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Advanced glycation endproduct-specific receptors in rat and mouse osteoblast-like cells: regulation with stages of differentiation

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Abstract Advanced glycation endproducts have been implicated in the development of diabetic complications. In addition, these products could also mediate certain bone alterations such as diabetic osteopenia. Several receptors specific for advanced glycation endproduct-modified proteins have been characterized in different cell types, contributing to the recognition and degradation of senescent proteins. In the present report, we investigated the possible presence of advanced glycation endproduct-binding proteins on osteoblast-like cells. Both UMR106 and MC3T3E1 cell lines express specific advanced glycation endproduct-binding sites, with an affinity constant between 0.4 and 1.7 • 10^6 M^{-1}, depending on the stage of osteoblastic differentiation; and with a receptor capacity of 1.5-2.0 • 10^7 sites/cell. Osteoblast-like cells were also found to participate both in the uptake and degradation of advanced glycation endproduct-modified bovine serum albumin at 37ºC. Radiolabelled ligand blotting studies confirmed the presence of several membrane binding proteins, with apparent molecular masses of 50, 45-40, 30, 25 and 18 kDa; the major bands corresponded to 30 and 25 kDa proteins. This study provides evidence of the presence of advanced glycation endproduct-specific binding sites, and for their regulation with the stage of differentiation, in two osteoblast-like cells in culture.

Key words Non-enzymatic glycosylation • Advanced glycation endproducts • Receptor for advanced glycation endproducts • Diabetes mellitus • Osteoblasts • Bone • Osteopenia

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Introduction

In poorly controlled diabetes mellitus, excessive non-enzymatic glycosylation (glycation) of proteins, lipids and nucleic acids occurs as a consequence of hyperglycaemia. During this process, a reducing sugar such as glucose initially reacts reversibly with a free amino group, resulting in the formation of a Schiff base. An intramolecular rearrangement then gives rise to a more stable, but still freely reversible, molecule: the Amadori product. With time, Amadori products can irreversibly progress to the formation of a family of cross-linked and fluorescent compounds, collectively known as advanced glycation endproducts (AGEs) [1, 2]. Since the formation of AGEs is essentially irreversible, they continue to accumulate over the years, particularly in long-lived structural proteins of insulin-independent tissues [3, 4].

Evidence has accumulated suggesting that AGEs could be involved in the pathogenesis of ageing and atherosclerosis, as well as in the appearance and progression of diabetic microangiopathies (nephropathy, retinopathy and neuropathy) [5]. Most studies have demonstrated that AGEs can induce structural and functional alterations of several proteins. In addition, incubation with protein-AGEs has been shown to elicit various modifications in the normal physiology of different tissues and cells in culture, the mechanisms of which are not fully understood. Similarly, high levels of AGEs have been found in the serum of diabetic and non-diabetic patients with end-stage renal disease, especially in association with peptides smaller than 10 kDa [6], which have been termed “second generation” AGEs. These highly reactive peptides have been implicated in the pathogenesis of haemodialysis-associated amyloidosis, through secondary AGE-modification of β-2 microglobulin [7].

Cell-surface receptors or binding proteins specific for AGE moieties have been detected on circulating monocyte/macrophages [8] and T-lymphocytes [9], and on endothelial cells [10], mesangial cells [11] and other cell types. These proteins, with or without receptor characteris-

tics, appear to contribute to diverse cellular functions (chemotaxis, adhesion, activation, cell growth and differentiation), as well as to the specific recognition and degradation of AGE-modified proteins, clotting and barrier function [1]. Several AGE-receptors have been characterized, such as p60 (OST-48) [12], p90 (80 K-H) [12], the macrophage scavenger receptor [13], a 35 kDa member of the immunoglobulin superfamily of receptors (RAGE) [14, 15], galectin-3 (a 32 kDa protein also known as Mac-2) [16], and a novel receptor that mediates AGE-induced chemotaxis in rabbit smooth muscle cells [17].

Several studies, both in vivo and in vitro, have suggested that AGEs could mediate bone alterations, such as osteopenia, which occur in poorly controlled diabetic patients. Recently, AGE-modified collagen has been detected in the cortical bone of diabetic and ageing rats [18-20]. In addition, Fong et al. [21] found that the formation of AGE on bone matrix diminishes its ability to induce bone formation. AGE-modified proteins have also been shown to enhance osteoclast-induced bone resorption [22], to stimulate interleukin-6 (IL-6) production by human bone-derived cells [23] and to dose-dependently inhibit the phenotypic expression of a primary culture of rat osteoblasts [20]. In addition, our group has demonstrated that AGE-modified proteins regulate the growth of mouse MC3T3E1 and rat UMR106 osteoblastic cells: an increase in cell proliferation and differentiation was observed after short incubation periods, while long-term cultures with protein-AGEs induced a depression in both parameters [24]. Our results suggested that AGEs directly regulate osteoblast growth. However, the mechanisms by which AGEs would regulate this growth were not studied.

In the present report, we investigated the possible presence of AGE-binding proteins on UMR106 and MC3T3E1 osteoblastic cells. We also compared the kinetic parameters and molecular masses of these receptors with others which have been previously described in different cell types, as well as their regulation throughout the different stages of osteoblastic differentiation.

Materials and methods

Materials

Bovine serum albumin (BSA), phenyl-methyl-sulphonyl-fluoride (PMSF), p-nitro-phenyl-phosphate (pNPP), glucose 6-phosphate (G6P), Iodo-Gen, tri-chloro-acetic acid (TCA), Triton X-100, prestained molecular weight standards, Kodak XAR-5 photographic film, Sephadex G-25 and G-50 were purchased from Sigma (St. Louis, MO., USA). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 and trypsin/EDTA were from Gibco (Life Technologies, Grand Island, N.Y., USA). Fetal bovine serum (FBS) was purchased from Gen (Buenos Aires, Argentina). Centricon 10 kDa cut-off filter cartridges were from Amicon Inc. (Beverly, Mass., USA). ^{125}I was from New England Nuclear. All other chemicals were of analytical grade.

Preparation of AGE-protein

AGE-BSA was prepared as we have previously described [24]. Briefly, BSA was incubated under sterile conditions in 150 mmol/L phosphate-buffered-saline (PBS), pH 7.4, with 100 mmol/L G6P at 37ºC for 6 weeks in the presence of protease inhibitors (1.5 mmol/L PMSF) and antibiotics (100 U/ml penicillin, 100 mg/l streptomycin). Control BSA was incubated under the same conditions without G6P. 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 AGE proteins was assessed by their characteristic fluorescence-emission maximum at 420 nm upon excitation at 340 nm, using an Aminco-Bowman SPF100 spectrofluorometer equipped with an off-axis Ellipsoidal Mirror Condensing System and an Aminco Ratio Photometer. The excitation source was an 150W Xenon arc lamp and the detector was an RCA 4837 photomultiplier tube. Standard 1 x 1 cm quartz cells were used and spectra were recorded on a Linseis 1600 *x-y* recorder. Quinine (1 μg/ml) was used as a fluorescence intensity standard to calibrate and monitor the performance of the fluorescence spectrophotometer. 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 17.6% relative fluorescence intensity/mg protein, as opposed to 2.2% for control BSA.

Cell culture

UMR106 rat osteosarcoma-derived cells were grown in 75 cm^2 plastic flasks at 37ºC 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 [25]. After 5-7 days, cells were sub-cultured using trypsin/EDTA and replated to begin the experiments. Cells were split at subconfluence and plated in 48-well plates ($2.5 \cdot 10^4$ cells/well) in 10% FBS-DMEM. Subconfluent cells were then serum deprived, and washed with RPMI-1640 before being submitted to the studies described below.

MC3T3E1 osteoblastic mouse calvaria-derived cells were grown in 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 for 10 days with medium supplemented by 5mmol/L β-glycerol-phosphate and 25 mg/L ascorbic acid. In addition, mineralization is achieved with these cells after extending this culture to 20 days. However, the cells only undergo active replication during the first 10 days of incubation [26]. Thus, in order to study the expression of AGE-receptors in the above stages of this cell line, different protocols were chosen. To obtain cells in active proliferation, cells were plated in 48-well plates ($2.0 \cdot 10^4$ cells/well) and cultured for 3 days in DMEM-10% FBS. In other experiments, MC3T3E1 cells were plated in 48-well plates ($2.0 \cdot 10^4$ cells/well), and cultured in 10% FBS-DMEM supplemented with 5 mmol/L β-glycerol-phosphate and 25 mg/l ascorbic acid for either 15 days (differentiated cells) or 25 days (mineralizing cells), changing the medium every 2 days. At the end of all incubations, cells were serum deprived, washed with RPMI-1640, and submitted to receptor binding studies as described below.

Alkaline phosphatase (ALP) determination

The cell monolayer from individual wells was solubilized in 0.5 ml 0.1% Triton X-100. Aliquots of total cell extract were then saved for protein determination using the Bradford method [27], and for measurement of the osteoblastic differentiation marker ALP as we have described previously [24].

Radiolabelling of AGE-BSA

AGE-BSA was iodinated with carrier-free ^{125}I by the Iodo-Gen method of Fraker and Speck [28]. 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 chromatography, and the fraction corresponding to ^{125}I-AGE-BSA was further purified by a Sephadex G-50 50 ml column, until more than 98% radioactivity was TCA precipitable. Protein concentrations were determined by the method of Bradford [27], using BSA as a standard. Specific radioactivity of labelled ^{125}I-AGE-BSA was between $1.1 \cdot 10^4$ and $1.8 \cdot 10^4$ cpm/ng protein.

Determination of protein/cell count standard curves

Standard curves were determined to evaluate the correlation between cell number and total protein content, for both UMR106 and MC3T3E1 cells. Briefly, confluent cells growing in a 75 cm^2 flask were trypsinized and seeded in serial dilutions in 24-well plates (8.0, 4.0, 2.0, and $1.0 \cdot 10^4$ cells/well, each condition in triplicate). They were then cultured in 10% FBS-DMEM for 48 h, at 37ºC and in a humidified 5% CO_2 atmosphere. Finally, individual wells were either trypsinized (for cell count determination with a haemocytometer), or washed with PBS and solubilized with 0.1% Triton X-100 for protein determination by the method of Bradford [27]. Cell count as compared to protein content was then evaluated by linear regression analysis for both MC3T3E1 osteoblasts ($r = 0.991$, $p < 0.001$) and UMR106 osteosarcoma cells ($r = 0.997$, $p < 0.001$).

Whole-cell homogenate and membrane preparation

Sub-confluent UMR106 or MC3T3E1 cells in active replication growing in 75 cm^2 flasks were washed with PBS, scraped, taken up in PBS and spun at $1000 \cdot g$ for 5 min. The supernatant was discarded, and the cellular pellet was solubilized in 0.1% Triton X-100. Bradford's method was used to determine protein concentration [27]. This whole-cell homogenate was saved for ligand blot studies as described below. In other experiments, osteoblast-like cells were grown to confluency in 150 cm^2 flasks. Cells were washed with PBS, resuspended in a homogenizing buffer (25 mmol/L Tris, 250 mmol/L sucrose, 2 mmol/L EDTA, 5 mmol/L β-mercapto ethanol, 1 mmol/L PMSF, pH 7.4), and sonicated three times at 60 psi for 15 s. The homogenate was centrifuged at $1\,000 \cdot g$ for 10 min, and the supernatant was further centrifuged at $10\,000 \cdot g$/4°C for 30 min. Membranes were then isolated from the supernatant by centrifugation at 105 000 x g for 60 min at 4°C. The resulting pellet was solubilized in 0.1% Triton X-100/PBS. Protein concentration was determined by the method of Bradford [27]. This material was then used for ligand blotting.

Binding studies

Radioligand binding studies were carried out with UMR106 and MC3T3E1 cells, cultured on 48-well plates under the conditions described above. These displacement studies were performed in 0.25 ml binding medium (RPMI-1640, 25 mmol/L Hepes, pH 7.4, 5 g/l BSA) at 4ºC, as previously described [9]. Cell monolayers were pre-incubated for 30 min with increasing concentrations of unlabelled AGE-BSA (0-1 000 µg protein/ml binding medium), following which 10^6 cpm of ^{125}I-AGE-BSA was added to each well. These doses were chosen since the activity of AGE-products on the differentiation and proliferation of these cell lines occurs between 100 and 1 000 µg protein/ml incubation medium, as we have described previously [24]. After incubating for 2 h at 4°C, the supernatant was aspirated and the cell layers were washed three 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 Hewlett Packard PRIAS gamma counter, and for protein determination according to the method of Bradford [27]. Non-specific binding of ^{125}I-AGE-BSA was determined in parallel incubations by adding an excess of unlabelled AGE-BSA. Specific binding was defined as the difference between total binding (cells incubated with radioligand and a known amount of unlabelled AGE-BSA) and non-specific binding (as defined above). ^{125}I-AGE-BSA was also co-incubated with an excess of control BSA. Protein concentrations were used to estimate cell number through the standard curves described above. At least three experiments were run for each experimental condition.

Uptake and degradation

Osteoblast-like cells were grown to confluency in 24-well plates in DMEM-10% FBS. Cells were washed with RPMI-1640 and incubated at 37°C for different periods of time with RPMI-1640/0.5% BSA/^{125}I-AGE-BSA, with or without an excess of unlabelled AGE-BSA. After these incubations, medium was taken up from each well, and degraded ^{125}I-AGE-BSA was determined by measuring TCA-non-precipitable radioactivity [9]. The remaining cell monolayers were washed as for binding experiments. Cells were then solubilized with 0.5ml 0.1% Triton X-100, and cell-associated radioactivity was determined. Specific uptake was defined by the same criteria as used for the binding studies.

Ligand blotting

In order to perform ligand blot studies for cellular AGE-binding proteins, UMR106 or MC3T3E1 whole-cell homogenates or membrane preparations (100 µg protein aliquots) were resuspended in 2 x Laemmli's sample buffer [29] containing β-mercapto ethanol and boiled for 3 min. Samples were electrophoresed on 12% SDS-PAGE, with prestained molecular weight standards processed in parallel. Overnight electroblotting onto a nitrocellulose filter was performed. After blocking for 2 h with a cold solution of PBS containing 1% BSA and 0.1% Triton X-100, the nitrocellulose filter was probed at 4ºC for 2 h with ^{125}I-AGE-BSA alone, or co-incubated with 1 000 µg/ml of either BSA or unlabelled AGE-BSA. The blot was washed twice with blocking solution, and then three times with PBS. After air drying, it was exposed to Kodak XAR-5 film at -70ºC, for autoradiographic evaluation [11].

Statistical analysis

Scatchard analysis [30] of the raw data from binding experiments was performed, in order to estimate receptor number and binding affinity constant, and to determine the standard error.

Results

^{125}I-AGE-BSA binding to osteoblast-like cells

Radioligand binding experiments were performed by incubating either UMR106 or MC3T3E1 cells with ^{125}I-AGE-BSA at 4°C for 2 h. Cell-associated ^{125}I-AGE-BSA was found to increase dose-dependently (Fig. 1). This binding was inhibited by an excess of unlabelled AGE-BSA, but not by control BSA. For instance, in the case of proliferating MC3T3E1 cells, the maximal binding was 0.86 % ± 0.07 % B/T per 100 µg protein; 0.25 % ± 0.03 % B/T per 100 µg protein in the presence of excess AGE-BSA; and 0.84 % ± 0.07 % B/T per 100 µg protein, with an excess of control BSA. Specific binding, as defined in Methods, is represented in Fig. 1 and shows a saturation pattern. Scatchard analysis of these specific binding data show results which are compatible with a one-site binding system (see inserts in Fig. 1).

It was apparent from the Scatchard analysis that MC3T3E1 cells, at the proliferative stage, showed a higher affinity of the receptor for ^{125}I-AGE-BSA when compared with the osteosarcoma line UMR106. However, no differences were found in receptor number, and both cell types showed a similar capacity for AGE binding to receptors (Table 1). These results suggested a relationship between AGE binding and the stage of osteoblastic differentiation. To further investigate this hypothesis, we studied the regulation of AGE receptors on MC3T3E1 osteoblast-like cells after induction of their differentiation by ascorbic acid/β-glycerol phosphate incubation, as described in Materials and methods. Cellular levels of ALP were determined to establish these stages of differentiation [26]. After culture for 72 h, the cells in the proliferative stage expressed undetectable levels of ALP. This marker was significantly higher after 15 days culture (ALP = 2.2 µmol pNP per min^{-1} per g protein^{-1}), which corresponds to the cells' differentiated stage. Furthermore, cell-associated specific activity of ALP reached a plateau after 25 days of culture (ALP = 8.5 µmol pNP per min^{-1} per g protein^{-1}) coinciding with the osteoblasts' mineralizing stage. ^{125}I-AGE-BSA binding was then investigated in each case, and the kinetic parameters found are shown in Table 1. These results indicate a decrease in specific binding as the MC3T3E1 cells become more differentiated. These changes can be accounted for by the observed decrease in the affinity constant, since no changes in the density of cellular receptors was found.

AGE-protein uptake and degradation

To assess whether osteoblast-like cells can internalize and degrade AGE proteins, cells were incubated with ^{125}I-AGE-

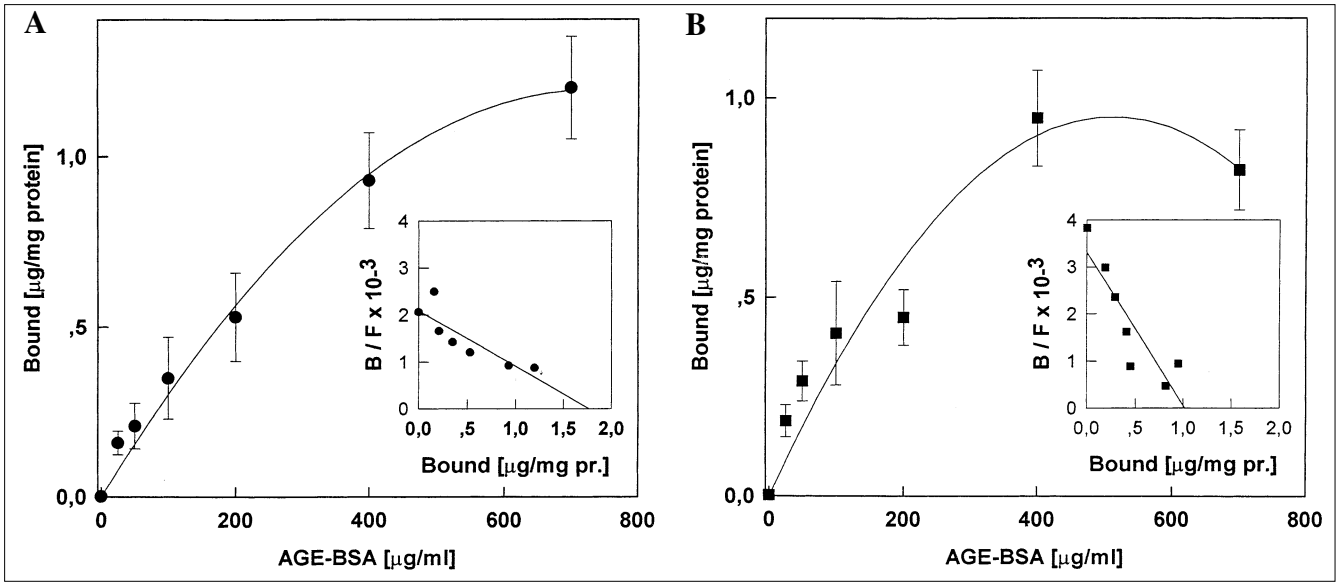

Fig. 1A, B Saturability of AGE-BSA binding to UMR106 (**A**) and proliferating MC3T3E1 (**B**) osteoblast-like cells. Specific binding versus the concentration of AGE-BSA is plotted. The figure shown corresponds to the mean ± SEM for three independent experiments, each performed by sextuplicate. Data were analysed by a non-linear least squares programme and the curve indicates the best fit line. *Insert*, Scatchard analysis of the binding data. Data from binding experiments were fit to the equilibrium binding equation B = nKA/(1+KA), where *B* is the amount of specifically bound ligand, *n* is the number of binding sites per cell, *A* is the concentration of free radioligand, and *K* is the association constant

Table 1 Kinetic parameters resulting from the Scatchard analysis of binding data of AGE-BSA to osteoblast-like cells. Data represent the mean of at least three independent experiments

Cell type	Stage of differentiation	K_a [M^{-1}]	R_o [sites/cell]
UMR106	$0.4 \pm 0.1 \cdot 10^6$	$2.0 \pm 1.2 \cdot 10^7$	
MC3T3E1	Proliferative	$1.7 \pm 0.8 \cdot 10^6$	$1.5 \pm 0.9 \cdot 10^7$
MC3T3E1	Differentiated	$1.4 \pm 0.5 \cdot 10^6$	$1.5 \pm 0.8 \cdot 0^7$
MC3T3E1	Mineralizing	$0.6 \pm 0.1 \cdot 10^6$	$1.8 \pm 0.5 \cdot 10^7$

BSA at 37°C for different periods of time. In the case of MC3T3E1 osteoblasts, specific cell-associated AGE-BSA was found to accumulate in a time-dependent manner throughout the 24 h incubation period (Fig. 2A). In addition to this ^{125}I-AGE-BSA uptake, TCA-soluble radioactivity derived from AGE-protein degradation was detected in the medium, and increased with incubation time up to 24 h (Fig. 2B). On the other hand, ^{125}I-AGE-BSA uptake and degradation by UMR106 cells up to 24 h exhibited both faster kinetics and a saturable pattern (Fig. 2A and B). In absolute terms, these two osteoblast-like cell lines attain a similar ^{125}I-AGE-BSA specific uptake after 24 h of culture, although more AGE-protein appears to be degraded by the osteosarcoma UMR106 line than by MC3T3E1 cells in the same period of time (Fig. 2B).

Ligand blot

To confirm and further characterize the presence of AGE binding sites on osteoblast-like cells, ligand blotting studies were carried out under reducing conditions using either whole cell homogenates or extracts of osteoblastic cell membranes. As shown in Fig. 3A, two AGE-binding proteins were observed in the homogenates of UMR106 and MC3T3E1 osteoblast-like cells, with apparent molecular masses of ~ 45 kDa (minor band) and 30 kDa (major band). However, cell membrane preparations showed several AGE-binding proteins: two major bands with apparent molecular masses of 30 and 25 kDa, and minor bands with molecular masses of 50, 40-45 and 18 kDa (Fig. 3B). The binding of ^{125}I-AGE-BSA to these sites, both in whole-cell homogenates and in membrane preparations, was specifically blocked by 1 000 µg/ml of unlabelled AGE-BSA (Fig. 3A and B, lanes 3 and 4). However, in the presence of an excess of control BSA, two bands of 45 and 30 kDa were observed with whole-cell extracts, showing similar intensity to those described above. Similar patterns of ^{125}I-AGE-BSA binding proteins were obtained for both UMR106 and MC3T3E1 osteoblastic lines (Fig. 3A and B, lanes 1 and 2).

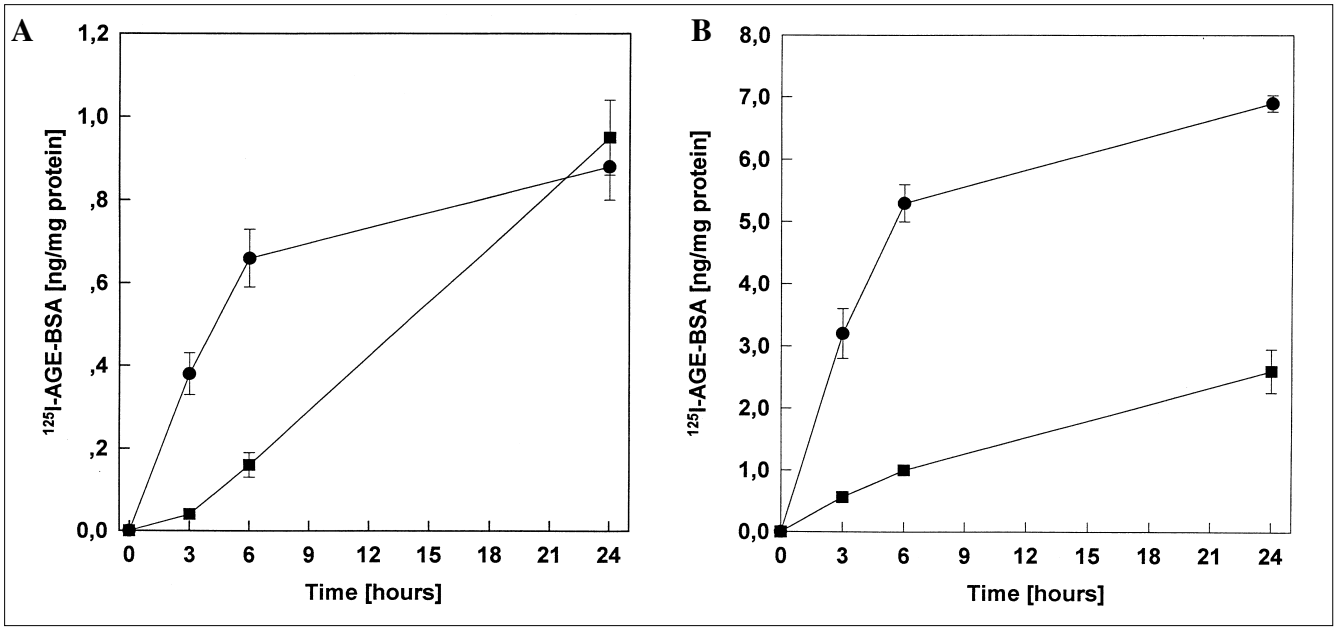

Fig. 2A, B Time-dependent AGE-specific uptake (**A**) and degradation (**B**) of ^{125}I-AGE-BSA by UMR106 (●) and MC3T3E1 (■) osteoblast-like cells. Data represent the mean ± SEM of two independent experiments, each performed in triplicate

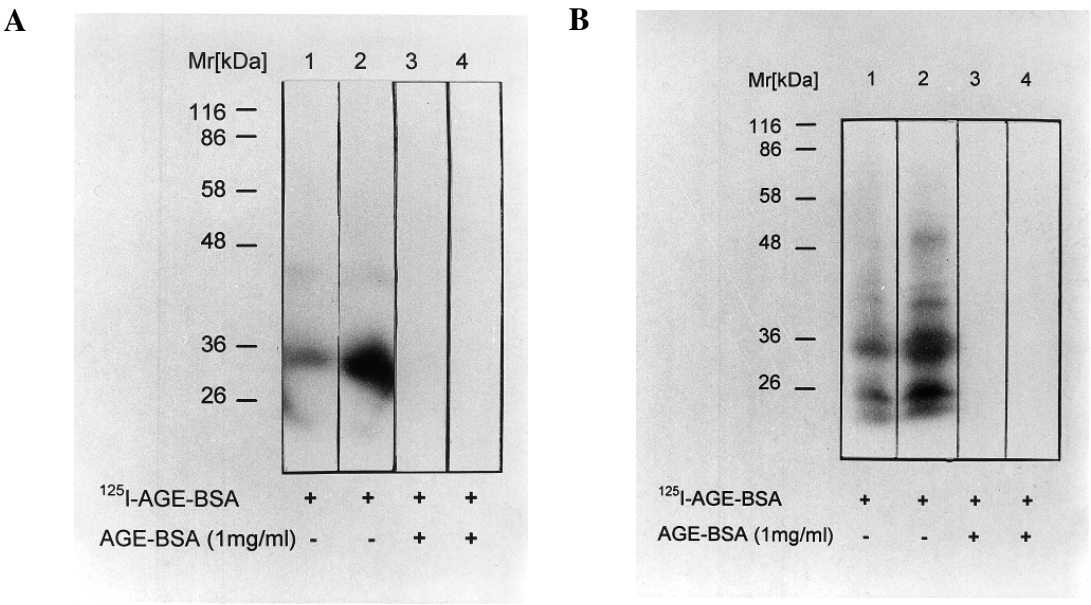

Fig. 3A, B Ligand blotting of ^{125}I-AGE-BSA to either whole-cell extracts (**A**) or membrane fractions (**B**) in the presence or absence of an excess of unlabelled AGE-BSA. In both cases, samples obtained from UMR106 cells (*lanes 1 and 3*) and MC3T3E1 cells (*lanes 2 and 4*) were processed in a similar manner. Samples were subjected to SDS-PAGE, followed by overnight electroblotting onto nitrocellulose membranes and incubated with ^{125}I-AGE-BSA as a probe ($2 \cdot 10^6$ cpm/ml incubation medium; $1.5 \cdot 10^4$ cpm/ng protein). Specific binding was determined in the presence of 0 or 1 000 µg/ml of unlabelled AGE-BSA, using autoradiographic detection. Pre-stained molecular weight markers were run in parallel lanes

Discussion

Advanced glycation endproducts have been implicated in the development of chronic complications of diabetes, such as nephropathy, retinopathy, neuropathy and macrovascular disease [1]. In addition, several lines of evidence have recently suggested that AGEs could also contribute to the osteopenia found in poorly compensated patients with type 1 diabetes. AGE-modified collagen has been shown to accumulate in the bones of diabetic rats, in relation to the duration of diabetes [18-20]. AGEs have also been found to enhance osteoclast-induced bone resorption [22], and to inhibit the phenotypic expression both of a primary culture of rat osteoblasts, and of MC3T3E1 and UMR106 osteoblast-like cell lines [20, 24]. Furthermore, AGEs have been shown to enhance the production of potent bone-resorption factor IL-6 in normal human bone-derived cells [23]. These results point towards a role for AGE-modified proteins in the regulation of osteoblast and/or osteoclast differentiation. However, the mechanisms by which AGE would exert these effects remain poorly defined.

Recent evidence has documented the existence of a family of AGE receptors/binding proteins, with different molecular masses and cellular functions [1]. Our present study indicates that both UMR106 and MC3T3E1 osteoblastic cells express binding sites which specifically recognize glucose-modified proteins. The evidence supporting the presence of AGE-receptors in these cells includes several findings. Both osteoblastic cell lines are able to bind AGE-BSA in a saturable manner, showing half-maximal binding between 35-120 µg AGE-BSA/ml. In addition, these cells show an affinity for AGE-BSA which is comparable to that of mesangial cells ($2.0 \cdot 10^6$ M^{-1}) [11], but an order of magnitude lower than that reported for other systems such as macrophage/monocytes, endothelial cells, T-lymphocytes and smooth muscle cells ($1.0\text{-}7.8 \cdot 10^7$ M^{-1}) [8-10, 17]. However, the number of AGE molecules bound per osteoblastic cell ranges between $1.5 \cdot 10^7$ and $2.0 \cdot 10^7$, which is one or two orders of magnitude greater than the cellular density reported for other cells [8-10, 17].

The kinetic parameters of the AGE-receptors described in this study suggest that osteoblastic cells could respond to AGE-modified proteins through these receptors. As we have previously described, the effects of AGE-BSA on osteoblastic proliferation and differentiation [24] are dose-dependent, being half-maximal at 50-200 µg/ml. This concentration range is approximately equal to the AGE-BSA doses needed for half-maximal binding to cell surface binding sites. Thus, at the concentration range where AGE-BSA exerts its bioactivity on osteoblast-like cells, its specific binding sites are significantly occupied and could mediate the previously described effects. However, direct evidence to prove this hypothesis deserves further studies using anti-receptor antibodies.

In recent studies, Tomasek et al. [18] showed that collagen-linked fluorescence was significantly increased in the cortical bone of diabetic rats (28.0 ± 2.7 in diabetic vs 15.3% ± 1.9% in control rats, expressed as a percentage of relative fluorescence per mg collagen). An increase in fluorescence was

also found to correlate with ageing (2.05-fold for 30-month-old vs 3-month-old rats). In addition, Katayama et al. [20] demonstrated that in streptozotocin-induced diabetic rats 8 weeks post-injection, fluorescent AGEs bound to collagen (obtained from the tibiae) increased significantly, reaching a level of about 20% relative fluorescence/mg collagen, compared to 8% in control rats. The levels of AGE formed on BSA in our system, as assessed by fluorescence analysis, are thus similar to those found in collagen obtained from bone samples of diabetic rats [18, 20]. The previous and present studies suggest that AGE-formation on several proteins may contribute to alterations observed in the bones of uncompensated diabetic patients.

In further experiments, ligand blot analysis confirmed the presence of several membrane proteins that bind ^{125}I-AGE-BSA, with apparent molecular masses of 50, 45-40, 30, 25 and 18 kDa. The binding of labelled AGE-BSA, either to osteoblast monolayers or to blotted membrane proteins, is specific for AGE-modified proteins, since no displacement was found in any case with an excess of unmodified BSA.

The major AGE binding site described in the present study, (30 kDa binding protein), may be structurally similar either to galectin-3, a 32 kDa AGE-receptor [16]; or to RAGE, the 35-kDa AGE-binding protein isolated from bovine lung [15]. In this context, it is interesting to note that RT-PCR analysis of total RNA extract from a primary culture of human bone-derived cells has demonstrated their expression of mRNA specific for RAGE [23]. On the other hand, ^{125}I-AGE-BSA ligand blotting of liver cell plasma membranes under reducing conditions has shown a pattern of binding proteins which is similar to our present results for osteoblastic cells [31]. In this latter study, the authors identified AGE-receptors p60 and p90 as OST-48 and 80 K-H proteins, respectively. Accordingly, several molecules capable of recognizing AGE-modified proteins could be present in osteoblast-like cell membranes.

The present study shows evidence for a differential regulation of AGE-receptors according to the stage of osteoblastic differentiation. UMR106, an established osteosarcoma cell line, expresses several markers of osteoblastic phenotype, namely bone-specific alkaline phosphatase, type I collagen synthesis and PTH-inducible cAMP levels [25]. In our experiments, we have found these cells to express AGE-receptors with a relatively low affinity for AGE-modified proteins. In comparison, MC3T3E1 cells in active proliferation show binding sites with a similar capacity but higher affinity for AGEs. However, proliferating MC3T3E1 cells have been reported to differentiate into osteoblastic cells morphologically and functionally, when they are cultured in a medium supplemented with β-glycerol-phosphate and ascorbic acid [26]. By using this model of sequential osteoblastic proliferation, differentiation and mineralization, we have found that the affinity of AGE-binding sites decreases with an increase in cellular differentiation (as assessed by their expression of osteoblast marker ALP). These results show for the first time a regulation of AGE-receptors with the stage of osteoblastic differentiation.

Similarly, in lung tissue cellular expression of RAGE has been associated with a mature phenotype. Katsuoka et al. [32] have shown that the presence of RAGE-mRNA correlates with an alveolar phenotype in lung cells, suggesting a role for these receptors in specific cellular functions. In addition, expression of RAGE (both mRNA and protein) was shown to be strongly reduced or absent in non-small-cell lung carcinoma, as opposed to normal lung tissue [33]. These investigators have suggested that the down-regulation of RAGE could be involved in lung tumour formation.

In addition to their ability to bind AGE-BSA, osteoblast-like cells also participate both in the uptake and degradation of AGE-BSA at 37°C. These cells specifically accumulate and slowly degrade ^{125}I-AGE-BSA, with kinetics which follow closely those of AGE-BSA uptake in each osteoblastic cell line. That osteoblast-like cells can bind, internalize and degrade AGE-modified proteins, suggests a role for their AGE-receptors in the turnover of bone and extracellular matrix proteins. A recent study has provided in vitro and in vivo evidence of AGE-protein enhanced bone resorption [22]. The effect of AGE-proteins obtained in vitro on the number of resorption pits formed on dentin slices was observed at 1 μmol/L AGE-protein concentration. These authors thus postulate the participation of AGEs in the remodelling of senescent bone matrix tissues, although the mechanisms by which AGEs enhance osteoclast-induced bone resorption were not investigated in their study. However, AGE-induced bone resorption, if it does occur physiologically, could also be partly accounted for by the AGE-dependent secretion of IL-6, which has been described for human bone-derived cells [23]. Moreover, these effects are in general agreement with our previous findings in osteoblastic cell lines, in which relatively long-term incubation with AGE-protein concentrations of 1.7-17 μmol/L inhibited cellular growth [24]. Thus, the presence of AGE-receptors on osteoblastic cells could contribute, at least in part, to the turnover of senescent bone and matrix proteins, which accumulate physiologically in ageing individuals, and at an increased rate in pathological situations such as diabetes.

In summary, this study has provided evidence for the presence of AGE-specific binding sites, and for their regulation with the stage of differentiation, in two osteoblast-like cells in culture.

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