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ORIGINAL CONTRIBUTION

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High glucose and N^{ε}-(carboxymethyl) lysine bovine serum albumin modulate release of matrix metalloproteinases in cultured human endothelial cells

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Summary *Background* Hyperglycaemia may contribute to endothelial dysfunction. Disturbances in endothelial functions include changes in the extracellular matrix underneath the cells. This may result from altered biosynthesis of matrix molecules or from modified biosynthesis and secretion of enzymes involved in the turnover of extracellular matrix. One important class of such enzymes are the matrix metalloproteinases (MMPs). *Aim of the study* The aim of this study was to investigate whether the condition of high glucose concentration relevant both to diabetes type 1 and 2 and metabolic syndrome, would affect the synthesis and release of MMPs in human umbilical cord endothelial cells (HUVEC) in vitro. *Methods* The HUVEC were isolated and cultured in vitro. The cells were exposed to medium with either low glucose (LG, 1 g/l) or high glucose (HG, 4.5 g/l) or the advanced glycation end product (AGE) N^{ε}-(carboxymethyl) lysine bovine serum albumin (CML-BSA), at a concentration of 10 μg/ml. The HUVEC-conditioned media were harvested and subjected to gelatin zymography and Western blotting. *Results* When HUVEC were incubated with HG or CML-BSA under serum free conditions a decreased secretion of pro MMP-2 was observed, both with gelatin zymography and Western blotting. The HUVEC also secreted MMP-9, but at lower levels, and effects of HG treatment were not significant. When HUVEC were stimulated with phorbol 12-myristate 13-acetate (PMA) secretion of pro MMP-2 was not increased, but the activation of pro MMP-2 into lower molecular forms increased, irrespective of culturing in LG, HG or CML-BSA. *Conclusion* The HUVEC exposed to high glucose or AGE exhibit decreased secretion of MMP-2. These findings may be relevant in understanding the altered turnover of the endothelial extracellular matrix observed in the diabetic state.

Key words diabetes – hyperglycaemia – matrix metalloproteinases – advanced glycation end products (AGEs) – endothelial cells

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Introduction

Diabetes can affect the vascular system, giving rise to micro- and macroangiopathy. The Diabetes Control and Complications Trial [1] demonstrated that chronic hyperglycaemia using glycated haemoglobin A_{1c} as indicator, is the most important factor for the development and progression of microvascular complications like nephropathy, retinopathy and peripheral neuropathy in type I and type II diabetes [1, 2]. Chronic hyperglycaemia modulates the glycosylation of protein by the classical enzymatic glycosylation pathways and non-enzymatic glycation reactions. The intermediate and late products of the glycation cascade are a class of heterogeneous sugar-amino acid adducts collectively named advanced glycation end products (AGEs). There are several types of AGEs, among which N^{ε}-(carboxymethyl)lysine (CML) and pentosidine are two of the best characterised [3, 4] The presence of AGEs is closely related to hyperglycaemia, and their properties make them potential effectors in the pathogenesis of diabetes-related complications. [2, 4, 5]. Importantly, plasma levels of pentosidine and CML are elevated in patients with end stage renal disease (ESRD) irrespective of glycaemic status [6].

High glucose levels have been shown in many cell types to trigger signalling cascades leading to changes in the balance between synthesis and degradation of extracellular matrix (ECM) [7–9]. Furthermore, hyperglycaemia leads to formation of AGEs on ECM protein, promoting cross-linking and changes in matrix assembly [10–11]. This may in turn lead to altered properties of the ECM proteins, including disturbed functional properties as well as increasing resistance to proteolytic turnover. As a result normal tissue remodelling and renewal are perturbed. These changes occur physiologically with advancing age and are accelerated in diabetes [11–14].

ECM degradation is important in the pathogenesis of atherosclerosis. At early stages of atherogenesis basement membrane components can be degraded by released proteolytic enzymes including the matrix metalloproteinases (MMPs) and serine proteases [15].

Both endothelial cells and macrophages release MMPs. Collagenase (MMP-1), gelatinase A (MMP-2) and stromelysin are predominantly secreted by endothelial cells [16], while human macrophages have the capacity to secrete several matrix metalloproteinases including interstitial collagenase, stromelysin, and 92-kDa gelatinase B (MMP-9) [17]. The levels of activities of these MMPs have been associated with several clinical manifestations of atherosclerosis including unstable angina and plaque rupture [17–19]. The biological basis of these associations is thought to be MMP-mediated degradation of ECM components in the cap of the atherosclerotic plaques.

MMPs are secreted in a latent proenzyme form and their activities are regulated at several levels, including gene expression, secretion of proenzymes that require activation by proteolysis and inhibition of the tissue inhibitors of metalloproteinases (TIMPs). MMP expression and activity can be modulated by glucose in some cell types such as mesangial cells and bovine aortic endothelial cells [20–21].

The aim of the present investigation was to use primary cultures of human endothelial cells to investigate whether exposure to high glucose or the AGE product CML-bovine serum albumin (CML-BSA) would affect endothelial synthesis and release of serine proteases, MMPs and TIMPs.

Materials and methods

Endothelial cell culture

HUVEC were isolated enzymatically from infant umbilical cords of normal pregnancies under sterile conditions and established as primary cell cultures. The cells were established at 37°C in 5% CO_2 in low glucose (LG) medium, i.e. DMEM with 1 g/l glucose (Sigma Aldrich, Oslo, Norway). The medium was supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 10% foetal bovine serum, 10 IU/ml heparin, 10 μg/ml Endothelial Cell Growth Supplement (ECGS), (BD Biosciences, Bedford, MA, USA). The cells were passaged 1–4 times and allowed to reach 80% confluency before being used in experiments.

Experimental design

The cells were exposed for 3 days to DMEM, with the above supplements but containing either LG; high glucose (HG), i.e. 4.5 g/l of glucose; or LG with in addition 10 μg/ml of CML-BSA, prepared as described [22]. Then from day 3 to 4, the cells were placed in the same three different media as described above, but without adding serum. After incubation for 20–24 h, the conditioned media were collected, centrifuged and stored at −20°C for further use. Media collected for zymography were supplemented with Hepes and $CaCl_2$ to a final concentration of 0.1 M Hepes and 0.1 M $CaCl_2$ before storage. For measuring of protein, cells in 6-well plates or flasks were stored at −20°C. In some experiments, the cells were also treated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) for 20 h.

Ethical approval for the use of human endothelial cells was obtained from the Human Research Ethics Committee.

Zymography

The SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gelatin-substrate zymography method was used to analyse gelatinolytic activity and urokinase in medium from HUVEC treated with LG, HG, and CML-BSA. To detect gelatinases, the SDS-PAGE gels contained 0.1% gelatin (Collagen type B from bovine skin, Sigma-Aldrich, Oslo, Norway). To detect urokinase activity, the gels were in addition loaded with plasminogen (8 μg/ml; Calbiochem, Cambridge, UK). As a positive control for proMMP-9 monomer (92 kDa) and homodimer (225 kDa), conditioned serum-free medium from the monocytic cell line THP-1 was used [23–24]. Furthermore, as positive control for active (62 kDa) and pro (72 kDa) forms of MMP-2, conditioned serum-free medium from an osteosarcoma cell line was used [25–26].

Two parts of medium fractions were mixed with one part of 2× sample buffer (2×=1 M Tris–HCl, pH 6.8 with 4% SDS, 20% glycerol and 2% bromophenol blue). The volume of conditioned medium loaded per lane was adjusted according to the protein content of the corresponding cell fraction. Samples were applied to wells of 7.5% gels containing either 0.1% gelatin, or 0.1% gelatin and 8 μg/ml plasminogen as substrates. The gels were run for 3 h. After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 50 min to wash out SDS, changing the solution twice. The gels were thereafter incubated in assay buffer (0.05 M Tris–HCl pH 7.4, 0.2 M NaCl, 0.005 M $CaCl_2$, 0.02% wt/vol Brij-35) overnight at 37°C to allow possible enzymes in the samples to degrade the gelatin matrix. In some experiments, the wash and assay buffers contained 10 mM EDTA, commonly used as a metalloproteinase inhibitor. To visualise regions of lysis, gels were then stained for 1 h with Coomassie blue (0.125% Coomassie G-250 wt/vol, 50% methanol vol/vol, and 1% acetic acid vol/vol), then destained with destaining solution (methanol: acetic acid: water (15:5:30) for 45 min. Areas containing gelatinolytic activity appeared as clear white zones on the blue-stained background.

To obtain quantitative information the areas containing gelatinolytic activity in the respective gels were analysed in a scanner (Eastman Kodak Co, Scientific Imaging System, Rochester, NY, USA). This was done for all the gels presented in this study. The staining of the gels varied, which gave variation in the numbers obtained by these analyses. Therefore the relative pixel unit values obtained in LG were used as reference to measure against HG and LG plus CML-BSA in the respective gels.

Western blotting

Western blotting was used to detect MMPs and TIMPs in medium from HUVEC treated with LG, HG and CML-BSA. The samples were mixed with Laemmli sample buffer and heated for 5 min at 100°C before electrophoresis in a 10% (to detect MMP-9 and MMP-2) or 15% (to detect TIMP-1 and TIMP-2) SDS-PAGE gel (readymade, from Bio-Rad, Hercules, CA, USA) for 1 h. The volume of conditioned medium loaded per lane was adjusted according to the protein content of the corresponding cell fraction. The proteins were electroblotted to a polyvinylidine difluoride membrane (PVDF) for 2 h at 1 A, after hydration with methanol and washing with dH_2O. Twenty-five millimolar of Tris–HCl with pH 7.5, 190 mM glycine, 20% methanol, was used as transfer buffer. The membranes were blocked with blocking buffer (5% non-fat dry milk in washing buffer) for 24–48 h at 4°C and subsequently washed with 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20 for 30 min at room temperature, changing the solution every 5 min, followed by incubation with monoclonal primary antibodies for 24 h at 4°C. The primary antibodies were diluted in washing buffer. The primary antibodies used were MMP-2, Ab-3, MMP-9, Ab-1 (from Biosciences Inc., San Diego, CA, USA) and TIMP-2, Ab-2, TIMP-1, Ab-2 (from Calbiochem, Cambridge, UK). Recombinant standards for TIMP-1 and TIMP-2 were obtained from Oncogene Research Products, San Diego, CA, USA. The blots were washed for 30 min, changing the solution every 5 min followed by incubation for 1 h at room temperature with the secondary antibody, which was diluted by 1: 3000 in washing buffer. The secondary antibody used was mouse Ig HRP-linked whole antibody from sheep (Amersham Biosciences, UK). The membranes were washed for 1 h, changing the solution every 5 min. The membranes were finally developed using detection reagents ECLTM (Amersham Biosciences, UK) and exposed to films (Hyper MP; Amersham, UK).

Determination of urokinase and plasmingen activator

Medium fractions were subjected to analyses using chromogenic substrates. Hundred microliter of medium samples from cells incubated in serum-free media with or without PMA stimulation, were incubated with 80 μl phosphate-buffered saline (PBS) and

20 µl (1.25 mg/ml) chromogenic substrates. S-2444 (Chromogenix-Instrumentation, Milano, Italy) is a chromogenic substrate for urokinase and S-2288 is a chromogenic substrate sensitive to a broad spectrum of trypsin-like serine proteases. The enzyme activities were recorded by reading the absorbance at 405 nm at different time points using a Titertek Multiscan spectrophotometer (Flow laboratories, Irvine, Scotland).

Detection of apoptosis

We used terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine (dUPT)-biotin nick-end labelling (TUNEL) for identification of the apoptotic cells in cultured primary HUVECs treated with LG, HG or CML-BSA, as described [27]. The cells were harvested by scraping and washed with PBS. The cells were then fixed with 1% paraformaldehyde in PBS for 10 min and centrifuged for 5 min at 1500 rpm. The cells were resuspended in 100% methanol and centrifuged again. The pellet was thereafter resuspended in PBS and centrifuged. The cell pellet was finally resuspended in 20 µl TUNEL reaction mixture, containing (400 U/µl) TdT (Roche Diagnostics, Mannheim, Germany), 0.05 mM dUPT (Roche), 5 mM $CoCl_2$, 0.1 mM DTT (Dithiothreitol, Sigma), reaction buffer and water, followed by incubation for 30 min at 37°C. The cells were then washed three times in PBS with 0.1% Triton X-100 and then stained with 50 µl Streptavidin-FITC (Amersham Biosciences) used at a concentration of 1 : 50 (in PBS with 0.1% Triton X-100 and saturated with 3% dry milk) for 30 min. After that 3 ml PBS with 0.1% Triton X-100 was added and the cells centrifuged for 5 min at 1500 rpm. The cells were resuspended again in 500 µl PBS with 0.1% Triton X-100. Samples of 50 µl were applied to slides (Super Frost Plus, Menzel, Germany) and covered with another cover slip and examined by fluorescence microscope (Axioplan 2, Zeiss, Germany).

Statistical methods

Comparison between groups of data was done by using box-plots and Mann–Whitney U test. Statistical significance was accepted with *p*-values less than 5%.

Results

Conditioned media from human umbilical cord endothelial cells cultured under serum free conditions were harvested and analysed for possible content of gelatinases by using SDS-PAGE gelatin zymography.

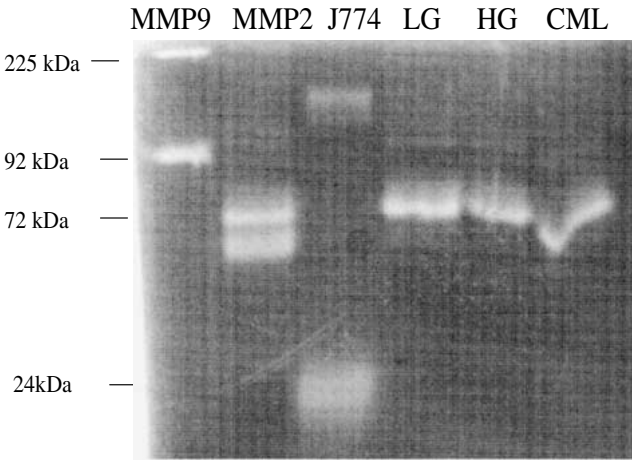

Fig. 1 SDS-PAGE zymography of medium from cultured HUVEC. HUVEC were incubated with LG, HG and CML-BSA (CML) for 3 days. From day 3 to 4, the cells were incubated under serum-free conditions as described in Materials and Methods. Medium fractions were subjected to gelatin substrate zymography. The migration position of the proMMP-2 (72 kDa), proMMP-9 (92 kDa), dimeric proMMP-9 (225 kDa) and supernatant from J774 cells, which contains urokinase (24 kDa), are shown at the left side of the panel

Bands with gelatinase activities were detected at positions that corresponded with proMMP-2 (72 kDa) in all samples. One representative gel is shown in Fig. 1. In some samples, bands with gelatinase activities were also detected at positions that corresponded to proMMP-9 (92 kDa) monomer standards. The amounts of proMMP-9, if any, were consistently lower, than those of proMMP-2 in all experiments. The MMP levels using zymography were found to vary with different donors of endothelial cells. Some of the samples contained MMP-9, whereas others were almost devoid of this enzyme. No bands could be detected in parallel gels with identical samples incubated in the presence of 10 mM EDTA, confirming

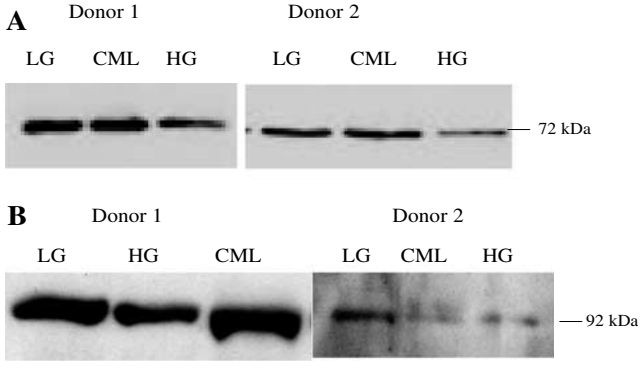

Fig. 2 Western blot analysis of medium from cultured HUVEC. HUVEC were incubated as described in legend to Fig. 1. Medium fractions from endothelial cells of different donors were analysed by Western blotting using antibodies against MMP-2 (**a**) and MMP-9 (**b**). Standard MMP-2 and MMP-9 were subjected to the same procedure

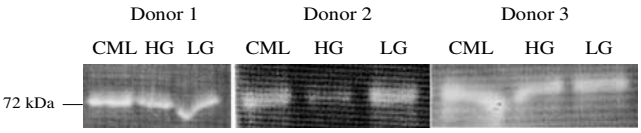

Fig. 3 SDS-PAGE zymography of medium from human cultured HUVEC. HUVEC obtained from three different donors were incubated as described in legend to Fig. 1 and subjected to gelatin substrate zymography

that the enzyme activities are of metalloproteinase nature (result not shown).

To further identify the bands at 72 and 92 kDa, Western blot analyses were performed on samples using antibodies against MMP-2 (Fig. 2 panel A) and MMP-9 (Fig. 2 panel B). This revealed that the 72-kDa band is proMMP-2 and the 92-kDa band is proMMP-9.

In medium from HUVEC, we observed that HG treatment led to a reduced activity in proMMP-2 when compared to medium from LG or CML-BSA-treated cells, run on the same gel. Fig. 3 shows zymography results from HUVEC of three different donors. The decrease observed was also confirmed by scanning analyses of the different bands to compare MMP-2 levels in the media from HG, LG and CML-BSA treated cells. Values obtained from LG data in these analyses were used as reference to measure against HG and CML-BSA. The relative values obtained from these analyses showed that MMP2 activity in HG medium and the corresponding value of MMP2 in medium from CML-BSA treated cells were lower than those from control cells, as can be seen in Fig. 4. The decrease observed after CML-BSA treatment was not statistically significant ($p = 0.16$), whereas the decrease after HG treatment had p-value of 0.006 in the samples analysed. Finally, by scanning analyses of data obtained by Western blotting a significant decrease in MMP-2 after HG treatment ($p = 0.008$) was observed, as can be seen in Fig. 5. The decrease after CML-BSA treatment was not statistically significant ($p = 0.69$).

In some experiments we also performed zymography analyses, with plasminogen in the gels, to detect the possible presence of urokinase, another enzyme capable of degrading ECM. Urokinase present in a sample will convert plasminogen to plasmin, which is capable of degrading gelatin. Such activity could not be detected in any of the samples, whether media were from cells treated with HG, CML-BSA or LG (Fig. 1).

To investigate whether there were different levels of TIMPs in samples from cells treated with LG, HG and CML-BSA, we performed Western blot analyses with antibodies against TIMP-1 and TIMP-2. We could not detect TIMP-1 and TIMP-2 in any of the samples even with 5 times concentrated samples, whether media were from cells treated with HG, CML-BSA or LG.

To investigate the effects of stimulating HUVEC with PMA, media were sampled from cells incubated in serum-free media with PMA for 24 h. The PMA-stimulated media were subjected to gelatin zymography. We observed that media from cells incubated with HG, LG and LG with CML-BSA added and stimulated with PMA, contained both proMMP-2 activity and an additional band with gelatinase activity at a position that corresponded to M_r of 66 kDa (Fig. 6). The pro MMP-2 activities did not seem to differ between control and PMA-stimulated cells. The 66-kDa band, however, could not be seen in

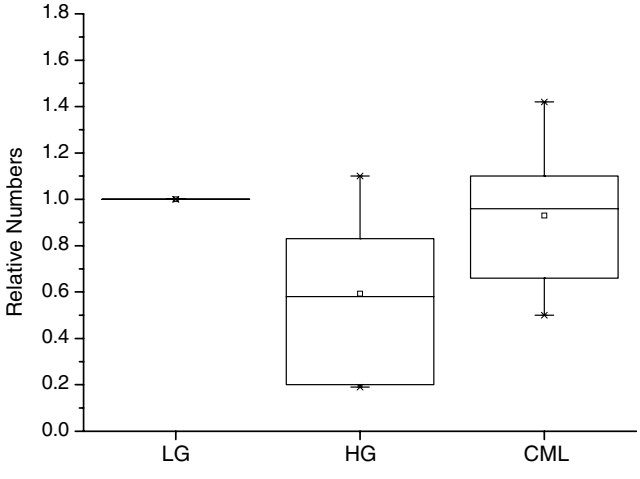

Fig. 4 Box plot analyses based on scanning analyses of zymography gels. The bands obtained after eight separate zymography gel experiments were subjected to scanning analyses. The numbers obtained were used to compare the data from cells cultured in LG, HG and LG with CML-BSA. Data from the LG group is used a reference and set to the value 1.0

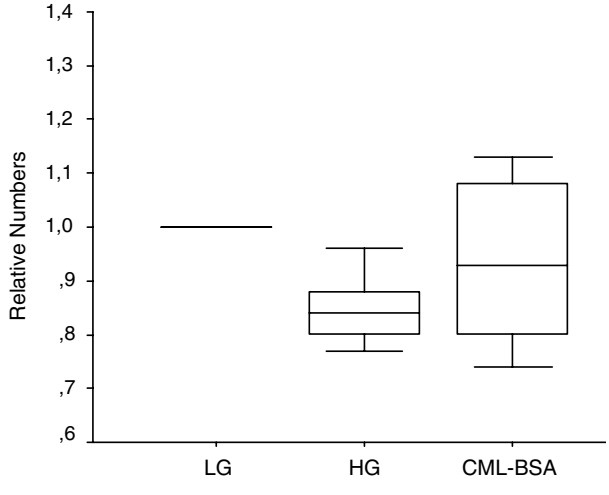

Fig. 5 Box plot analyses based on scanning analyses of Western blots of MMP-2. The bands obtained after five separate Western blots for MMP-2 detection were subjected to scanning analyses. The numbers obtained were used to compare the data from cells cultured in LG, HG and LG with CML-BSA. Data from the LG group are used a reference and set to the value 1.0

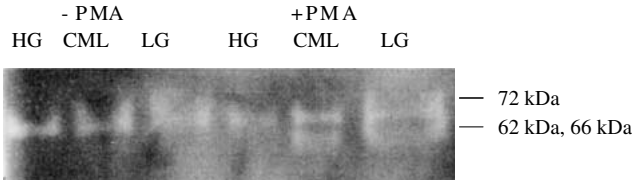

Fig. 6 The effect of PMA on MMPs' gelatinolytic activities in cultured HUVEC. HUVEC were incubated as described in legend to Fig. 1, but in addition stimulated with PMA for 24 h under serum free conditions. Medium samples from PMA-stimulated were subjected to gelatin substrate zymography

control cells. The results, therefore, indicate increased activation of proMMP-2 in the stimulated cells, irrespective of incubation in media with LG, HG or LG/CML-BSA conditions.

Media samples from cells incubated in serum-free media with and without PMA stimulation were subjected to analyses using chromogenic substrates S-2288 and S-2444. Such activities, if any, were consistently low, in all samples, irrespective of treatment with LG, HG or LG with added CML-BSA, with or without PMA stimulation (results not shown). These data show that HUVEC secrete low, if any amounts of urokinase and trypsin-like enzymes under the conditions used in these studies.

To determine if the different culture conditions would lead to apoptosis HUVEC were subjected to the TUNEL assay. As shown in Fig. 7 a somewhat higher degree of apoptosis was observed after treatment with HG. However, the higher degree of apoptosis in HG was not consistent in all cell cultures. The degree of apoptosis, therefore, seemed to vary between donors. These data indicate that a small degree of apoptosis could be observed in cell cultures, irrespective of incubation with LG, HG or CML-BSA in serum free media for 24.

Discussion

In the present study we show that primary cultures of HUVEC decreased the secretion of MMP-2 when cultured in the presence of high glucose. We also found that the advanced glycation end product CML decreased MMP-2 secretion, although to a lesser extent. Further, incubation of HUVEC in medium with a high glucose concentration also decreased the secretion of MMP-2. We also observed a decrease in MMP-2 amounts by Western blotting in cells exposed to high glucose. Some studies have previously shown that high glucose exposure leads to decrease in MMP secretion in HUVEC and mesangial cells [28–29]. In one study of endothelial cells, increased MMP-2, but decreased MMP-3 levels were observed [30]. However, the results are not directly comparable as the experimental design was different from our study. Furthermore, in another study using primary HUVEC, no change in MMP-2 secretion was observed after treatment with high glucose medium, but the level of TIMP-2 was found to increase [9]. In contrast, treating primary HUVEC with nitric oxide resulted in decreased MMP-2 expression [31]. An interesting finding was the effect of CML-BSA. Although the decrease in MMP-2 activity was low, it was consistent in all cultures used. The concentration of CML-BSA employed was physiologically relevant [22] and the decrease was not the result of pharmacological effects. The CML-BSA used in this study is modified to a higher degree than what is seen in subjects with diabetes [22]. Still, the effects observed in our in vitro studies, are relevant to changes in endothelial cell basement membranes seen in diabetes. Stimulation of HUVEC with PMA resulted in increased activation of MMP-2, leading to the appearance of processed forms of the enzyme. This result suggests that hyperglycaemia combined with an inflammatory/activated state may contribute to further changes in matrix turnover, with the increased activation of MMP-2 observed. This may be one effect of hyperglycaemia during development of vascular dysfunction.

Matrix components undergo continuous turnover through biosynthesis and degradation of the individual matrix components. MMPs are the major regulators of ECM turnover. Activated enzymes are capable of degrading many extracellular matrix components including laminin, collagen IV and

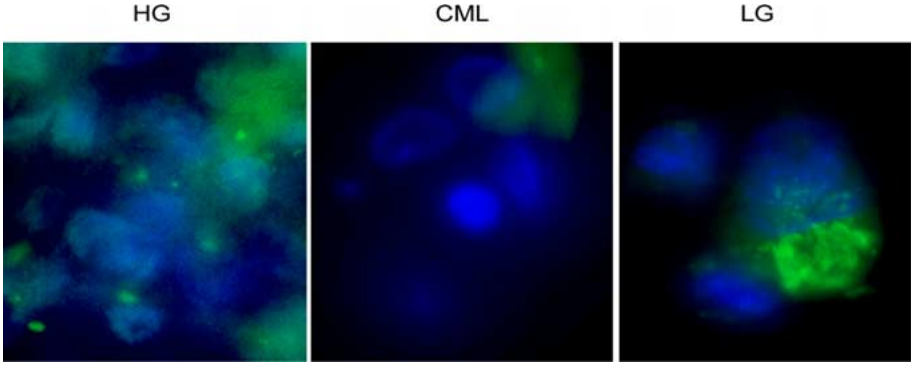

Fig. 7 Apoptosis in cultured HUVEC. HUVEC were incubated as described in legend to Fig. 1. Apoptotic cells were identified by the TUNEL assay. Green fluorescence indicates presence of apoptotic cells

fibronectin. They also act as regulatory molecules, both by functioning in enzyme cascades and by processing matrix proteins, cytokines, growth factors and adhesion molecules to generate fragments with enhanced or reduced biological activities. The MMPs have been implicated in atherosclerotic plaque ruptures, but also in processes of wound healing. Whether an up or down regulation of MMP expression is beneficial or not may depend on the process in question [32]. A further understanding of the regulation of MMP expression in the vasculature is therefore relvant to both atherosclerosis and diabetes [33–34].

Diabetic microangiopathy is characterised ultrastructurally by the thickening of BM in the body. Changes in the vascular basement membranes of retinal vessels in diabetic retinopathy may develop along with or without diabetic nephropathy. Diabetic nephropathy is characterised by accumulation of mesangium matrix and thickening of basement membrane within the glomeruli. Microangiopathy also leads to thickening of retinal capillary basement membranes and loss of intramural pericytes. It has been demonstrated that high glucose exposure can decrease MMP-2 expression in mesangial cells, which can possibly contribute to mesangial matrix expansion in diabetic nephropathy [35–36]. Furthermore, ECM remodelling also takes place throughout the different phases of atherosclerosis as part of an injury and inflammatory response. Inflammatory mediators and oxidized lipoproteins can stimulate the expression and activation of MMPs, promoting ECM degradation [33,37]. Changes in matrix turnover are, therefore, associated both with atherosclerosis and diabetes.

Taken together, these finding suggest that exposure to AGE or a high glucose concentration causes a reduction in the activities of MMPs in the endothelial cells, which may lead to reduced matrix turnover. This reduction can also affect fibronectin, laminin, and type IV collagen and matrix-associated growth factors, and contribute to qualitative changes of matrix. Our data show that decreased endothelial MMP activities may be associated with hyperglycaemia and exposure to AGEs.

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