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Advanced glycation end-products (AGEs) induce concerted changes in the osteoblastic expression of their receptor RAGE and in the activation of extracellular signal-regulated kinases (ERK)

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Abstract

An increase in the interaction between advanced glycation end-products (AGEs) and their receptor RAGE is believed to contribute to the pathogenesis of chronic complications of Diabetes mellitus, which can include bone alterations such as osteopenia. We have recently found that extracellular AGEs can directly regulate the growth and development of rat osteosarcoma UMR106 cells, and of mouse calvaria-derived MC3T3E1 osteoblasts throughout their successive developmental stages (proliferation, differentiation and mineralisation), possibly by the recognition of AGEs moieties by specific osteoblastic receptors which are present in both cell lines. In the present study we examined the possible expression of RAGE by UMR106 and MC3T3E1 osteoblastic cells, by immunoblot analysis. We also investigated whether short-, medium- or long-term exposure of osteoblasts to extracellular AGEs, could modify their affinity constant and maximal binding for AGEs (by ^{125}I-AGE-BSA binding experiments), their expression of RAGE (by immunoblot analysis) and the activation status of the osteoblastic ERK 1/2 signal transduction mechanism (by immunoblot analysis for ERK and P-ERK). Our results show that both osteoblastic cell lines express readily detectable levels of RAGE. Short-term exposure of phenotypically mature osteoblastic UMR106 cells to AGEs decrease the cellular density of AGE-binding sites while increasing the affinity of these sites for AGEs. This culture condition also dose-dependently increased the expression of RAGE and the activation of ERK. In proliferating MC3T3E1 pre-osteoblasts, 24–72 h exposure to AGEs did not modify expression of RAGE, ERK activation or the cellular density of AGE-binding sites. However, it did change the affinity of these binding sites for AGEs, with both higher- and lower-affinity sites now being apparent. Medium-term (1 week) incubation of differentiated MC3T3E1 osteoblasts with AGEs, induced a simultaneous increase in RAGE expression and in the relative amount of P-ERK. Mineralising MC3T3E1 cultures grown for 3 weeks in the presence of extracellular AGEs showed a decrease both in RAGE and P-ERK expression. These results indicate that, in phenotypically mature osteoblastic cells, changes in ERK activation closely follow the AGEs-induced regulation of RAGE expression. Thus, the AGEs-induced biological effects that we have observed previously 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. (Mol Cell Biochem **000**: 000–000, 2003)

Key words: advanced glycation end-products, receptor for advanced glycation end-products, extracellular signal-regulated kinases, osteoblasts, bone

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Introduction

The accumulation of advanced glycation-end-products (AGEs) has been postulated to be involved in ageing, Alzheimer's disease and the chronic complications of Diabetes mellitus [1, 2]. AGEs, which are formed in part as a consequence of the non-enzymatic glycosylation of proteins, can be taken up and removed by specific receptors present in a variety of cell types. The occupation of these receptors by AGEs may also induce alterations in cell homeostasis by various mechanisms, which include regulation of cell surface molecules (tissue factor, V-CAM, I-CAM), of proteases or their inhibitors (MMPs, PAI-1), of extracellular matrix protein production (collagen types I and IV, fibronectin, vitronectin), or of cytokine and growth factor secretion (IL-1 and –6, IGF-I, PDGF, VEGF, to name a few). Three classes of AGE-receptors have been identified to date: RAGE (receptor for AGEs), a 45–50 kDa member of the immunoglobulin superfamily of proteins; the AGE-receptor-complex, which consists of oligosaccaryl-transferase-48 (OST-48), 80K-H (an 80–87 kDa substrate for protein kinase C) and galectin-3 (a 30–35 kDa S-lectin); and the class A macrophage scavenger receptor [3–5].

In particular, RAGE-mediated recognition of AGEs has been shown to regulate various cellular processes. This pathway is initiated by AGE-RAGE interaction, and can be followed by oxidation of $p21^{ras}$ through locally produced reactive oxygen species (ROS), recruitment of phosphatidyl-inositol-3′-kinase (PI3K), activation of extracellular signal-regulated kinases (ERK), nuclear translocation of the transcription factor NF-κB, and finally regulation of the expression of tissue-specific subsets of NF-κB-inducible genes. Interestingly, the gene which encodes for RAGE protein possesses cis-acting recognition elements for NF-κB in its promoter region. Thus, long-term activation of RAGE by its ligands – as could occur with diabetic AGE-accumulation – has been postulated to up-regulate the expression of this receptor, thereby exacerbating its cellular effects [3].

Our group and other investigators have shown that AGE-modified proteins can exert direct effects on osteoblast-like cells in culture [6–12]. In particular, we have found that soluble AGE-proteins regulate osteoblastic growth in a biphasic manner: while initially eliciting an increase in cellular proliferation and differentiation, a relatively long-term incubation of these cells with AGE-modified proteins induces a significant decrease in both parameters of osteoblast development [9]. These effects are probably mediated by membrane-associated AGE-specific receptors, which we have recently described in osteoblastic cells [13]. The affinity constant of these AGE-receptors is differentially regulated throughout the successive stages of osteoblastic development [13], and this is concordant with the maturation-dependent effects induced by AGE on osteoblasts in culture [9, 10, 14].

Recently we have also shown that the accumulation of AGE on collagen regulates osteoblastic growth, probably through the modulation of inducible and endothelial nitric oxide synthase (NOS) expression as well as by intracellular generation of reactive oxygen species (ROS) [14].

The object of this study was to further investigate the role of AGE receptors in the AGE-induced modulation of osteoblastic development. To achieve this aim, we initially performed AGE-binding experiments with osteoblasts which had been exposed either to control or AGE-modified proteins. This allowed us to evaluate possible AGE-induced changes both in the maximal binding of AGEs to cells, and in the global affinity constant of the osteoblastic AGE receptors/binding proteins. In other experiments, we assessed the expression of RAGE by osteoblast-like cells in culture. We also investigated the regulation of RAGE expression as a consequence of short-, medium- or long-term osteoblastic exposure to extracellular AGEs, as well as the involvement of ERK activation as a possible signal transduction pathway for the recognition of AGEs by receptors such as RAGE.

Materials and methods

Materials

Bovine serum albumin (BSA), ribose, glucose-6-phosphate (G6P), Iodo-Gen, Triton X-100, tri-chloro-acetic acid (TCA), prestained molecular weight standards, Kodak XAR-5 photographic film, Sephadex G-25 and G-50 and rat tail acid-soluble type I collagen were purchased from Sigma (St. Louis, MO, USA). Centricon 10 kDa cutoff filter cartridges were purchased from Amicon Inc. (Beverly, MA, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA and fetal bovine serum (FBS) were obtained from Gibco (Life Technology, Argentina). Tissue culture disposable material was from Nunc. Goat polyclonal anti-RAGE and anti-ERK1/2 antibodies, as well as a monoclonal anti-P-ERK antibody, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). ^{125}I was from New England Nuclear. All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

Preparation of AGE-modified type I collagen for cell matrices and of AGE-modified BSA

AGE-BSA was prepared as we have previously described [9]. Briefly, BSA was incubated for 3–6 weeks in sterile conditions in 150 mM phosphate-buffered-saline (PBS), pH 7.4, with 100 mM G6P or ribose at 37°C. G6P or ribose were used as glycating sugars instead of glucose, to speed up non-enzymatic glycosylation. Control BSA was incubated in the

same conditions without sugar. At the end of the incubation period, BSA and AGE-BSA were separated from non-covalently-bound low molecular weight molecules by centrifugation/filtration with Centricon filter cartridges. The formation of protein-AGEs was assessed by their characteristic fluorescence-emission maximum at 420 nm upon excitation at 340 nm [9]. Fluorescence values of test substances were expressed as percentage relative fluorescence. Thus, the estimated level of AGE-BSA obtained in this *in vitro* incubation was 18.5% relative fluorescence intensity/mg protein, as opposed to 3.2% for control BSA.

Type I collagen was solubilized in sterile 0.02 N acetic acid (2.5 mg/ml) (pH 3.0), poured into plastic dishes (50 μg/cm^2) and incubated overnight at 37°C. Collagen formed a thin film during incubation [7]. The film was washed with phosphate-buffered saline (PBS) and further incubated in PBS with or without 100 mM ribose at 37°C for 3 weeks in sterile conditions [15]. Finally, the plates were washed thoroughly with DMEM and the cells were plated for different experiments. AGE-formation on collagen was monitored by measuring AGE-specific fluorescence emission (356 nm excitation wavelength and 440 nm emission wavelength), as we have previously described [14]. After incubating 3 weeks with ribose, collagen contained 4-fold more AGE-associated fluorescence than control collagen incubated with PBS.

Cell culture

UMR106 rat osteosarcoma-derived cells were grown in 75 cm^2 plastic flasks at 37°C in a humidified 5% CO_2 atmosphere in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/L streptomycin. This cell line has been shown to conserve certain characteristics of differentiated osteoblastic phenotype [160]. After 5–7 days, cells were sub-cultured using trypsin/EDTA and replated on plastic, on control collagen or on glycated collagen to begin the experiments. MC3T3E1 non-transformed osteoblastic mouse calvaria-derived cells were grown at 37°C in 5% CO_2/DMEM/10% FBS and antibiotics, and passaged every 4–6 days. Previous studies have demonstrated that expression of osteoblastic markers begins after culturing these cells with medium supplemented by β-glycerol-phosphate and ascorbic acid [17, 18]. When cells are cultured in these conditions on collagen-coated plates, ALP begins to be expressed after 5 days. In addition, mineralisation is achieved with these cells after extending this culture to 20 days. However, the cells only undergo active replication during the first 5 days of incubation.

To study the effect of AGE-modifies proteins on UMR106 and MC3T3E1 osteoblasts, cells were grown under the conditions that we have previously reported [9, 13, 14] for the times indicated in each experiment.

Radiolabelling of AGE-BSA

AGE-BSA was iodinated with carrier-free ^{125}I by the Iodo-Gen method as we have previously described [13]. Briefly, 30 μg of AGE-BSA in PBS was incubated with 0.5 mCi carrier-free ^{125}I in an Iodo-Gen-coated vial at room temperature for 20 min. To separate free from bound ^{125}I, the sample was fractionated by Sephadex G-25 column cromatography, and the fraction corresponding to ^{125}I-AGE-BSA was further purified by a Sephadex G-50 50 ml column, until > 98% radioactivity was TCA precipitable. Specific radioactivity of labelled ^{125}I-AGE-BSA was between $1.1.10^4$ and $1.8.10^4$ cpm/ng protein.

^{125}I-AGE-BSA binding studies

Radioligand binding studies were carried out with UMR106 and MC3T3E1 osteoblastic cells, cultured on collagen- or AGE-collagen-coated 48-well plates in the conditions described in the figures. These displacement studies were performed in 0.25 ml binding medium (RPMI-1640, 25 mM Hepes, pH 7.4, 5 g/l BSA) at 4°C, as previously described [13]. Cell monolayers were pre-incubated for 30 min with increasing concentrations of unlabelled AGE-BSA (0–1000 μg protein/ml binding medium), following which 10^6 cpm of ^{125}I-AGE-BSA was added to each well. After incubating for 2 h at 4°C, the supernatant was aspirated and the cell layers were washed 3 times each with cold PBS/0.5% BSA, and cold PBS alone. Cell monolayers were then solubilized in 0.5 ml 0.1% Triton X-100, which was transferred to tubes for counting in a Packard PRIAS gamma counter. Non-specific binding of ^{125}I-AGE-BSA was determined in parallel incubations by adding an excess of unlabeled AGE-BSA. Specific binding was defined as the difference between total binding (cells incubated with radioligand and a known amount of unlabeled AGE-BSA) and non-specific binding (as defined above). Specificity of binding for AGE moieties was confirmed in certain incubations by adding an excess of control BSA.

Western blot analysis of RAGE and P-ERK

We next evaluated the expression of RAGE by both cell lines, the possible effect of an exposure to AGE-modified proteins on the expression of this receptor, as well as the activation status of the ERK 1/2 transduction pathway. In these experiments, osteoblastic cells growing for different periods of time on control or AGE-modified collagen matrices, or growing on plastic in serum-free medium with the addition of either BSA or AGE-BSA, were lysated in Laemmli's buffer [19] and

the protein content was evaluated by the method of Lowry [20]. These lysates were heated at 100°C for 3 min and 30 µg of protein subjected to 12% SDS-PAGE. The separated proteins were then transferred to nitrocellulose membranes. After washing and blocking, the membranes were incubated with anti-RAGE polyclonal antibodies, a monoclonal anti-P-ERK antibody, or a polyclonal antibody that recognizes both phosphorylated and unphosphorylated ERK1/2. Blots were developed using chemiluminescence reagents. The intensity of the specific bands was quantified by densitometry after scanning of the photographic film. Images were analysed using the Scion-beta 2 program.

Statistical analysis

Three independent experiments were run for each experimental condition. Results are expressed as the mean ± S.E.M. Statistical analysis of the data was performed by Student's *t*-test. Scatchard analysis [13] of the raw data from binding experiments was performed using the LIGAND program of NIH, in order to estimate receptor number and binding affinity constants.

Results

Binding of ^{125}I-AGE-BSA in osteoblastic cells

We have previously reported that UMR106 and MC3T3E1 cells can specifically bind, take up and degrade AGE-BSA in a saturable and time-dependent manner. In addition, we have shown that these osteoblastic cells possess membrane-associated 18–50 kDa receptors/binding proteins with affinity for ^{125}I-AGE-BSA [13]. To investigate the possible regulation of these receptors by osteoblastic exposure to AGE-modified matrices, cells were grown either on control collagen or on AGE-collagen for 72 h. Radioligand binding studies were then performed with both cell lines, using ^{125}I-AGE-BSA as specific ligand. Scatchard analysis of the data showed different results for each cell line (Fig. 1 and Table 1). In the case of the UMR106 osteosarcoma-derived cell line, exposure to extracellular AGEs halved the cellular density (Ro) of AGE-binding sites, but doubled the affinity constant (K_a) of these sites for AGEs. For the non-transformed MC3T3E1 preosteoblastic cells, a more complex effect was observed: the cells grown on AGE-modified collagen, apparently expressed two kinds of AGE-binding sites, which could be distinguished on the basis of their affinity for AGE. However, the total number of binding sites per cell was essentially the same as for MC3T3E1 cells grown on control collagen. These results suggest that the expression of various AGE-binding receptors could be modulated by the exposure of osteblast-like cells to extracellular AGE-modified proteins.

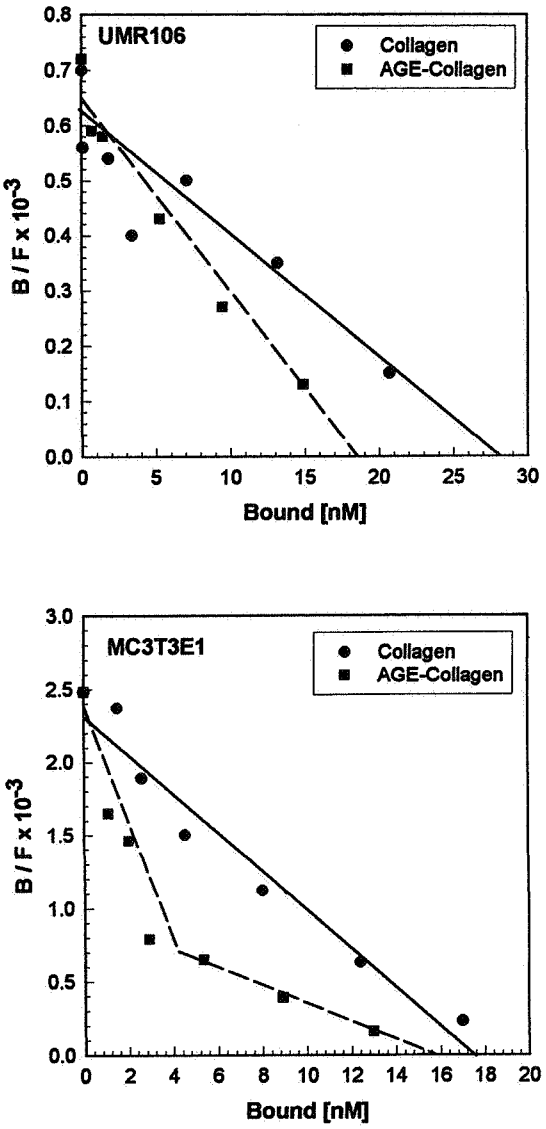

Fig. 1. Effect of AGE-modified collagen on ^{125}I-AGE-BSA binding to osteoblastic cells. UMR106 and MC3T3E1 cells were grown on collagen or AGE-collagen for 72 h. After this incubation period, ^{125}I-AGE-BSA binding was assayed as described in 'Materials and methods'. Scatchard plots for UMR106 and MC3T3E1 cells represent the mean of 3 independent experiments performed by triplicate.

Basal and AGE-modulated expression of RAGE in osteoblast-like cells

In other experiments with UMR106 and MC3T3E1 osteoblastic cells, we assessed the basal expression of RAGE

Table 1. Kinetic parameters resulting from the Scatchard analysis of binding data of ^{125}I-AGE-BSA to osteoblast-like cells

Cell type	Collagen matrix	K_a^1 (M^{-1})	K_a^2 (M^{-1})	Ro (sites/cell)
UMR106	Control	$0.82\ 10^5$		$2.5\ 10^7$
	AGE-modified	$1.80\ 10^5$		$1.2\ 10^7$
MC3T3E1	Control	$0.23\ 10^6$		$5.1\ 10^7$
	AGE-modified	$1.7\ 10^6$	$0.11\ 10^6$	$4.0\ 10^7$

Data represent the mean of 3 independent experiments. KA – affinity constant; Ro – number of sites/cell.

by immunoblot analysis. The expression of RAGE was also measured after culturing both cell lines for 24 h in the presence of different concentrations of BSA or AGE-BSA. Representative Western blots with RAGE-immunoreactive bands are shown in Fig. 2. The antibody recognized a major band of approximately 50 kDa, which is consistent with the results of other authors [21]. After 24 h, AGE-BSA significantly increased RAGE protein expression in UMR106 osteosarcoma cells, in a dose-response manner (Fig. 2A). However, no AGE-induced difference in RAGE expression was observed in the proliferating MC3T3E1 pre-osteoblasts, when they were submitted to the same conditions (Fig. 2B).

To further investigate the expression of RAGE protein in the different developmental stages of the MC3T3E1 line, these cells were grown either on control or AGE-modified collagen for various periods of time. The immunoblotting of MC3T3E1 cellular lysates (Fig. 3A), obtained after having cultured the cells on control or glycated matrices for 24 h (proliferating pre-osteoblasts), 1 week (differentiated stage), or 3 weeks (mineralizing stage), indicated a development- and AGE-dependent regulation of RAGE expression in this cell line (Fig. 3). In the proliferating stage, cells expressed similar levels of RAGE when grown on control or AGE-modified collagen. These results are in agreement with the experiments indicated above, performed with MC3T3E1 pre-osteoblasts in the presence of BSA or AGE-BSA for 24 h (Fig. 2). However, when MC3T3E1 cells were cultured on AGE-collagen for 1 week, a significant increase was observed in the expression of RAGE. Finally, when cultures were extended to 3 weeks (mineralising osteoblasts), AGE-modified collagen induced a statistically significant decrease in the expression of RAGE (Fig. 3).

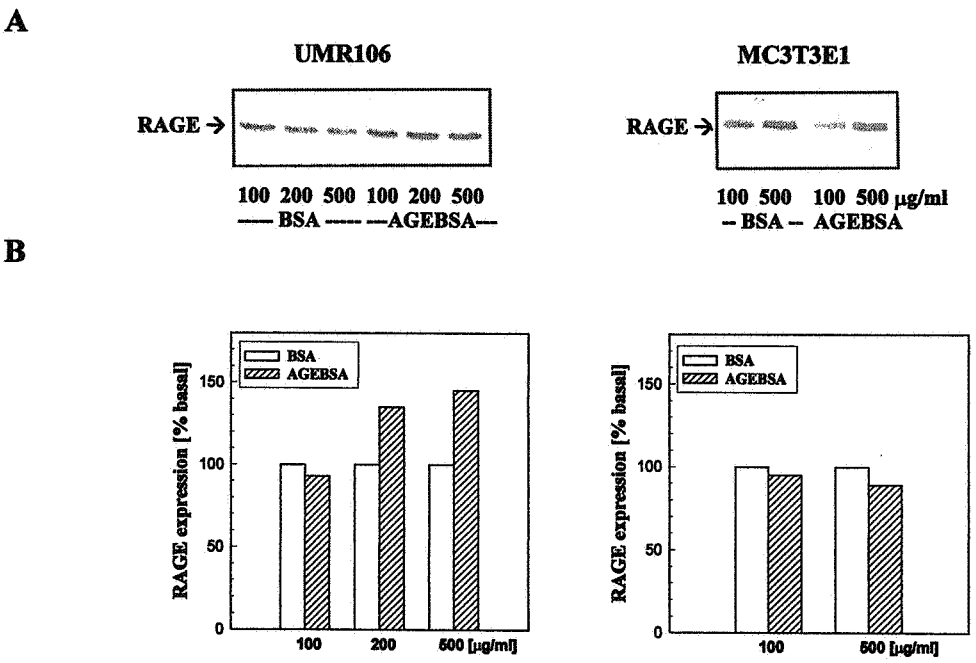

Fig. 2. Expression of RAGE in osteoblastic cells. UMR106 and MC3T3E1 osteoblasts were incubated with different concentrations of BSA or AGE-BSA for 24 h. After this incubation period, RAGE expression was analyzed by Western blot using a specific anti-RAGE antibody at a dilution of 1:1500. Blot were developed using a colorimetric reagent. (A) Bands corresponding to 50 kDa of RAGE protein. (B) Quantitation of RAGE protein was analyzed using the Scion-beta 2 program.

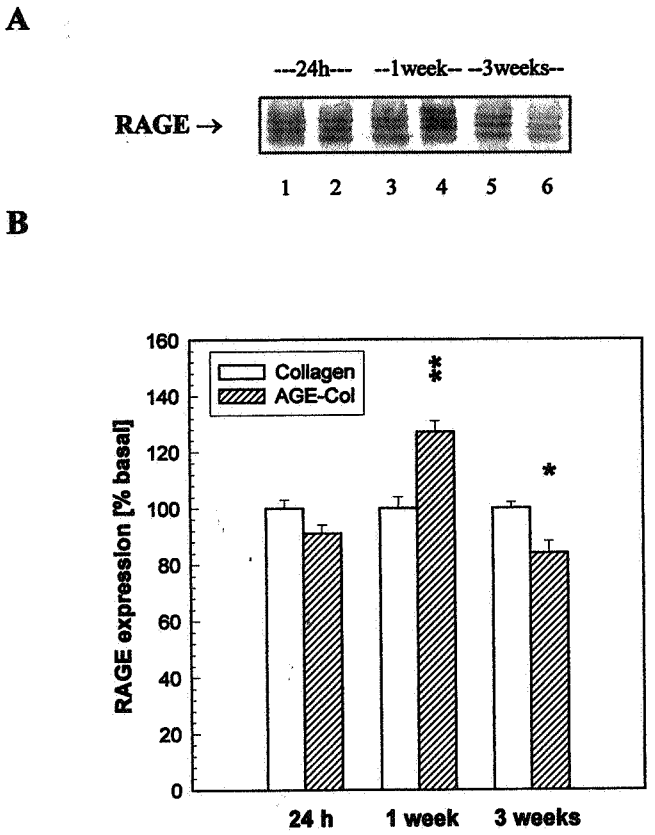

Fig. 3. Regulation of RAGE expression throughout the MC3T3E1 osteoblast developmental stages. MC3T3E1 cells were grown on collagen or AGE-modified collagen for 24 h, 1 week or 3 weeks. At the end of the culture period, RAGE expression was analyzed as described in 'Materials and methods'. (A) Bands corresponding to RAGE proteins were developed using a chemoluminescent method. Lanes corresponding to cells grown on collagen are 1, 3 and 5; lanes corresponding to cells grown on AGE-collagen are 2, 4 and 6. (B) Quantitation of RAGE protein was analyzed using the Scion-beta 2 program. Results are expressed as mean ± S.E.M. of 5 independent experiments. Differences between collagen and AGE-collagen are: *$p < 0.002$; **$p < 0.001$.

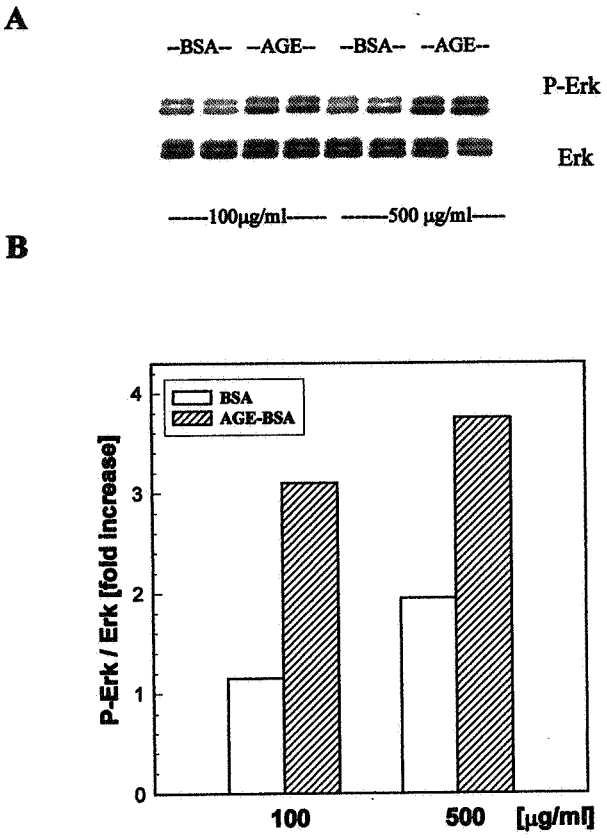

Fig. 4. Activation of ERK in osteoblast-like cells. UMR106 cells were grown for 24 h in the presence of different doses of BSA or AGE-BSA. Cells were lysated and extracts were submitted to Western blotting to analyze P-ERK and ERK expression. (A) Bands corresponding to 42/44 kDa proteins are indicated. (B) Quantitation of bands was carried out by densitometry and analyzed using the Scion-beta 2 program. The activation of ERK is expressed as the ratio between phosphorylated and unphosphorylated forms of ERK.

AGEs regulate ERK activation in osteoblast-like cells

To determine the activated status of ERK 1/2, Western blots with anti-phospho-ERK (P-ERK) and anti-ERK were performed, and the ratio of P-ERK to total ERK was determined for each experimental condition. As can be seen in Fig. 4, AGE-modified BSA induced the activation of ERK in UMR106 osteoblasts cultured for 24 h, in a dose-dependent manner. A similar pattern was observed in the AGE-induced up-regulation of RAGE expression (Fig. 2A).

In MC3T3E1 cells, AGE-induced regulation of ERK activation was found to be dependent on the stage of osteoblastic development (Fig. 5). In the proliferative (pre-osteoblastic) stage, no differences were observed in the relative amount of P-ERK when cells were grown on control or AGE-modified collagen for 24 h. However, after culturing the osteoblasts for 1 week, AGE-collagen increased the relative amount of P-ERK in differentiated MC3T3E1 cells. In the end, when the osteoblasts reached their mineralising stage (3-week cultures), AGE-collagen significantly decreased the activation of ERK. These results suggest that AGE moieties can modulate the activation of ERK, in a manner which is dependent on the degree of osteoblastic maturation. In addition, this regulation of ERK activation by AGE-modified collagen follows the same pattern as the AGE-induced changes in RAGE expression, throughout the developmental stages of MC3T3E1 osteoblasts.

Discussion

The present study shows that short-, medium- or long-term exposure of osteoblast-like cells to extracellular AGE-modi-

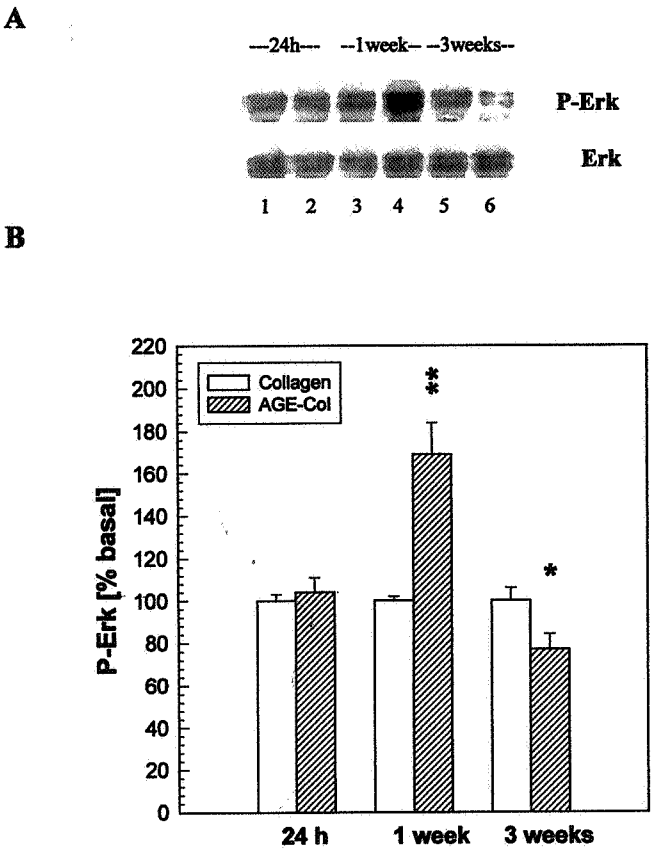

Fig. 5. Modulation of relative amounts of P-ERK throughout the developmental stages of MC3T3E1 cells. Osteoblasts were grown on collagen or AGE-modified collagen for 24 h, 1 week or 3 weeks. At the end of the culture period, ERK activation was analyzed as described in 'Materials and methods'. (A) Bands corresponding to P-Erk and Erk proteins are indicated. Lanes 1, 3 and 5 correspond to cells grown on collagen; lanes 2, 4 and 6 correspond to cells grown on AGE-collagen. (B) Quantitation of P-Erk and Erk was analyzed using the Scion-beta 2 program. Results are expressed as mean ± S.E.M. of 3 independent experiments. Differences between collagen and AGE-collagen are: *$p < 0.05$; **$p < 0.02$.

fied proteins regulates the expression of RAGE, the affinity constant and cellular density of membrane-associated AGE-specific receptors. It also modulates the activation of the signal transduction pathway involving ERK. In earlier studies, we had found that incubation of osteoblastic cells with AGE-BSA or AGE-collagen induced changes in their growth, development and mineralising potential, and in their secretion of IGF-I and IGFBPs [9, 10, 14]. In addition, we had also found that osteoblasts growing on plastic dishes (not coated with collagen) expressed 18–50 kDa AGE-binding proteins, and that maturation of these cells through their successive developmental stages (proliferation/differentiation/mineralisation) induced a 4-fold decrease in the receptors' affinity constant for AGEs, but did not change the total number of AGE-binding sites per cell [13]. Put together, these previous results led us to hypothesize that several AGE receptors could be present in osteoblastic cells, any (or all) of which could be mediating the biological effects of AGEs on osteoblasts.

In this study, we initially investigated the cellular density and affinity constant of AGE-binding sites in osteoblastic cells grown for 72 h either on control or AGE-modified collagen matrices. Surprisingly, we found that the affinity constants of AGE-binding sites in osteoblasts grown on unmodified collagen, were significantly lower than the corresponding (same culture time) affinity constants which we had observed in previous studies with osteoblasts growing on plastic dishes [13]. Thus, we found a 5-fold decrease K_a in the case of UMR106 cells, and a 7-fold decrease in the case of MC3T3E1 pre-osteoblasts. However, the cellular density of AGE-binding sites was similar in osteoblasts grown on collagen (present study) and plastic (previous study). These results probable reflect a regulation of AGE-binding sites due to the interaction of type-I collagen with osteoblastic receptors such as integrins.

When osteoblasts were grown 72 h on AGE-modified collagen, cell-line specific effects were observed (Fig. 1, Table 1). In the case of UMR106 transformed cells, growth on AGE-collagen vs. control collagen halved the cellular density of AGE-binding sites while doubling their affinity constant for AGE. However, a more complex pattern was observed for the MC3T3E1 non-transformed pre-osteoblasts: cells grown on AGE-modified collagen showed two kinds of AGE-binding sites, which could be distinguished according to their affinity constants for AGEs. The higher affinity sites possessed an affinity constant 7-fold greater than that of MC3T3E1 cells grown on control collagen. Moreover, the lower affinity sites had an affinity constant approximately half of that observed in cells growing on unmodified-collagen. On the other hand, the total number of binding sites per cell was approximately the same for MC3T3E1 pre-osteoblasts grown on control or AGE-modified collagen.

All of the observed changes in the kinetic parameters of AGE-binding sites of the osteoblastic cells grown on AGE-collagen vs. control collagen, may be attributed to multiple causes. Thus, they could be the consequence of an increase in the interaction between extracellular AGEs and their specific osteoblastic receptors, or the consequence of a decrease in the interaction of other cellular receptors (such as integrins) and their consensus sequences on type-I collagen (which may be blocked by AGEs moieties) as we have previously suggested [14]. Additionally, the differences observed in the effect of AGE-collagen on UMR106 and MC3T3E1 cells might be due to different responses to AGEs of transformed vs. non-transformed, and/or phenotypically mature vs. immature, cell lines. Whatever the causes, ultimately all of the changes in the kinetic parameters of osteoblastic AGE-binding sites which we have observed in the present study (and, for that matter, in previous studies [13]), could merely be

reflecting a modification in the relative abundance of the different receptors which might be present (e.g. RAGE, galectin-3). In this sense, recent results obtained by Takagi *et al.* pointed to RAGE as a plausible mediator for AGE-induced effects on osteoblastic cells [8]. These investigators found that primary cultures of human osteoblasts expressed mRNA which was specific for RAGE (although they did not actually demonstrate the presence of RAGE protein in these cultures). They also found that AGE-BSA induced the nuclear translocation of the transcription factor NF-κB in MC3T3E1 pre-osteoblastic cells (proliferative stage).

The receptor RAGE has been shown to be expressed by a number of cell types, including neurons, microglia, endothelial cells, mononuclear phagocytes and vascular smooth muscle cells [22]. Engagement of RAGE by AGEs can result in enhanced expression of adhesion molecules, cytokines and growth factors. Its binding also modulates gene expression by triggering a signal transduction cascade involving $p21^{ras}$, ERK1/2, CDC42-Rac and activation (nuclear translocation) of NF-κB [23, 24]. Incubation of RAGE with AGEs induces the up-regulation of RAGE, possibly as a consequence of the interaction of NF-κB with two recognition sites for this transcription factor which are present in the promoter region of the RAGE gene [25].

In this study we were able to confirm that UMR106 and MC3T3E1 osteoblastic cells do in fact express RAGE. The next step consisted in investigating whether the exposure of these osteoblasts to extracellular AGEs could modulate the expression of RAGE. In our culture conditions, we found that the presence of extracellular AGEs directly modified osteoblastic RAGE expression, in a manner which was dependent on the concentration of AGE, on cell type, and on the stage of osteoblastic development. After a 24 h incubation with UMR106 osteosarcoma cells, soluble AGEs (AGE-BSA) induced an increase in the expression of RAGE at doses of 200 and 500 μg protein/ml, concentrations which we have previously shown to stimulate UMR106 expression of alkaline phosphatase (ALP) [9]. In the case of the MC3T3E1 calvaria-derived cells, the effect of AGEs on RAGE expression depended on the stage of osteoblastic maturation. After a 24 h culture with either AGE-BSA or AGE-collagen (proliferating pre-osteoblasts), no effect was observed regarding MC3T3E1 expression of RAGE when compared with unmodified BSA or collagen. To evaluate the medium- and long-term effect of AGEs on RAGE expression, MC3T3E1 cells were grown either on control or AGE-modified collagen. Type I collagen was selected since this protein alone accounts for > 85% of bone extracellular matrix protein. In addition, we have recently shown that the accumulation of AGEs on type I collagen can regulate osteoblastic growth [14]. In the present study, after MC3T3E1 osteoblasts were cultured for one week (differentiated osteoblasts) AGE-collagen induced an increase in the expression of RAGE, compared to control collagen. Interestingly, exposure of these osteoblasts to AGE-collagen for one week enhances their expression of ALP, as we have previously described [14]. Finally, when MC3T3E1 cultures in this study were extended to 3 weeks (mineralising cultures), osteoblasts grown on AGE-modified collagen vs. control collagen showed a decrease in their expression of RAGE. In our previous study [14], this kind of exposure to AGE-collagen was associated with a decrease in the number of surviving cells, and with a decrease in the formation of nodules of mineralisation.

We next addressed the issue of the possible signal transduction pathways which could be activated in osteoblasts as a consequence of AGEs/RAGE interaction. In this sense, Bierhaus *et al.* have recently proposed that a difference must be made between acute (min to h) and chronic (days to weeks) activation of RAGE by its ligands [26]. In the case of acute AGEs/RAGE interactions, stimulus-induced degradation of IκBα and IκBβ allows free NF-κB p65 to translocate to the nucleus and activate gene transcription (however, IκBα transcription is also increased and eventually limits the availability of free NF-κB p65, closing an autoregulatory loop). In the case of chronic activation of RAGE, an increase in the transcription and protein synthesis of NF-κB p65 eventually overrides the acute autoregulatory loop, producing sustained NF-κB activation. ERK-1 and ERK-2 can promote NF-κB activity (and eventually its transcription) independently of IκBα degradation, most probably by modulating NF-κB binding to DNA. In this way, ERK 1/2 is probably important in both acute and chronic phases of RAGE activation.

Our present results show that soluble and extracellular matrix AGE-modified proteins can activate osteoblastic ERKs by inducing their phosphorylation. We found this activation of ERK to parallel the AGE-induced regulation of RAGE expression. In the case of UMR106 osteosarcoma-derived cells, a 24 h incubation with AGE-BSA dose-dependently induced an increase in the activation of ERK. When MC3T3E1 cells were studied, an AGE-associated regulation of ERK activation was observed, which was dependent on the osteoblastic stage of development. As mentioned above, these cells were cultured on either control or glycated type I collagen matrices. In the case of MC3T3E1 pre-osteoblasts (24 h), no differences could be observed in ERK activation between control and AGE-modified collagen. However, in phenotypically differentiated MC3T3E1 cultures (1 week), AGE-modification of collagen was associated with an increase in the relative amount of P-ERK. When the osteoblasts finally achieved their mineralising stage (3 weeks), ERK activation was decreased in cells growing on AGE-collagen.

In the case of the pre-osteoblastic MC3T3E1 cells (24 h culture), although in the present experiments we were unable to observe AGE-induced alterations in ERK activation, previous studies have demonstrated that in these same culture conditions AGE-collagen can transiently increase MC3T3E1

cell attachment and proliferation [14]. These findings suggest that the early effects of AGE-modified proteins on pre-osteoblastic MC3T3E1 cells could be mediated by AGE-receptors other than RAGE (or, if RAGE is activated, it could be through a signal transduction pathway which does not involve ERK). Interestingly, by preliminary immunoblot studies we have found that both UMR106 and proliferating MC3T3E1 osteoblastic cells express readily detectable levels of galectin-3 (our own unpublished observations).

Overall, our results seem to indicate that although RAGE is expressed in all stages of osteoblastic development, a regulation in the activation of ERK due to the presence of AGEs can only be observed at the later stages of this development (that is, when osteoblasts are phenotypically mature). In these later stages, and according to the results of other authors, AGEs/RAGE interaction would be expected to increase ERK activation in every experimental condition (this is what we have observed, for example, with 24 h cultures of UMR106 cells). However, most studies by other authors (and our own with UMR106 cells) were performed with relatively short-term cell cultures, and so the results actually correspond to the effects of acute phase RAGE activation. In fact, the effect of the chronic activation of RAGE on the relative amount of P-ERK has not been clearly established by other investigators. In our experiments with mature MC3T3E1 osteoblasts cultured 1–3 weeks in permanent contact with AGE-collagen, which are more akin to a setting of chronic AGEs/RAGE interaction, we observed that the level of ERK activation correlates with the osteoblastic expression of RAGE, but not directly with the actual presence of extracellular AGEs (although AGEs is probably ultimately responsible for the regulation of RAGE expression). Whatever the precise mechanism responsible for the regulation of osteoblastic ERK activation, we have found that P-ERK is increased by AGEs in parallel with an AGE-induced enhancement of ALP expression (24 h UMR106 cultures, and 1 week MC3T3E1 cultures) [14]. We have also observed that AGEs provoke a decrease in osteoblastic ERK activation, in culture conditions which are associated with a decrease both in cell survival and in the formation of nodules of mineralisation (3 week MC3T3E1 cultures) [14]. In this context, it is interesting to note that a decrease in ERK activation would induce a relative inhibition of NF-κB DNA-binding capacity, and that NF-κB inhibition has been directly linked to apoptosis [27]. Thus, AGE-induced inhibition of P-ERK at this late stage of osteoblastic development could contribute to a decrease in cell survival, and so to a decrease in osteoblastic bone-forming capacity.

The possible significance of these in vitro findings could be related to the pathological accumulation of AGEs that has been described in the cortical bone of diabetic and ageing rats [7, 28]. Our results could also help to explain the decrease in osteoblast recruitment and bone-forming capacity that has been observed *in vivo* in patients with type 1 and type 2 diabetes mellitus [29]. These processes could be partly mediated by the presence of RAGE on osteoblasts, as we are describing in the present study. The accumulation of AGEs in bone extracellular matrix, and the generation of signal transduction pathways regulating ERK activation, would lastly affect bone turnover and could play a role in the pathogenesis of the osteopenia present in poorly controlled diabetic patients.

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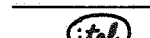
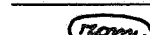
	Change to italics	— Under matter altered
	Change to roman type	Encircle matter altered
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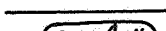	Make spacing equal		Between items
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