Molecular and Cellular Biochemistry **170:** 43–51, 1997.

Effects of advanced glycation end-products on the proliferation and differentiation of osteoblast-like cells

Antonio D. McCarthy,[1] Susana B. Etcheverry,[1] Liliana Bruzzone[2] and Ana M. Cortizo[1]

[1]*Cátedra de Bioquímica Patólogica,* [2]*División Química Analítica, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115 (1900) La Plata, Argentina*

Received 11 March 1996; accepted 28 October 1996

Abstract

Two different lines of osteoblast-like cells were used to investigate the effect of advanced glycation end-products of bovine serum albumin on cell proliferation and differentiation. These parameters were found to be both dose- and time-dependent. Cell proliferation remained unchanged after a 24 h incubation period, it increased after intermediate periods of incubation with advanced glycation end-products, but was found to be depressed after several days incubation. Cellular alkaline phosphatase activity followed a similar pattern: an initial increase induced by advanced glycation end-products was generally followed, after relatively long incubation periods, by a slight but significant decrease in this parameter. $^{45}Ca^{2+}$ uptake was only significantly inhibited by advanced glycation end-products after 24 h incubation. These results suggest that advanced glycation end-products directly regulate osteoblast proliferation and differentiation in a dose and time dependent manner. (Mol Cell Biochem **170**: 43–51, 1997)

Key words: advanced glycation endproducts, diabetes mellitus, osteoblastic cells, cell proliferation, cell differentiation, bone

Introduction

In young developing patients with insulin-dependent Diabetes mellitus (IDDM), chronic hyperglycaemia is associated with a deficit in bone mass [1–8]. Delay in maturation and growth impairment have also been described in the most severely affected patients [2]. These defects appear to be related with a failure to acquire sufficient endosteal bone during growth, and to nutritional, hormonal and vascular factors [9–12]. Animal models have been used to study the influence of Diabetes mellitus on bone metabolism [13], however, its effect on individual bone-derived cell types is poorly understood.

One of the mechanisms involved in the development of diabetic complications is the accelerated non-enzymatic glycosylation (glycation) of proteins, which leads to an excessive formation of advanced glycation endproducts (AGE) [14–17]. These AGE products are irreversibly attached to their associated proteins, altering both their structure and bioactivity. The AGE moieties are specifically recognized by plasma membrane receptors in cells from various tissues [18, 19]. The occupation of these specific receptors by AGE induces the secretion of cytokines and growth factors, which in turn lead to an alteration in the proliferation and differentiation of nearby cells [20–22]. The excessive accumulation of protein-AGEs in diabetic non-insulin-dependent tissues could thus contribute to the proliferative/degenerative alterations typical of diabetic complications [23, 24].

It has recently been shown that collagen-linked fluorescence, a measure of nonenzymatic collagen cross-linking due to AGE formation, increases significantly in rat cortical bone both with diabetes and age [25]. Locatto *et al.* [26] have also found increased bone collagen glycation, and increased resistance of bone collagen to collagenase hydrolysis, in alloxan-induced diabetic rats. The effect of AGE on a model

Address for offprints: A.M. Cortizo, Cátedra de Bioquímica Patológica, Facultad de Ciencias Exactas, UNLP, 47 y 115 (1900) La Plata, Argentina

of demineralized bone-matrix-induced endochondral bone formation was investigated by Fong *et al*. [27]. They found that the formation of AGE on bone matrix alters its ability to induce bone formation. This experimental evidence suggests that AGE-formation, as a consequence of high glucose levels either *in vivo* or *in vitro*, may be an important factor affecting bone development. However, the direct effect of protein-AGEs on bone-derived cell types in culture has not been studied previously.

In this study we investigate the effect of AGE products on the proliferation and differentiation of osteoblast-like cells in culture.

Materials and methods

Preparation of AGE-protein

Fatty acid-free bovine serum albumin (BSA) (Sigma) was incubated in phosphate buffered saline (PBS) pH 7.4, with 100 mM glucose-6-phosphate (G-6-P) at 37°C for 6 weeks in the presence of protease inhibitor 1.5 mM phenylmethylsulfonyl fluoride, and antibiotics (100 U/ml penicillin – 100 μg/ml streptomycin). This procedure has been previously applied using alternatively glucose or G-6-P [18, 28, 29]. Control BSA was incubated under the same conditions without G-6-P. At the end of the incubation period, BSA and AGE-BSA were separated from low molecular weight molecules by centrifugation/filtration with Centricon 10 kDa cut-off filter cartridges. The formation of AGE was assessed by its 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 × 1 cm quartz cells were used and spectra were recorded on a Linseis 1600 x-y recorder [14]. 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.

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 Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. This cell line has been shown to conserve certain characteristics of osteoblast phenotype [30]. After 5–7 days, the cells were subcultured using trypsin-EDTA to replate the cells and begin the experiments. Cells were split at subconfluence and plated in 24-well plates (2.5 10^4 cells/well) in 10% FBS-DMEM. Subconfluent cells were serum deprived, washed with DMEM and incubated in 0.5 ml DMEM/well with either BSA or AGE-BSA (50–1000 μg/ml), for different incubation periods. These doses were chosen since the bioactivity of AGE-products occur at these concentrations in cells which have been demonstrated to possess AGE receptors [20, 21, 31]. Similarly, 5–1000 μg/ml AGE-BSA have been previously used for the displacement of ^{125}I-AGE-BSA in radioreceptor competition studies [18, 32]. At the end of each incubation, cells were washed twice with PBS and processed for differentiation and proliferation assays.

MC3T3E1 mouse calvaria-derived cells were grown in DMEM-10% FBS and antibiotics, and passaged every 3–4 days. Previous studies have demonstrated that expression of osteoblastic markers begins after incubating these cells for 15 days with medium supplemented by 5 mM β-glycerol phosphate and 25 μg/ml ascorbic acid [33]. However, the cells only undergo active replication during the first two weeks of incubation. Thus, in order to test the effect of AGE on the proliferation and differentiation of this cell line, different protocols were chosen. For studies characterizing the effect of protein-AGE on cell proliferation, cells were plated in 24-well plates (2.0 10^4 cells/well) and cultured for 2 days in 10% FBS-DMEM. After this period, they were serum deprived and incubated with either BSA or AGE-BSA as described for UMR106 cells. To assess the effect of protein-AGE on MC3T3E1 differentiation, cells were cultured in 10% FBS-DMEM media supplemented with 5 mM β-glycerol phosphate and 25 μg/ml ascorbic acid for 4 weeks, changing the medium every 3 days. This medium was then replaced for DMEM without serum and different concentrations of either BSA or AGE-BSA, and incubated for different periods of time as before. Osteoblastic phenotype was evaluated by alkaline phosphatase activity.

Cell proliferation bioassay

The crystal violet mitogenic bioassay [34] was carried out as we have described previously [35]. Briefly, cells were fixed with 5% glutaraldehyde and stained with 0.5% crystal violet. Absorbance was read at 540 nm after dye extraction with glycine/HCl buffer, pH 3.0/30% methanol. We have previously shown that the absorbance values obtained with the crystal violet assay correlate directly with the cell count ($r = 0.897$, $p < 0.001$) [35].

Cell differentiation assay: Alkaline phosphatase specific activity (ALP)

The cell monolayer was solubilized in 0.5 ml 0.1% Triton-X100. Aliquots of the total cell extract were saved for protein determination using the Bradford method [36], and for measurement of ALP as we have described previously [35]. ALP is known to be a marker of mature osteoblast phenotype and this method has been extensively used to assess differentiation in these cell types [35, 37].

Calcium uptake

$^{45}Ca^{2+}$ uptake was measured by incubating cells with $^{45}CaCl_2$ (28 Ci/mmol, DUPONT), 0.5 μCi/ml at 37°C/30 min. After this incubation, cells were rinsed twice with cold PBS and solubilized with 0.1% Triton-X100. An aliquot was saved for protein determination. The remaining extract was counted by liquid scintillation counting [33].

Statistical analysis

At least three experiments were run by triplicate for each experimental condition. Results are expressed as percentage of control BSA, X ± SEM, n = number of cases. Statistical analysis of the data was performed employing Student's *t* test.

Results

The incubation of BSA with 100 mM G-6-P for prolonged periods of time, resulted in the formation of AGE-modified BSA (AGE-BSA). The appearance of this adduct was determined spectrofluorometrically and typical fluorescence-emission spectra for BSA and AGE-BSA are shown in Fig. 1. Cultured osteoblast-like cells were subsequently exposed to either BSA or AGE-BSA at different doses (50–1000 μg/ml), and for different periods of time (24, 48, 72 or 120 h). Cell proliferation and differentiation were evaluated at the end of each incubation, as described in Materials and methods.

Effect of AGE on osteoblast-like cell proliferation

To assess the time-dependent patterns of AGE-BSA effect on cell proliferation, UMR106 and MC3T3E1 osteoblast-like cells were incubated for different periods of time after exposure to 200 μg/ml BSA or AGE-BSA as described in Materials and methods. Figure 2 shows that AGE induced a biphasic effect: cell proliferation was stimulated as early as 48 h after exposure to AGE-BSA in both cell lines. This effect disappeared with longer incubations: a slight but statistically significant inhibition of UM106 cell proliferation was detected after 3 days of incubation with AGE-BSA, while a similar effect occured after 5 days of incubation in the case of MC3T3E1 cells.

The effect of different AGE-BSA concentrations on cell proliferation was investigated after 2 days of incubation. AGE-BSA (100–500 μg/ml) significantly stimulated UMR106 cell proliferation in a biphasic manner, ranging from 112–118 % of control BSA (Fig. 3A). On the other hand, AGE-modified BSA induced a dose-dependent increase in MC3T3E1 cell proliferation (Fig. 3B), ranging from 107–112% of control BSA.

Effect of AGE on osteoblast-cell differentiation

In order to evaluate the effect of AGE-protein on the differentiation of osteoblast phenotype, cells were incubated for 1–3 days in the presence of either BSA or AGE-BSA at different doses.

After 24 h incubation, AGE-BSA increased UMR106 cell differentiation in a dose-response manner, as assessed by the specific activity of ALP (Fig. 4A). This effect was in the range of 125–245% over control BSA. After 48 h incubation, there were no significant differences between the effect of BSA and AGE-BSA on cell differentiation. A longer incubation period (i.e. 72 h) in the presence of AGE-BSA induced a significant inhibition of ALP activity, ranging from 83–85% of control BSA ($p < 0.01$) (data not shown).

The effect of AGE-protein was also investigated in the normal MC3T3E1 osteoblast-like cells. In this line, a biphasic stimulation of cell differentiation was observed after 72 h incubation in the presence of 100–500 μg/ml AGE-BSA (Fig. 4B). However, no differences were found between the effect of AGE-BSA and BSA on MC3T3E1 cells at 24 and 48 h incubation (data not shown).

Effect of AGE on calcium uptake

Calcium uptake experiments were performed to test the participation of Ca^{2+} in the regulation of the proliferation and/or differentiation of UMR106 osteoblast-like cells by protein-AGEs. After 24 h incubation, AGE-BSA inhibited $^{45}Ca^{2+}$ uptake at 100 and 200 μg protein/ml, compared to BSA (Table 1). A slight though non-significant decrease was observed at 500 and 1000 μg/ml, suggesting a biphasic regulation of calcium uptake. These changes in $^{45}Ca^{2+}$ uptake were inversely associated with the ALP activity of UMR106 cells incubated under the same conditions. A similar study was performed after incubating the cells for 48 and 72 h with either BSA or AGE-BSA in the media. No significant differences

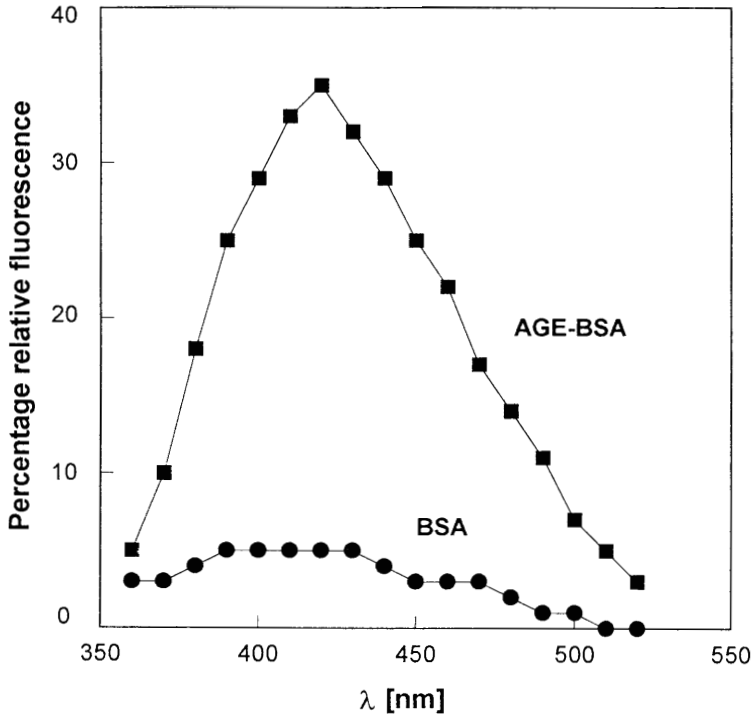

Fig. 1. Fluorescence-emission spectra of BSA and AGE-BSA ($\lambda_{excitation}$ = 340 nm).

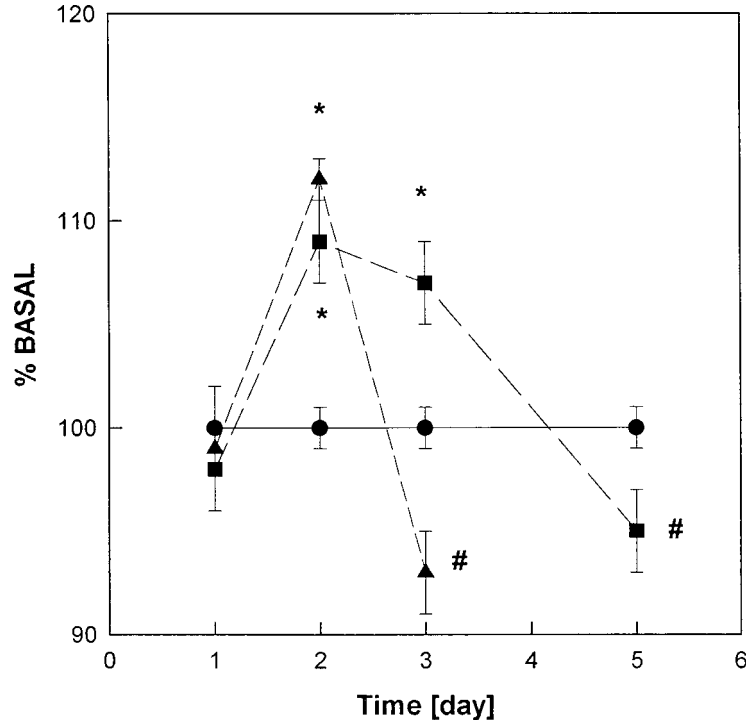

Fig. 2. Time course of AGE-BSA effect on UMR106 and MC3T3EI cell proliferation. Cultures were exposed to 200 µg/ml of control BSA (●) or AGE-BSA for either UMR106 (▲) or MC3T3E1 (■) osteoblast-like cells, for different incubation periods. After this incubation, cell proliferation was assessed by the cristal violet bioassay. Differences between AGE-BSA and control BSA are: $^{\#}p < 0.05$; $^{*}p < 0.001$.

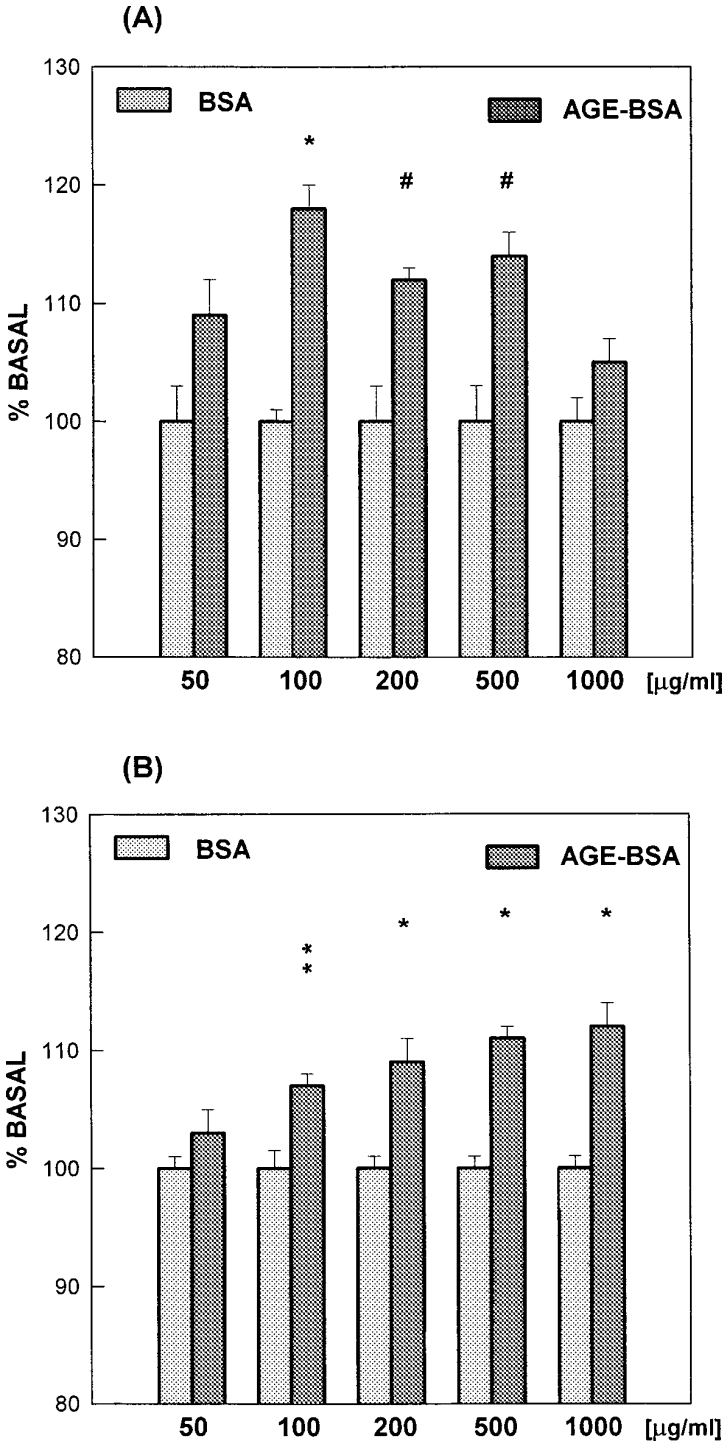

Fig. 3. Cell proliferation of UMR106 (A) and MC3T3E1 (B) osteoblast-like cells incubated with BSA or AGE-BSA for 48 h (n = 9), Differences between BSA and AGE-BSA are: $^{\#}p < 0.01$, $^{**}p < 0.002$, $^{*}p < 0.001$.

were found for these incubation periods and over the range of protein concentrations tested. However, a trend by AGE-BSA to increase $^{45}Ca^{2+}$ uptake was observed after 48 h incubation at all the protein concentrations assayed (112–140% control BSA).

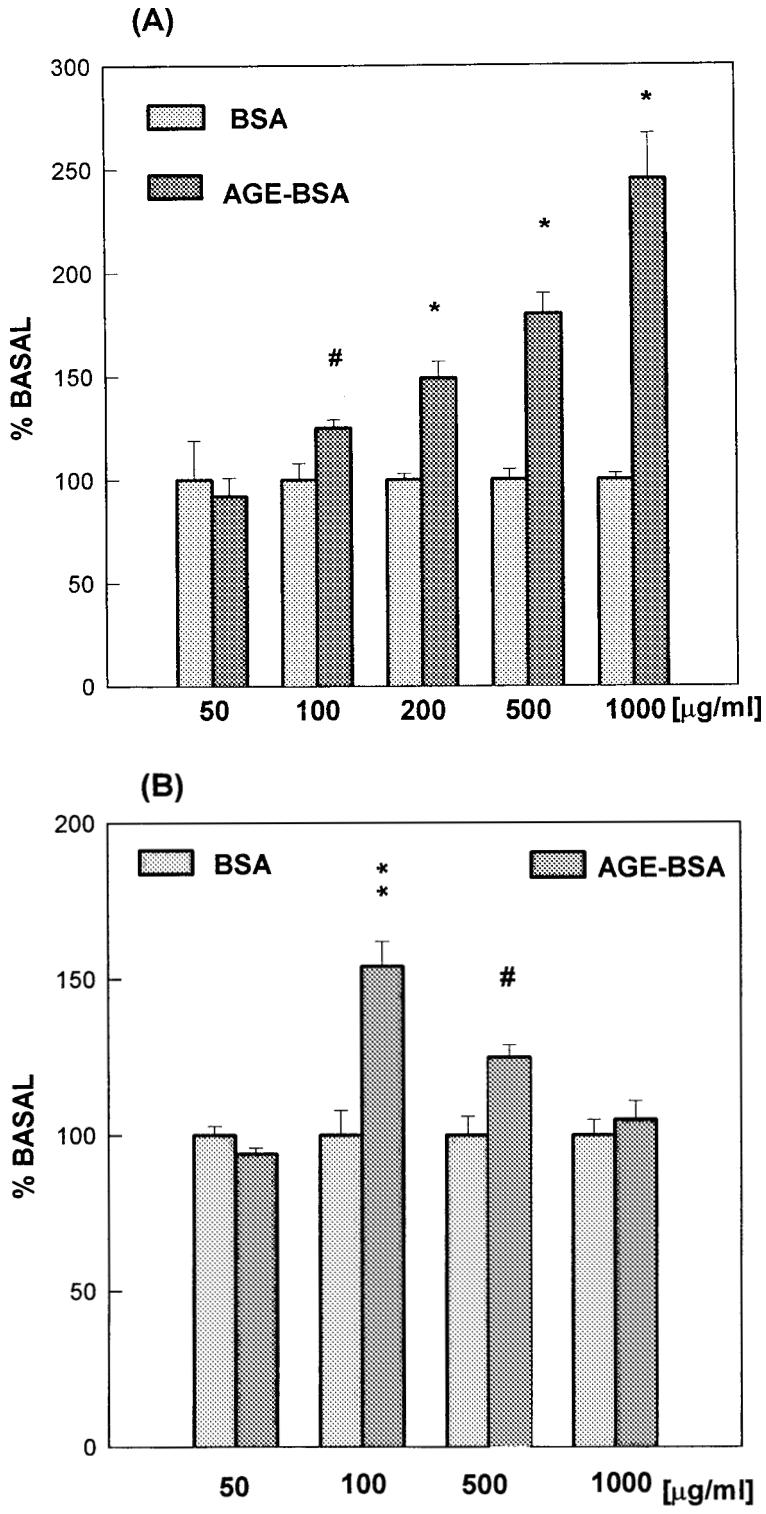

Fig. 4. Effect of BSA or AGE-BSA on ALP specific activity of UMR106 (A) and MC3T3E1 (B) osteoblast-like cells incubated for 24 h (n = 12) and 72 h (n = 6), respectively. Differences between BSA and AGE-BSA are: $^{\#}p < 0.02$, $^{**}p < 0.01$, $^{*}p < 0.001$.

Table 1. $^{45}Ca^{2+}$ uptake by UMR106 cells cultured with AGE-BSA for 24 h

AGE-BSA [μg/ml]	% control BSA (n = 9)	p values (vs control BSA)
100	42 ± 10	< 0 001
200	62 ± 8	< 0 05
500	73 ± 7	N.S.
1000	80 ± 8	N.S.

N.S.: not significant.

Discussion

Our present results show a direct effect of AGE-BSA on the proliferation and differentiation of cultured osteoblast-like cells. Despite intense research, few AGE molecular structures are known in detail, and of these fewer still have been demonstrated to exist *in vivo*. We chose to prepare AGE-protein by means of a prolonged incubation of BSA and Glucose-6-phosphate, because this leads to the formation of a mixture of intermediate and late AGEs that is believed to be representative of the spectrum of adducts existing *in vivo* [38]. Moreover, AGE-BSA has been extensively used to study the changes induced by protein advanced glycation endproducts on several cell lines and tissues [18, 28, 29, 38].

The effect of AGE-BSA on osteoblast-like cell proliferation, assayed by the crystal violet method, was time-dependent (Fig. 2): it remained unchanged after relatively short incubation periods (24 h), increased after intermediate periods (2 or 3 days for UMR106 or MC3T3E1 cells, respectively), but was found to be depressed after 3 or 5 days incubation. These results suggest that AGE-BSA induces an early and transient mitogenic effect on osteoblast-like cells, followed by an impaired osteoblastic progression. Furthermore, the increase in cell proliferation induced by protein-AGE was dependent on its concentration in the culture media (Fig. 3). This stimulation was observed in a narrow range of concentrations (100–500 μg/ml) in the UMR106 osteosarcoma-derived cells, whereas it was dose-dependent in the normal MC3T3E1 osteoblast-like cultures.

When ALP activity was studied, cells exposed to AGE-BSA initially increased their ALP specific activity. This was observed when UMR106 or MC3T3E1 cells were incubated for 24 or 72 h respectively (Fig. 4). However, longer culture periods neutralized this short-term increase and finally lead to a decrease in UMR106 ALP activity (72 h). Analysis of the dose relationship shows a difference between the two cell lines. A dose-dependent response was observed in the osteosarcoma line whereas a biphasic response to AGE-BSA was detected in the normal osteoblastic cell line.

With the exception of the increase in alkaline phosphatase specific activity after 24 or 72 h for UMR106 or MC3T3E1 cells, respectively, all of the observed effects were relatively small. However, they are of the same order as those observed after the incubation of UMR106, MC3T3E1 [35, 41] or primary osteoblast cultures with growth factors and insulin-mimetic compounds [35, 41, 42]. Thus, these results seem to indicate that AGE-proteins could act as weak mitogens in the absence of serum in osteoblastic cells in culture.

The binding of protein-AGE to specific plasma membrane receptors has been demonstrated in different systems. For instance, endothelial cells [31], mesangial cells [19], fibroblasts [24] and mouse monocyte-derived RAW 264.7 cells have been shown to possess these specific receptors. In this last cell line [40], the measured binding constant was identical to that of murine peritoneal macrophages ($3.1\ 10^7\ M^{-1}$) [18, 32]. In these experiments, concentrations of AGE-BSA between 5 and 1000 μg/ml were used to displace an ^{125}I-AGE-BSA radioligand. In our experiments with osteoblast-like cultures, the AGE-BSA doses we used were in the same range as those of the competition studies above mentioned. Even though the presence of an AGE-receptor has not been demonstrated in osteoblastic cells, the pattern of certain observed effects such as the time-course, dose-dependence and the nature of AGE-protein employed, are similar to those observed in cells known to possess AGE-receptors [19, 21, 24, 31].

Several studies have established that in vitro AGE-modified proteins regulate cell proliferation. Thus, AGE induces monocytes to synthesise and release beta interleukin-1 (IL-1β), alpha tumor necrosis factor (TNF-α), platelet-derived growth factor (PDGF) and insulin-like growth factor I (IGF-I) [21]. These factors seem to mediate a growth promoting pathway initiated by the binding of AGE to its specific receptor. From time-dependent course studies, it was determined that the IGF-I mRNA increase appeared after 48 h of AGE-exposure. Furthermore, this induction of IGF-I mRNA was dependent on the concentration of AGE-BSA added, up to 250 μg/ml. Since IL-1β and TNF-α appeared earlier and disappeared within 12 h of cell exposure to protein-AGE, these authors suggest that AGE induces IGF-I indirectly through the release of other cytokines. This mechanism could also occur in osteoblast-like cells, in which IGFs are known to be secreted and are important regulators of cell growth [35, 39]. Similarly, studies carried out with fibroblasts have demonstrated an increase in the mitogenicity of these cells after exposure to AGE modified matrix [24]. In a rat smooth muscle cell culture, an increase in cell proliferation was observed after the specific binding of AGE-modified proteins to their plasma membrane receptors [24]. It has been suggested that these effects could occur via cytokines or growth factors secreted by the cells.

We examined the role of Ca^{2+} in the regulation of differentiation and proliferation of UMR106 osteoblast-like cells by AGE-BSA. Our results indicate that low concentrations of AGE-BSA (100–200 μg/ml) decrease calcium uptake after 24 h incubation. These results support the AGE-induced

decrease of calcium uptake reported by Fong *et al*. in a model of demineralized bone-matrix-induced endochondral bone formation [27]. We found no significant changes in calcium uptake at longer incubation periods. Nevertheless, the alterations induced by AGE-BSA in this parameter appear to follow the same temporal sequence as found in the proliferative effect in UMR106 cells, namely, an increase from 24–48 h while decreasing after longer incubation periods. Since this assay represents a balance between Ca^{2+} intake mechanisms (via calcium channels) and Ca^{2+} extrusion pathways (Ca^{2+}-ATPase and to a lesser extent Na^{+}/Ca^{2+} exchange) [43], any of these mechanisms could be the target of AGE-protein regulation in UMR106 cells.

Our present study is compatible with a long-term deleterious effect of AGEs on osteoblast development. Altogether, our data are in agreement with the observation that *in vitro* formation of AGE on bone matrix leads to an inhibition of osteoblast maturation in a model of demineralized bone-matrix-induced endochondral bone formation [27], and with the reported defects in growth velocity in diabetic animals [13] as well as in human diabetic subjects [1–8].

Our results open the question of the physiological relevance and the possible role of AGE-protein accumulation in diabetic bone matrix. Recently, Tomasek *et al*. [25] demonstrated an increase of collagen-linked fluorescence in cortical bone of diabetic versus control rats. This increase in fluorescence intensity was approximately two-fold. However, the concentration of collagen-AGE in this tissue cannot be accurately estimated by this method, since apparently the most important fluorophores in the AGE family are its early intermediate compounds [32]. This could be done by using either polyclonal immunoassays or a radio-receptor assay for AGE [32]. Nevertheless, the impact of protein-AGE on bone turnover, if it is relevant *in vivo*, will not only depend on its concentration but also on the time of exposure of the bone cells to the AGE products. Our results seem to indicate that the normal balance between bone resorption and formation could be perturbed by exposure of osteoblast cells to an excess of AGE-protein adducts.

Protein advanced glycation endproducts have been strongly implicated in the initiation and acceleration of chronic complications in diabetes [24]. Recently, it has been suggested that AGE-induced damage is initiated, in most cases, by an interaction with cell-membrane receptors specific for AGE [18, 19, 24, 31, 32, 40]. Although such a receptor has not been described in osteoblast-like cells, the effects which we now report could be accounted by such a mechanism.

In summary, this study has provided evidence for a direct effect of advanced glycation products on osteoblast-like cells, suggesting a role for protein-AGEs in the pathogenesis of bone alterations in uncompensated diabetic patients.

Acknowledgments

SBE is a member of the Carrera del Investigador, CONICET, Argentina, and AMC is a member of the Carrera del Investigador, CICPBA, Argentina. This work was supported by grants from Facultad de Ciencias Exactas, Universidad Nacional de La Plata.

References

1. Levin ME, Boisseau VC, Avioli LV: Effects of diabetes mellitus on bone mass in juvenile and adult-onset diabetes. N Engl J Med 294: 741–245, 1976
2. Rosenbloom AL, Lezotte DC, Weber FT, Gudat J, Heller DR, Weber ML, Klein S, Kennedy BB: Diminution of bone mass in childhood diabetes. Diabetes 26: 1052–1055, 1977
3. Santiago JV, McAlister WH, Ratzan SK, Bussman Y, Haymond MW, Schackelford G, Weldon, W: Decreased cortical thickness and osteopenia in children with diabetes mellitus. J Clin Endocrinol Metab 45: 845–848, 1977
4. Shore RM, Chesney RW, Mazess RB, Rose PG, Bargman GJ: Osteopenia in juvenile diabetes. Calcif Tissue Int 33: 455–457, 1981
5. Hui SL, Epstein S, Johnston CC Jr: A prospective study of bone mass in patients with type I diabetes. J Clin Endocrinol Metab 60: 74–80, 1985
6. McNair P, Madsbad S, Christensen MS, Christiansen C, Faber OK, Binder C, Transbol I: Bone mineral loss in insulin-treated diabetes mellitus: studies on pathogenesis. Acta Endocrinol (Copenh) 90: 463–472, 1979
7. Mathiassen B, Nielsen S, Ditzel J, Rodbro P: Long-term bone loss in insulin-dependent diabetes mellitus. J Intern Med 227: 325–327, 1990
8. Krakauer JC, McKenna MJ, Buderer NF, Rao DS, Whitehouse FW, Parfitt AM: Bone loss and bone turnover in diabetes. Diabetes 44: 775–782, 1995
9. Wiske PS, Wentworth SM, Norton JA, Epstein S, Johnston CC: Evaluation of bone mass and growth in young diabetics. Metabolism 31: 848–854, 1982
10. Kayath MJ, Dib SA, Vieiaa JG: Prevalence and magnitude of osteopenia associated with insulin-dependent diabetes mellitus. J Diabetes Complic 8: 97–104, 1994
11. Renzende AA, Petenusci SO, Urbinati EC, Leone FA: Kinetic properties of osseous plate alkaline phosphatase from diabetic rats. Com Biochem Physiol A 104: 469–474, 1993
12. Fargcs S, Halmos T, Salumon F: Bone changes in diabetes mellitus. Isr J Med Sci: 782–783, 1972
13. Weiss RE, Reddi AH: Influence of experimental diabetes and insulin on matrix-induced cartilage and bone differentiation. Am J Physiol 238: E200–E207, 1980
14. Monnier VM, Cerami A: Nonenzymatic browning *in vivo*: possible process for aging of long-lived proteins. Science 211: 491–493, 1981
15. Monnier VM, Vishwanath V, Frank KE, Elmets CA, Dauchot P, Kohn RR: Relation between complications of type I diabetes mellitus and collagen-linked fluorescence. N Engl J Med 314: 403–408, 1986
16. Monnier VM, Kohn RR, Cerami A: Accelerated age-related browning of human collagen in diabetes mellitus. Proc Natl Acad Sci USA 81: 583–587, 1984
17. Beisswenger PJ, Makita Z, Curphey TJ, Moore LL, Jean S, Brinck-Johnsen T, Bucala R, Vlassara H: Formation of immunochemical advanced glycosylation end products precedes and correlates with early

manifestations of renal and retinal disease in diabetes. Diabetes 44: 824–829, 1995
18. Vlassara H, Brownlee M, Cerami A: High-affinity receptor-mediated uptake and degradation of glucose-modified proteins: a potential mechanism for the removal of senescent macromolecules. Proc Natl Acad Sci USA 82: 5588–5592, 1985
19. Skolnik KY, Yang Z, Makita Z, Radoff S, Vlassara H: Human and rat mesangial cell receptors for glucose-modified proteins: potential role in kidney tissue remodeling and diabetic nephropathy. J Exp Med 174: 931–939, 1991
20. Vlassara H, Brownlee M, Manogue KR, Dinarello CA, Pasagian A: Cachectin/TNF and IL-1 induced by glucose modified proteins: role in normal tissue remodeling. Science 240: 1546–1548, 1988
21. Kirstein M, Aston C, Hintz R, Vlassara H: Receptor-specific induction of insulin-like growth factor I in human monocytes by advanced glycosylation end product-modified proteins. J Clin Invest 90: 439–446, 1992
22. Imani F, Horii Y, Suthanthiran M, Skolnik KY, Makita Z, Sharma V, Sehajpal P, Vlassara H: Advanced glycosylation end product-specific receptors on human and rat T-lymphocytes mediate synthesis of interferon γ: role in tissue remodeling. J Exp Med 178: 2165–2172, 1993
23. Brownlee M: Glycation and diabetic complications. Diabetes 43: 836–841, 1994
24. Vlassara H, Bucala R, Striker L: Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. Lab Invest 70: 138–151, 1994
25. Tomasek JJ, Meyers SW, Basinger JB, Green DJ, Shew RL: Diabetic and age-related enhancement of collagen-linked fluorescence in cortical bones of rats. Life Sci 55: 855–861, 1994
26. Locatto ME, Abrazon H, Caferra D, Fernandez MC, Alloatti R, Puche RC: Growth and development of bone mass in untreated alloxan diabetic rats. Effects of collagen glycosylation and parathyroid activity on bone turnover. Bone Miner 23: 129–144, 1993
27. Fong Y, Edelstein D, Wang EA, Brownlee M: Inhibition of matrix-induced bone differentiation by advanced glycation end-products in rats. Diabetologia 36: 802–807, 1993
28. Vlassara H, Moldawer L, Chan B. Macrophage/monocyte receptor for nonenzymatically glycosylated proteins is upregulated by cachectin/tumor necrosis factor. J Clin Invest 84, 1813–1820, 1989
29. Yang Z, Makita Z, Horii Y, Brunelle S, Cerami A, Sehajpal P, Suthanthiran M, Vlassara H. Two novel rat liver membrane proteins that bind advanced glycosylation endproducts: relationship to macrophage receptor for glucose-modified proteins. J Exp Med 174: 515–524, 1991
30. Partridge NC, Alcorn D, Michelangeli VP, Ryan G, Martin TJ: Morphological and biochemical characterization of four clonal osteogenic sarcoma cell lines of rat origin. Cancer Res 43: 4308–4312, 1983
31. Esposito C, Gerlach H, Brett J, Stern D, Vlassara H. Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties. J Exp Med 170: 1387–1407, 1989
32. Radoff S, Makita Z, Vlassara H. Radioreceptor assay for advanced glycosylation end products. Diabetes 40: 1731–1738, 1991
33. Quarles LD, Yahay DA, Lever LW, Caton R, Wenstrup RJ: Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an *in vitro* model of osteoblast development. J Bone Min Res 7: 683–692, 1992
34. Okajima T, Nakamura K, Zhang H, Ling N, Tanabe T, Yasuda T, Rosenfeld RR: Sensitive colorimetric bioassay for insulin-like growth factor (IGF) stimulation of cell proliferation and glucose consumption: use in studies of IGF analogs. Endocrinology 130: 2201–2212, 1992
35. Cortizo AM, Etcheverry SB: Vanadium derivatives act as growth factor-mimetic compounds upon differentiation and proliferation of osteoblast-like UMR106 cells. Mol Cell Biochem 145: 97–102, 1995
36. Bradford M: Rapid and sensitive method for quantitation of microgram quantities ofprotein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254, 1976
37. Stein GS, Lian JB: Molecular mechanism mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocrine Rev 14: 424–442, 1993
38. Vlassara H, Fuh H, Makita Z, Krunghrai S, Cerami A, Bucala R: Exogenous advanced glycosylation endproducts induce complex vascular dysfunction in normal animals: a model for diabetic and aging complications. Proc Natl Acad Sci USA 89: 12043–12047, 1992
39. Baylink DJ, Finkelman RD, Mohan S: Growth factors to stimulate bone formation. J Bone Min Res 8: Supp 2, S565–S572, 1993
40. Radoff S, Vlassara H, Cerami A,: Characterization of a solubilized cell surface binding protein on macrophages specific for protein modified nonenzymatically by advanced glycosylation endproducts. Arch Biochem Biophys 263: 418–423, 1988
41. Davidai G, Lee A, Schuartz Y, Hazum E: PDGF induces tyrosine phosphorylation in osteoblast-like cells: relevance to mitogenesis. Am J Physiol 263: E205–E209, 1992
42. Lau K-HW, Tanimoto H, Baylink DJ: Vanadate stimulates bone cell proliferation and bone collagen synthesis in vitro. Endocrinology 123: 2858–2867, 1988
43. Exton JH: Mechanisms of action of calcium-mobilizing agonists: some variations on a young theme. FASEB J 2: 2670–2676, 1988