Inhibitory Effect of Peptide-Free Forms of Advanced Glycation End Products on the Proliferation and Extracellular Matrix Protein Production of Cultured Cells

Kazuhiro Yoshihara, Tomohide Okumura, Tomonobu Yoshida, and Masatoshi Beppu*

School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan

(Received January 29, 2001; Accepted February 16, 2001)

Advanced glycation end products (AGEs), generated by the Maillard reaction, accumulate in long-lived proteins like collagen and are presumed to be involved in aging and the pathogenesis of complications in diabetes. We investigated the effect of peptide-free forms of AGEs that can be released by the catabolism of AGE-proteins on cellular activities using cultured cells and synthetic AGEs, pentosidine and pyrraline. The exposure of cultured fibroblasts to 15 or 30 μM of pentosidine resulted in the partial inhibition of cell proliferation and production of extracellular matrix (ECM) proteins. Pentosidine also inhibited the proliferation and ECM protein production of chondrocytes, but its effect on cell proliferation was weak while that on ECM production was prominent, as compared with the effect of pentosidine. A similar inhibitory effect on cell proliferation was observed when fibroblasts or chondrocytes were exposed to pyrraline. Pentosidine and pyrraline did not cause cell death in these cells. Pentosidine appeared to enter the cells during the culture. These results suggest that peptide-free forms of AGEs affect cell growth and cell metabolism by acting both extracellularly and intracellularly.

Key words —— advanced glycation end product, pentosidine, pyrraline, fibroblast, chondrocyte, collagen

INTRODUCTION

Long-lived proteins are highly susceptible to advanced glycation processes by way of the Maillard reaction.[1,2] The advanced glycation end products (AGEs) formed on such proteins have been implicated as one of the major factors responsible for the pathogenesis of various complications in diseases such as diabetes.[3,4] Collagen is one of the long-lived proteins and is a major constituent of bone, cartilage and dermal tissue. Recent studies have demonstrated that cartilage accumulates pentosidine,[5] a well-identified AGE, compared with other tissues, and its level increases linearly with age after 20 years, whereas it is relatively low in dermal tissue.[6] It is known that collagen in tissues acts as a substratum for cell spreading, and that it modulates various cell activities such as adhesion, morphology, growth, and differentiation.[7] The cell growth-supporting property of collagen is lost by glycation,[8] but the mechanism underlying the restriction of cell proliferation as a result of glycation of collagen has not been clarified. Glycated collagen was found to be metabolized, and some of the AGEs are released, in peptide-free forms, into the circulatory system.[9] Thus, it is of interest to determine whether such free forms of AGEs exert any disadvantageous effect on cells.

In this study, we investigated the effect of the peptide-free form of pentosidine[10] on cultured fibroblasts and chondrocytes *in vitro*, particularly on their growth and extracellular matrix (ECM) protein production. The effect of another AGE, pyrraline,[11] was also examined.

MATERIALS AND METHODS

Reagents ——Dulbecco's modified Eagle's Medium (DMEM), penicillin G-streptomycin sulfate mixed solution, fetal bovine serum (FBS), and 0.05% trypsin-0.53 mM EDTA solution were purchased from GIBCO BRL (Grand Island, NY, U.S.A.). Collagenase S-1 was obtained from Nitta Gelatin Co. (Japan). A Cell Counting Kit-8 was purchased from

*To whom correspondence should be addressed: Laboratory of Public Health, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan. Tel.: +81-426-76-4495; Fax: +81-426-76-4499; E-mail: beppum@ps.toyaku.ac.jp

Dojindo Laboratories (Japan). [6-^{3}H] Thymidine was purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K.).

Preparation of Pentosidine and Pyrraline —— Pentosidine was prepared according to the method previously reported.[12] Pyrraline was prepared according to the method of Portero-Otin *et al*. with slight modifications.[13] These synthesized products were confirmed to be pure compounds according to ^{1}H-nuclear magnetic resonance (NMR) spectrometry and electrospray ionization (ESI) mass spectrometry.

Cell Preparation and Culture —— Human epidermal fibroblasts were purchased from Asahi Techno Glass (Japan). Articular chondrocytes were isolated from the knee and shoulder joint cartilage of a New Zealand white rabbit (6-week-old) after it had been administered an anesthesia according to the method of Green[14] with slight modifications. The handling of animals to obtain the chondrocytes was approved by the local animal ethics committee at the School of Pharmacy, Tokyo University of Pharmacy and Life Science. Fibroblasts and chondrocytes were cultured in DMEM supplemented with 1% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate at 37°C in a humidified 5% CO_2 atmosphere using 96-well culture plates (2×10^5 cells/well/0.2 ml) and 24-well culture plates (1×10^4 cells/well/ml), respectively. All cells used in this study were taken from the culture between passages 2 and 4.

Measurement of Cell Proliferation —— Cell proliferation was assessed by the measurement of viable cells or [^{3}H] thymidine incorporation.

The number of viable cells in culture was assessed using a Cell Counting Kit-8 which colorimetrically measured intracellular NADH,[15] according to the manufacturer's instruction.

[^{3}H] Thymidine Incorporation into cell DNA was measured as described below. On day 1, 3 or 5, 1 μCi (3.7×10^4 Bq) of [6-^{3}H] thymidine (74 GBq/mmol) was added to the medium of fibroblasts in culture. After 12 hr, the medium was removed and the remaining cells were dissolved with 1 ml of 0.2 M NaOH for 30 min. Then, 20 μl of 1% bovine serum albumin (BSA) solution and 500 μl of ice-chilled 20% trichloroacetic acid (TCA) were added. The TCA precipitate was collected onto a glass fiber filter (GF/C, Whatman) and was successively washed with 10% TCA and methanol. The radioactivity on the filter was measured in a scintillator (Econofluor®-2, Packard Instrument Co., CT, U.S.A.) using a liquid scintillation counter (LSC-3500, Aloka Co., Japan).

Determination of Extracellular Matrix Content —— The amounts of collagen and proteoglycan produced by fibroblasts and chondrocytes into the culture medium were determined using a test kit (Biocolor, U.S.A.) according to the manufacturer's instructions.

Evaluation of Intracellular Pentosidine Concentration —— The intracellular pentosidine content of fibroblasts following its internalization was determined by high-performance liquid chromatography (HPLC).[15] After the cells cultured with or without pentosidine were washed with 10 mM phosphate-buffered saline, pH 7.3 (PBS), several times, they were destroyed by ultrasound sonication. Immediately thereafter, the destroyed cells were transferred to a microtube and then centrifuged at $9000 \times g$ for 20 min. The supernatant obtained was subjected to HPLC analysis for pentosidine.

Morphological Evaluations —— The morphology of the cells treated with pentosidine was observed by phase-contrast microscopy. The occurrence of apoptosis was determined by staining the cell nuclei using Hoechst 33258, according to the method of Oberhammer *et al*.[16]

Statistical Analysis —— The data obtained were expressed as the mean ± S.D. of 3 determinations. An unpaired *t*-test was used to assess differences between the control and AGEs treatment. A p value of less than 0.05 was considered statistically significant.

RESULTS

Human fibroblasts proliferated linearly in proportion to the cultivation period, up to 5 days, as assessed by the intracellular NADH content of viable cells (Fig. 1A, circle). The rate of cell proliferation was significantly decreased following treatment with 15 or 30 μM pentosidine for 3 to 5 days (Fig. 1A, triangle and square). The inhibitory effect of pentosidine on the proliferation of fibroblasts was confirmed by [^{3}H] thymidine incorporation into the growing fibroblasts. The radioactivity incorporated into the fibroblasts treated with 15 or 30 μM of pentosidine was significantly lower than that in the control (Fig. 1B). The production of ECM protein collagen and proteoglycan from the fibroblasts into the culture medium was significantly inhibited by pentosidine (Fig. 2A and B). We then investigated whether another type of cell is susceptible to the in-

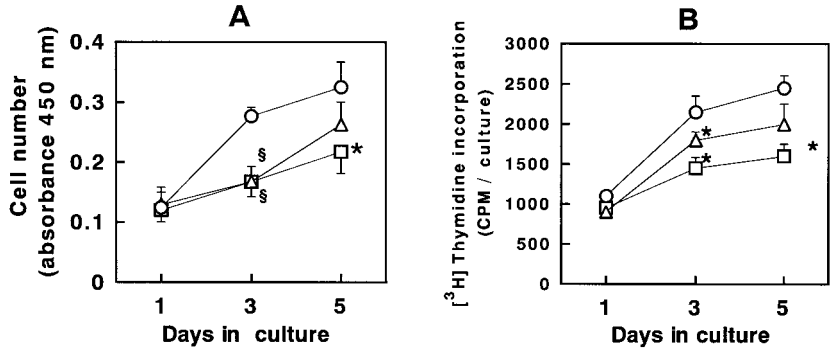

Fig. 1. Effect of Pentosidine Exposure on the Proliferation of Fibroblasts

Human fibroblasts were cultured in the absence or presence of pentosidine for the indicated days. Pentosidine was added to the culture medium at the time of seeding of the cell. Cell proliferation was assessed by the colorimetric method (A) and [^{3}H] thymidine incorporation (B), as described in MATERIALS AND METHODS. ○, control; △, 15 μM pentosidine; □, 30 μM pentosidine. Each point and bar indicates the mean value of triplicate determinations and standard deviations, respectively. *$p < 0.05$ vs. control, §$p < 0.01$ vs. control.

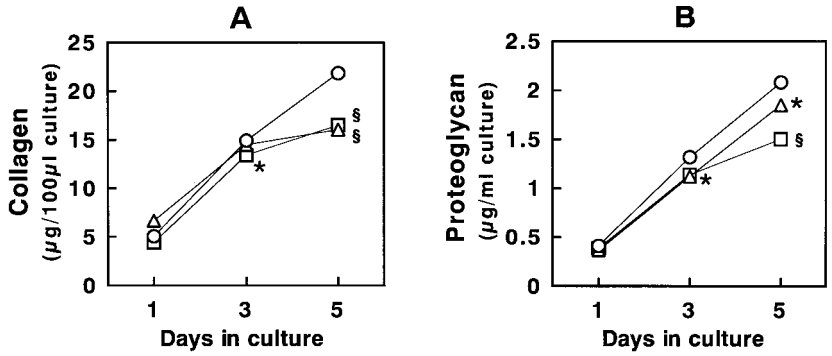

Fig. 2. Effect of Pentosidine Exposure on ECM Protein Production by Cultured Fibroblasts

A, collagen; B, proteoglycan. ○, control; △, 15 μM pentosidine; □, 30 μM pentosidine. Data are presented as described in the legend to Fig. 1.

hibitory action of pentosidine, using rabbit articular chondrocytes. The proliferation of chondrocytes was only slightly inhibited by pentosidine at 30 μM (Fig. 3). In contrast, the production of collagen and proteoglycan was strongly inhibited by pentosidine at 15 and 30 μM (Fig. 4A and B). A peptide-free form of pyrraline, another AGE, was also examined for growth inhibitory action on fibroblasts and chondrocytes. The cell proliferation of fibroblasts was strongly inhibited by 15 and 30 μM of pyrraline throughout the culture period, except at 15 μM on day 5 (Fig. 5A). However, such an inhibitory effect was not observed in the case of chondrocytes (Fig. 5B).

Neither morphological changes nor apoptosis were observed for the fibroblasts and the chondrocytes cultured in the presence of pentosidine or pyrraline (data not shown). Hence, the cells were not likely to be dying under the conditions employed.

The extracellularly added peptide-free form of pentosidine appeared to enter the cells, since a significant amount of pentosidine was found to be associated with the cells (0.95 pmol/1 $\times$ 10^5 cells)

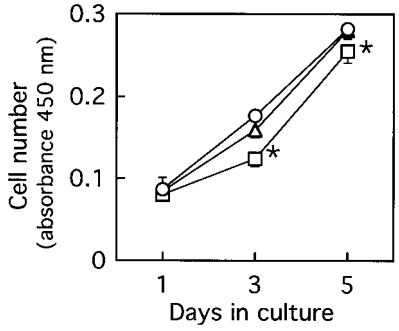

Fig. 3. Effect of Pentosidine Exposure on the Proliferation of Chondrocytes

Rabbit chondrocytes were cultured in the absence or presence of pentosidine, and their proliferation was assessed by the colorimetric method as described in MATERIALS AND METHODS. ○, control; △, 15 μM pentosidine; □, 30 μM pentosidine. Data are presented as described in the legend to Fig. 1.

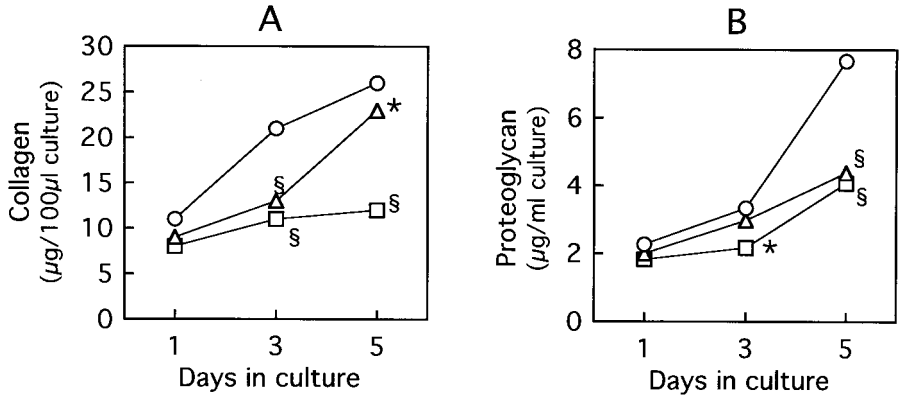

Fig. 4. Effect of Pentosidine Exposure on ECM Protein Production by Cultured Chondrocytes
A, collagen; B, proteoglycan. ○, control; △, 15 μM pentosidine; □, 30 μM pentosidine. Data are presented as described in the legend to Fig. 1.

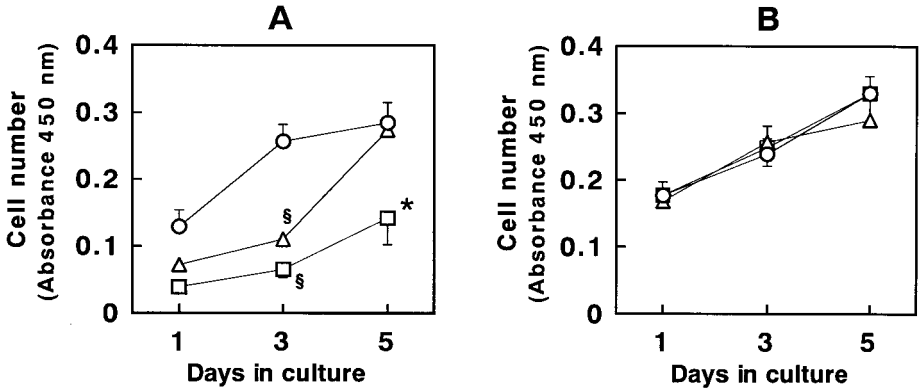

Fig. 5. Effect of Pyrraline Exposure on the Proliferation of Fibroblasts (A) and Chondrocytes (B)
Human fibroblasts and rabbit chondrocytes were cultured in the absence or presence of pyrraline, and their proliferation was assessed by the colorimetric method as described in MATERIALS AND METHODS. Data are presented as described in the legend to Fig. 1.

when the fibroblasts were exposed to 15 μM of pentosidine for 5 days.

DISCUSSION

The present study is the first to examine the effect of peptide-free forms of AGEs on cellular activities. Pentosidine was used as a glycoxidation product,[17] an AGE generated by glycation involving oxidative pathways, and pyrraline was used as an AGE formed via a non-oxidative pathway.[18]

Until now, the effects of AGEs *in vitro* have been evaluated using AGE-proteins. AGE-BSA was reported to have an inhibitory effect on the calcium signaling of glomerular cells,[19] and to increase the expression of mRNA for vascular endothelial growth factor in human retinal pigment epithelial cells.[20] Kawano *et al.*[8] demonstrated that the proliferation of fibroblasts *in vitro* was suppressed when they were plated on AGE-collagen. It was also reported that the exposure of human fibroblasts to AGE-modified β_2-microglobulin and albumin resulted in a decrease in collagen synthesis.[21] Although the mechanisms of the inhibitory actions of AGE-proteins have not been elucidated, it is possible that AGE-proteins affected the cellular activities through binding to the cell surface, since receptors for AGEs have been reported to be present on various types of cells.[22]

The present study indicated that peptide-free forms of AGEs also suppress cellular activities. Free pentosidine was inhibitory against growth and ECM protein production in fibroblasts and chondrocytes. In the case of fibroblasts, while growth inhibition was observed on day 3 and later, the decreased production of collagen and proteoglycan was observed only on day 5. Therefore, it is conceivable that the decrease in the production of the extracellular matrix proteins is simply due to the decrease in cell growth. On the other hand, in the case of

chondrocytes, the decrease in the production of ECM proteins was much greater than that in cell growth. Hence, the production of these ECM proteins may have been suppressed by the inhibition of protein synthesis and/or their secretion.

Chondrocytes were less susceptible to the growth inhibitory action of pentosidine and pyrraline than fibroblasts. The susceptibility of cells to the toxic actions of AGEs may be different by cell type.

Free pentosidine was suggested to enter the cells. Thus, it is possible that the observed inhibitory effects of pentosidine resulted from the binding of pentosidine to some intracellular components regulating cell growth and metabolism, as well as from binding to cell-surface receptors for AGEs.

The level of the peptide-free form of pentosidine in plasma is very low. We previously reported that the total concentration of plasma pentosidine, including peptide-bound and -free forms, was below 500 nM in the healthy control.[12] On the other hand, plasma pyrraline in the peptide-free form amounted to 80 ± 30 nM in healthy control subjects and 440 ± 173 nM in uremic patients.[23] Therefore, the micromolar order concentration of the free forms of AGEs may not arise in biological fluids under physiological conditions. However, oxidative stress can cause and accelerate pentosidine formation, as seen in rheumatoid arthritis,[24,25] uremic[26–28] and diabetic subjects,[27,29] in which markedly high AGE levels in plasma were observed. Therefore, the concentrations of AGEs employed in the present study could occur locally under unhealthy conditions. Further investigation is necessary to elucidate the role of peptide-free AGEs in the etiology of abnormalities in cell behavior, growth and metabolism.

Acknowledgements We thank Akiko Hayashi for her technical assistance. This work was supported in part by a grant for private universities provided by the Japan Private School Promotion Foundation.

REFERENCES

1) Maillard, L. C. (1912) Action des acides amines sur les sucres: Formation des melanoidines par voie methodique. *C. R. Acad. Sci.*, **154**, 66–68.
2) Nagaraj, R. H. and Sady, C. (1996) The presence of a glucose-derived Maillard reaction product in the human lens. *FEBS Lett.*, **382**, 234–238.
3) Vlassara, H., Bucala, R. and Striker, L. (1994) Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Lab. Invest.*, **70**, 138–151.
4) Schleicher, E. D., Wangner, E. and Nerlich, A. G. (1997) Increased accumulation of the glycoxidation product N(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. *J. Clin. Invest.*, **99**, 457–468.
5) Bank, R. A., Bayliss, M. T., Lafeber, F. P., Maroudas, A. and Tekoppele, J. M. (1998) Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage. The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage. *Biochem. J.*, **330**, 345–351.
6) Takahashi, M., Hoshino, H., Kushida, K. and Inoue, T. (1995) Direct measurement of crosslinks, pyridinoline, deoxypyridinoline, and pentosidine, in the hydrolysate of tissues using high-performance liquid chromatography. *Anal. Biochem.*, **232**, 158–162.
7) Kleinman, H. K., Klebe, R. J. and Martin, G. R. (1981) Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.*, **88**, 473–85.
8) Kawano, E., Takahashi, S., Sakano, Y. and Fujimoto, D. (1990) Nonenzymatic glycation alters properties of collagen as a substratum for cells. *Matrix*, **10**, 300–305.
9) Portero-Otin, M., Pamplona, R., Bellmunt, M. J., Bergua, M., Nagaraj, R. H. and Prat, J. (1997) Urinary pyrraline as a biochemical marker of non-oxidative Maillard reactions in vivo. *Life Sci.*, **60**, 279–287.
10) Sell, D. and Monnier, V. M. (1989) Structure elucidation of a senescence cross-link from human extracellular matrix: Implication of pentoses in the aging process. *J. Biol. Chem.*, **264**, 21597–21602.
11) Njoroge, F. G., Sayre, L. M. and Monnier, V. M.(1987) Detection of D-glucose-derived pyrrole compounds during Maillard reaction under physiological conditions. *Carbohydr. Res.*, **167**, 211–220.
12) Yoshihara, K., Nakamura, K., Kanai, M., Nagayama, Y., Takahashi, S., Saito, N. and Nagata, M. (1998) Determination of urinary and serum pentosidine and its application to elder patients. *Biol. Pharm. Bull.*, **21**, 1005–1008.
13) Portero-Otin, M., Nagaraj, R. H. and Monnier, V. M. (1995) Chromatographic evidence for pyrraline formation during protein glycation in vitro and in vivo. *Biochem. Biophys. Acta*, **1247**, 74–80.
14) Green, W. T., Jr. (1971) Behavior of articular chondrocytes in cell culture. *Clin. Orthop.*, **75**, 248–260.
15) Ishiyama, M., Miyazono, Y., Sasamoto, K., Ohkura, Y. and Ueno, K. (1997) A highly water-soluble

disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta*, **44**, 1299–1305.
16) Oberhammer, F. A., Pavelka, M., Sharma, S., Tiefenbacher, R., Purchio, A. F., Bursch, W. and Schulte-Hermann, R. (1992) Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta 1. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 5408–5412.
17) Fu, M.X., Wells-Knecht, K. J., Blackledge, J.A., Lyons, T.J., Thorpe, S. R. and Baynes, J. W. (1994) Glycation, glycoxidation, and cross-linking of collagen by glucose: Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. *Diabetes*, **43**, 676–683.
18) Hayase, F., Nagaraj, R. H., Miyata, S., Njoroge, F. G. and Monnier, V. M. (1989) Aging of proteins: Immunological detection of a glucose-derived pyrrole formed during Maillard reaction *in vivo*. *J. Biol. Chem.*, **264**, 3758–3764.
19) Mene, P., Pascale, C., Teti, A., Bernardini, S., Cinotti, G. A. and Pugliese, F. (1999) Effects of advanced glycation end products on cytosolic Ca^{2+} signaling of cultured human mesangial cells. *J. Am. Soc. Nephrol.*, **10**, 1478–1486.
20) Lu, M., Kuroki, M., Amano, S., Tolentino, M., Keough, K., Kim, I., Bucala, R. and Adamis, A. P. (1998) Advanced glycation end products increase retinal vascular endothelial growth factor expression. *J. Clin. Invest.*, **101**, 1219–1224.
21) Owen, W. F., Jr., Hou, F. F., Stuart, R. O., Kay, J., Boyce, J., Chertow, G. M. and Schmidt, A. M. (1998) β_2-microglobulin modified with advanced glycation end products modulates collagen synthesis by human fibroblasts. *Kidney Int.*, **53**, 1365–1373.
22) Thornalley, P. J. (1998) Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. *Cell. Mol. Biol.*, **44**, 1013–1023.
23) Odani, H., Shinzato, T., Matsumoto, Y., Takai, I., Nakai, S., Miwa, M., Iwayama, N., Amano, I. and Maeda, K. (1996) First evidence for accumulation of protein-bound and protein-free pyrraline in human uremic plasma by mass spectrometry. *Biochem. Biophys. Res. Commun.*, **224**, 237–241.
24) Miyata, T., Ishiguro, N., Yasuda, Y., Ito, T., Nangaku, M., Iwata, H. and Kurokawa, K. (1998) Increased pentosidine, an advanced glycation end product, in plasma and synovial fluid from patients with rheumatoid arthritis and its relation with inflammatory markers. *Biochem. Biophys. Res. Commun.*, **244**, 45–49.
25) Rodriguez-Garcia, J., Requena, J. R. and Rodriguez-Segade, S. (1998) Increased concentrations of serum pentosidine in rheumatoid arthritis. *Clin. Chem.*, **44**, 250–255.
26) Takahashi, M., Kushida, K., Kawana, K., Ishihara, C., Denda, M., Inoue, T. and Horiuchi, K. (1993) Quantification of the cross-link pentosidine in serum from normal and uremic subjects. *Clin. Chem.*, **39**, 2162–2165.
27) Taneda, S. and Monnier, V. M. (1994) ELISA of pentosidine, an advanced glycation end product, in biological specimens. *Clin. Chem.*, **40**, 1766–1773.
28) Odetti, P., Fogarty, J., Sell, D. R. and Monnier, V. M. (1992) Chromatographic quantitation of plasma and erythrocyte pentosidine in diabetic and uremic subjects. *Diabetes*, **41**, 153–159.
29) Gugliucci, A. and Menini, T. (1998) Circulating advanced glycation peptides in streptozotocin-induced diabetic rats: evidence for preferential modification of IgG light chains. *Life Sci.*, **62**, 2141–2150.