) and tissue culture disposable material was obtained from Nunc (Buenos Aires, Argentina). Centricon 10 kDa cutoff filter cartridges were purchased from Amicon Inc. (Beverly, MA, USA). Annexin V-FITC, Annexin V-PE, and propidium iodide (PI) were from Molecular Probes (Eugene, OR). BSA, ribose, Triton X-100, goat anti-rabbit IgG-FITC were from Sigma (St.Louis, MO, USA). Goat polyclonal anti-RAGE was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). A rabbit polyclonal anti-galectin-3 antiserum was made against recombinant galectin-3, and immunoglobulins purified on a Protein A-Sepharose column (Ahmed, H. and Vasta, G.R., unpublished). The specificity of the galectin-3 antibody was confirmed by the identification of a single band corresponding to galectin-3 in MC3T3E1 cell extract (see Results, Fig. 4A-Inset). Vectashield-DAPI mounting media was from Vector Laboratories, Inc. (Burlingame, CA, USA). All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

Preparation of AGEs

AGEs were prepared as we described previously [11]. Briefly, AGE-BSA was prepared by incubating under sterile condition, 10 mg/ml BSA with 100 mM ribose in 150 mM phosphate-buffered saline (PBS), pH 7.4 at 37°C for 3 weeks. Ribose was used as the glycating sugar instead of glucose to speed up non-enzymatic glycosylation. Control BSA was incubated in the same conditions without sugar. Unbound sugar was removed by centrifugation/filtration with centricon filter cartridges. Preparation was tested for endotoxin using an E-Toxate kit and was found to be below the limit of detection (<0.5 U/ml). The formation of AGEs was assessed by their characteristic fluorescence-emission maximum at 420 nm upon excitation at 340 nm [12]. The estimated levels of AGE-BSA obtained in this in vitro incubation were 18.5% relative fluorescence intensity/mg protein, in contrast to 3.2% for control-BSA.

Cell culture and experimental treatments

UMR106 rat osteosarcoma cells and MC3T3E1 mouse calvaria-derived cells were grown in high glucose-DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin, at 37°C in a 5% CO_2 atmosphere [12]. Cells were seeded on 75 cm^2 flasks, sub-cultured using trypsin-EDTA, and replated on multi-well plates. The UMR106 cell line has been shown to conserve certain characteristics of a differentiated osteoblastic phenotype [18]. In the case of non-transformed MC3T3E1 line, the cells only undergo active replication during the first 5 days of incubation [19]. Cells were incubated in serum-free DMEM with several doses of control unmodified BSA or AGE-BSA for the periods of time indicated in the legends of the figures. For immunofluorescence studies, cells were seeded onto glass coverslips and incubated as described before.

Evaluation of cell death

The mechanism of cell death induced by AGEs was evaluated using an annexin V-FITC/propidium iodide (PI) assay as previously described [20]. Early apoptotic stages were characterized by annexin V-FITC-positive/PI-negative

Table 1

Gene	Forward primer	Reverse primer
Mouse RAGE	5′CCTTGACCTGTGCCATCTCT3′	5′GGGTGCACCATCCTTTATCCA3′
Rat RAGE	5′CCCTGACCTGTGCCATCTCT3′	5′GGGTGTGCCATCTTTTATCCA3′
Mouse galectin-3	5′CAGGAAAATGGCAGACAGCTT3′	5′CCCATGCACCCGGATATC3′
Rat galectin-3	5′CAGGAAAATGGCAGACGGCTT3′	5′CCCATGCACCAGGCCATC3′
18S rRNA	5′CGGCTACCACATCCAAGGGAA3′	5′GCTGGAATTACCGCGGCT3′

(V^+/PI^-) staining. Cell numbers were determined by counting the cells per field in 30 fields per coverslip.

Alternatively, apoptosis was also evaluated by flow cytometric analysis using Annexin V conjugated to phycoeritrin (PE). After treatment with BSA or AGE-BSA, cells were washed with PBS, trypsinized. The pellets obtained by centrifugation were suspended in 300 μl of binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Cells were stained with 2 μl Annexin V-PE by incubation for 30 min at room temperature in the dark. The apoptosis ratio (Annexin V positive cells/total cells) was analyzed quantitatively using FACCalibur (BD) and CellQuest software.

Immunofluorescence microscopy

Sub-confluent osteoblasts grown on glass coverslips were washed in PBS, fixed with 4% paraformaldehyde in PBS (10 min at room temperature), and permeabilized with methanol for 4 min at −20°C [17]. Non-specific binding sites were blocked with 1% BSA in PBS for 2 h. Cells were then incubated with rabbit polyclonal anti-galectin-3 or goat polyclonal anti-RAGE antibodies (1:100 in blocking buffer) overnight at 4°C. After washing, cells were exposed to a goat anti-rabbit or rabbit anti-goat IgG-FITC (1:200) for 1 h at room temperature. Cells were mounted in 80% glycerol in PBS and observed under a fluorescence microscope.

Quantitative real-time RT-PCR measurements of gene expression

Total RNA was extracted from cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions. The quantity and integrity of RNA was determined spectrophotometrically (280 and 260 nm). Total RNA (2 μg) was used in the RT-PCR reaction. The RNA was converted to single stranded DNA with 200 units of M-MLV reverse transcriptase (Promega) in 25 μl of reaction mixture containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 μg random hexamers (Gibco 48190-011), 10 mM of each dNTP (Invitrogen), and 20 units RNAse inhibitor (Promega). The

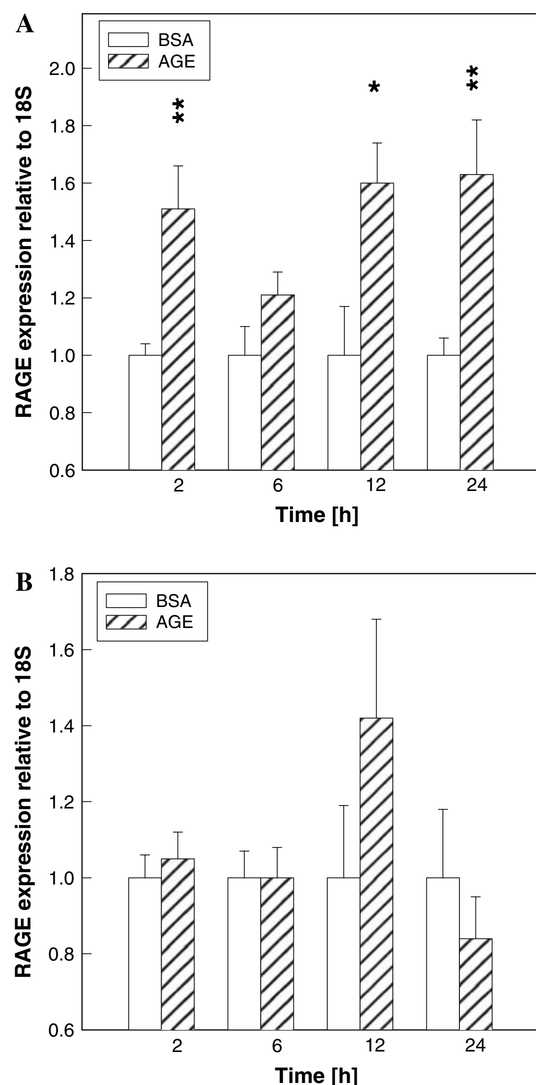

Fig. 1 Effect of AGEs treatment on RAGE mRNA expression in MC3T3E1 (**A**) and UMR106 (**B**) osteoblast-like cells. Total RNA was extracted from osteoblasts at specified time intervals after treatment with 200 μg/ml of unmodified BSA or AGEs-BSA. RAGE mRNA expression was assessed by real time RT-PCR and normalized to 18S. Bars represent mean ± SEM derived from three independent experiments performed in sextuplicate. Differences versus BSA are: $*P < 0.05$, $**P < 0.01$

mixture was incubated at 37°C for 30 min, then at 42°C for 30 min and at 70°C for 10 min. The cDNA was used as a template for amplification in a polymerase chain reaction (PCR) using sequence-specific primers (Invitrogen) for RAGE, galectin-3, and 18S ribosomal RNA. cDNA primer sequences are shown in Table 1. Real time PCR on a ABI Prism 7700 Sequence Detection System (PE Biosystems) was performed utilizing a SYBR Green PCR core reagents detection system (Applied Biosystems). PCR was performed with an initial step of 2 min at 50°C, then 10 min at 95°C followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The levels of RAGE and galectin-3 expression were normalized to 18S. In each series of PCR reactions, the standards used were dilutions of a pool of total RNA extract from osteoblasts samples (16, 8, 4, 2, 1, 0.5, 0.25, and 0 ng of total cDNA/25 µl reaction tube).

Statistical analysis

Three independent experiments were run for each experimental condition. Results are expressed as the mean ± SEM. Statistical analysis of the data was performed by Student's *t* test. A *P* value < 0.05 was considered significant for all statistical analyses.

Results

AGEs regulate RAGE mRNA and protein expression

MC3T3E1 and UMR106 osteoblastic cells were exposed to 200 µg/ml AGE-BSA or control unmodified-BSA. Expression of RAGE mRNA was analyzed by real time RT-PCR in cells exposed to AGEs during 6–24 h. In the MC3T3E1 pre-osteoblastic line, AGE-BSA significantly up-regulated the RAGE mRNA transcript over basal levels (Fig. 1A). Real time PCR performed on RNA extract of UMR106 osteosarcoma cells showed that expression of RAGE was not altered after 6–24 h treatment with 200 µg/ml AGEs (Fig. 1B). The expression of RAGE protein was analyzed in both osteoblastic lines by immunofluorescent staining of RAGE with a specific antibody. RAGE-

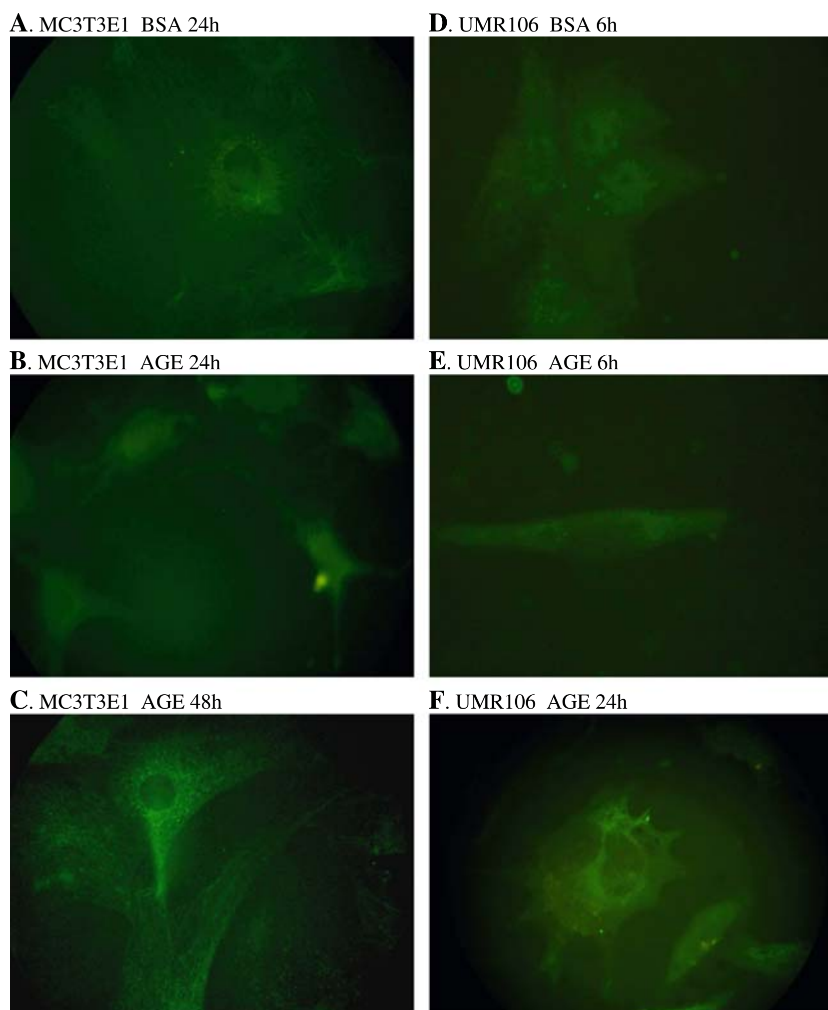

Fig. 2 RAGE-associated immunofluorescence in MC3T3E1 (**A–C**) and UMR106 (**D–F**) cells. Osteoblasts were cultured in DMEM with 200 µg/ml of unmodified BSA or AGEs-BSA for different periods of times. Cells were fixed and stained with an anti-RAGE antibody followed by FITC-conjugated secondary antibody. Specimens were examined by fluorescence miscroscopy, Obj 100×

associated immunofluorescence was present in both cell lines with a diffuse staining pattern (Fig. 2). Treatment of MC3T3E1 cells for 6–24 h with 200 μg/ml AGEs did not modify in this pattern (Fig. 2A, B). However, longer incubations of these cells with AGEs (48–72 h) revealed a clear increase in the immunofluorescence of RAGE (Fig. 2C). On the other hand, short-term (6–24 h) AGE-treatment of UMR106 cells induced an increase in RAGE associated fluorescence (Fig. 2D–F).

Effect of AGEs on Galectin-3 mRNA and protein expression

Galectin-3 transcript levels were analyzed after treating cells with control BSA or AGE-BSA for 2–24 h. In the MC3T3E1 pre-osteoblasts, 200 μg/ml AGE significantly increased the levels of galectin-3 mRNA (Fig. 3A). In contrast, the expression of this AGE-receptor in the UMR106 osteosarcoma line remained unchanged after incubation with AGE (Fig. 3B). The expression of galectin-3 protein was analyzed in both osteoblastic lines by immunofluorescent staining of galectin-3 with a specific antibody. Galectin-3 was present in both osteoblastic lines, mainly associated with the cytoplasm (Fig. 4). In MC3T3E1 osteoblasts, 200 μg/ml AGE-BSA increased galectin-3 associated immunofluorescence after 24–48 h culture (Fig. 4A–C). AGE-BSA treated UMR106 cells also showed an increase in galectin-3 immunofluorescence when cells were incubated for 6–24 h.

In vitro induction of apoptosis by AGEs

Studies were carried out to assess potential pro-apoptotic effects of AGEs on osteoblastic cells. Apoptosis of rat osteosarcoma UMR06 and mice pre-osteoblastic MC3T3E1 cells was measured by annexin-V binding to externalized phosphatidyl serine by immunofluorescence microscopy. After 24 h incubation, AGE-BSA increased the proportion of apoptotic cells in both osteoblastic cell lines. This effect was doses-dependent, and varied with the cell line investigated. An aliquot of 100 and 200 μg/ml AGE-BSA significantly increased the number of apoptotic cells in the MC3T3E1 line (160% and 156% of BSA control, Fig. 5A). In the UMR106 cells, AGE-BSA induced apoptosis in a dose-response manner with maximal effect noted at 500 μg/ml (205% of BSA control, $P < 0.02$; Fig. 5B).

Concurrent with examination by microscopy, the exposure of phosphatidyl serine on plasma membrane cells treated with 200 μg/ml unmodified-BSA or AGE-BSA was also evaluated by flow cytometry. In control treated-UMR and -MC3T3E1 cells, the mean apoptotic population was 10.5% and 9.7% respectively, which increased to 18% and 21% after 24 h treatment with AGE-BSA (Fig. 5C).

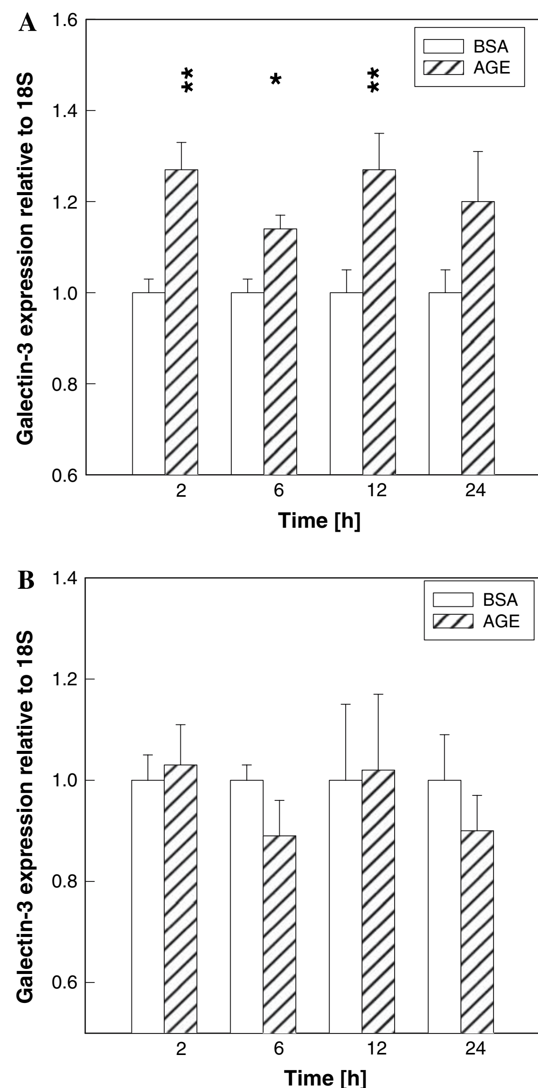

Fig. 3 Galectin-3 mRNA expression in MC3T3E1 (**A**) and UMR106 (**B**) osteoblast-like cells. Total RNA was extracted from osteoblasts at specified time intervals after treatment with 200 μg/ml of unmodified BSA or AGEs-BSA. Galectin-3 mRNA expression was assessed by real time RT-PCR and normalized to 18S. Bars represent mean ± SEM derived from three independent experiments performed in sextuplicate. Differences versus BSA are: $^{*}P < 0.01$, $^{**}P < 0.001$

Discussion

AGEs play an important role in the pathophysiological processes affecting patients with Diabetes mellitus, Alzheimer's disease, and aging. Some of these AGEs structures display toxic bioactivities initiated by the interaction with different receptors. In previous studies we have shown that osteoblasts express two types of AGE receptors:

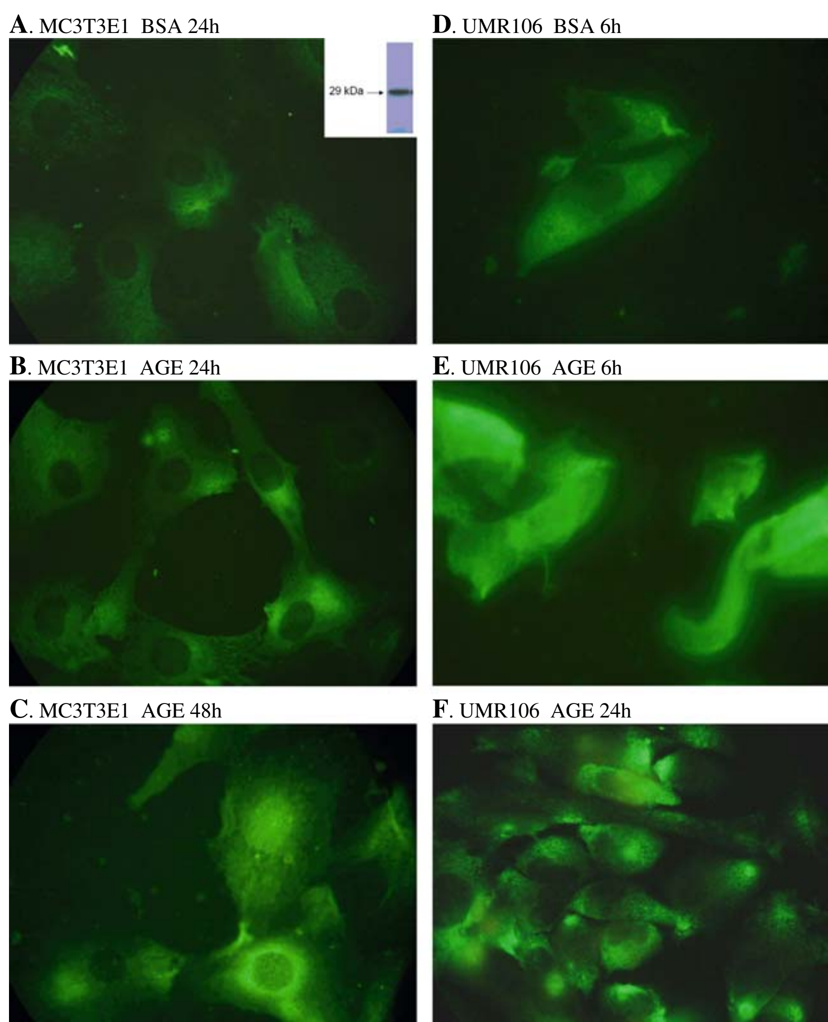

Fig. 4 Galectin-3-associated immunofluorescence in MC3T3E1 (**A**–**C**) and UMR106 (**D**–**F**) cells. Osteoblasts were cultured in DMEM with 200 μg/ml of unmodified BSA or AGEs-BSA for different periods of times. Cells were fixed and stained with an anti-Galectin-3 antibody followed by FITC-conjugated secondary antibody. Specimens were examined by fluorescence miscroscopy, Obj 100×. A-Inset: Specificity of anti-gal3 antibodies. Crude extract from mouse calvaria-derived osteoblasts (MC3T3E1) was tested with the anti-galectin-3 antibodies on western blot that resulted single band

RAGE and AGE-R3/galectin-3 and that the expression of these proteins is regulated by the levels of AGEs.

To further investigate the mechanisms through which AGEs up regulate their receptors, we examined the effect of AGEs on RAGE and galectin-3 mRNA expression by use of real time RT-PCR and the expression of the AGE-receptor proteins by immunofluorescence. In MC3T3E1 preosteoblasts, AGEs significantly increased both RAGE mRNA (Fig. 1A) and protein expression. However, the timing of protein expression (Fig. 2A–C) was delayed 24 h in this cell line in comparison with mRNA levels. Similar doses of AGEs have been previously shown to inhibit the proliferation and differentiation of osteoblasts and to engage AGE-binging sites [12–14]. In previous studies using western blot, we showed that in MC3T3E1 cells, RAGE protein levels remain unmodified after 24 h treatment with AGE-BSA [17]. Our present results also shown that RAGE protein was up-regulated in UMR106 osteosarcoma cells incubated with AGEs for 6–24 h (Fig. 2D–F), in agreement with our previous observation of RAGE expression as assessed by western blot [16]. However, this effect was not accompanied by an enhancement of RAGE mRNA expression in this cell line (Fig. 1B). Forbes [21] also found up-regulation of RAGE protein expression in diabetic kidney; real time RT-PCR, however, showed no significant difference in renal RAGE expression between control and diabetic groups. Thus, the up-regulation of the RAGE protein has to be explained by other mechanisms, such as the refolding of the protein, intracellular re-distribution, or other posttranslational events.

The process of bone remodeling is altered in the development of osteoporosis, and the AGE-RAGE interaction can play a role in bone loss associated with diabetes and aging [22]. Further, AGEs are present in osteoporotic bone specimens [23] and increase with aging [24]. These effects could be mediated by the RAGE present in osteoblasts.

Galectin-3, another receptor for AGEs, has been proposed to participate in the clearance of toxic AGE structures via endocytosis, as well as some biological

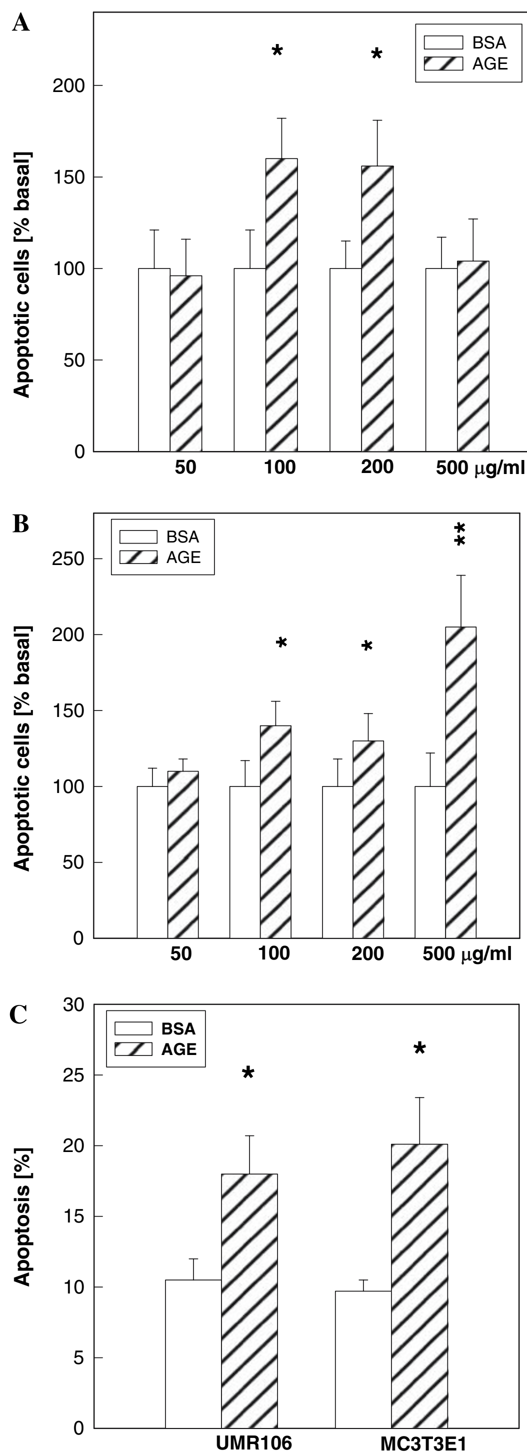

Fig. 5 AGEs-induced apoptosis in MC3T3E1 (**A**) and UMR106 (**B**) osteoblasts. Cells were incubated in serum-free DMEM with 50–500 (**A** and **B**) or 200 μg/ml (**C**) of control unmodifed-BSA or AGEs-BSA for 24 h. Apoptosis was evaluated by the method of FITC-Annexin-V-PI (**A** and **B**) and by flow cytometry using Annexin-V-PE as described in Materials and methods. Each bar represents the mean ± SEM of 3–4 independent experiments. Differences versus BSA are: $*P < 0.05$, $**P < 0.02$

effects of AGEs [25, 26]. In the present study, we showed that in MC3T3E1 osteoblasts galectin-3 transcript levels were increased by 2–12 h incubation with AGEs (Fig. 3A). Further, and in agreement with our previous study by western blot [17], galectin-3 protein levels were also enhanced by 24–48 h treatment with AGEs, as assessed by immunofluorescence (Fig. 4A–C). Although galectin-3 mRNA expression was not modified by the presence of AGEs in UMR106 osteosarcoma cells, galectin-3 protein expression was enhanced after 6–24 h (Fig. 4D–F). This is in agreement with our previous results for galectin-3 measured by western immunoblot after incubating UMR106 cells with AGEs for 48 h [17].

Finally, we demonstrated that a 24 h exposure to AGEs induced apoptosis in both cell lines (Fig. 5). Previously we shown that 24 h of incubation with 200 μg/ml AGE-BSA induced a decrease in IGF-I and an increase in IGFBPs secretion into the media [15], which can be associated with the increment in the proportion of apoptotic MC3T3E1 cells observed in the present study (Fig. 5A). The AGEs-induced temporal regulation of AGE receptors in the MC3T3E1 line, suggest that galectin-3 could be responsible for the early effects of AGEs in this cell line, whereas RAGE could be involved in the long-term detrimental effects of AGEs (ROS production, inhibition of growth, and differentiation). However, additional studies using neutralizing antibodies or shut-down genes for RAGE and galectin-3 are needed in order to verify this hypothesis.

In our previous studies with UMR106 cells, 24 h exposure to AGEs resulted in increase in cell proliferation, alkaline phosphatase expression, and ERK activation [12, 13, 16]. However, chronic exposure of UMR106 cells to AGEs induced a decrease in cell proliferation, inhibition of alkaline phosphatase and enhancement of ROS production [13]. In this cell line, both RAGE and galectin-3 receptors appear to be involved in the AGEs-induced effects. However, since galectin-3 expression is substantially lower than RAGE expression in UMR106 cells, RAGE could be the most physiologically relevant AGEs-receptor in these cells.

In conclusion, we provide evidence for direct effects of AGEs on the regulation of gene and protein expression of RAGE and galectin-3 in osteoblastic cells, showing that the AGEs are able to enhance their expression, and to increase apoptosis in osteoblasts. These observations, together with our previous reports, suggest that these AGEs-receptors may play important roles in the bone alterations described in aging and diabetic patients.

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