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RESEARCH ARTICLE

Thiamine and benfotiamine prevent apoptosis induced by high glucose-conditioned extracellular matrix in human retinal pericytes

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Abstract

Background Early and selective loss of pericytes and thickening of the basement membrane are hallmarks of diabetic retinopathy. We reported reduced adhesion, but no changes in apoptosis, of bovine retinal pericytes cultured on extracellular matrix (ECM) produced by endothelial cells in high glucose (HG). Since human and bovine pericytes may behave differently in conditions mimicking the diabetic milieu, we verified the behaviour of human retinal pericytes cultured on HG-conditioned ECM.

Methods Pericytes were cultured in physiological/HG on ECM produced by human umbilical vein endothelial cells in physiological/HG, alone or in the presence of thiamine and benfotiamine. Adhesion, proliferation, apoptosis, p53 and Bcl-2/Bax ratio (mRNA levels and protein concentrations) were measured in wild-type and immortalized human pericytes.

Results Both types of pericytes adhered less to HG-conditioned ECM and plastic than to physiological glucose-conditioned ECM. DNA synthesis was impaired in pericytes cultured in HG on the three different surfaces but there were no differences in proliferation. DNA fragmentation and Bcl-2/Bax ratio were greatly enhanced by HG-conditioned ECM in pericytes kept in both physiological and HG. Addition of thiamine and benfotiamine to HG during ECM production completely prevented these damaging effects.

Conclusions Apoptosis is strongly increased in pericytes cultured on ECM produced by endothelium in HG, probably due to impairment of the Bcl-2/Bax ratio. Thiamine and benfotiamine completely revert this effect. This behaviour is therefore completely different from that of bovine pericytes, underlining the importance of establishing species-specific cell models to study the mechanisms of diabetic retinopathy.

Keywords human retinal pericytes; extracellular matrix; diabetic retinopathy; high glucose; thiamine; apoptosis

Introduction

Thickening of the capillary basement membrane is among the first events occurring in the development of diabetic microangiopathy [1–3] and was also described in other forms of hyperhexosaemia, such as galactose-fed rats, which develop a diabetic-like retinopathy [4,5]. The onset and evolution of diabetic retinopathy is influenced by qualitative and quantitative changes in the extracellular matrix (ECM) of the capillary basement membrane. In particular, alterations of some ECM components were

shown to influence the characteristics of endothelium [6] and ECM-associated growth factors [7], although the mechanisms are still unclear.

Endothelium is the major contributor to the synthesis of capillary ECM material. Loss of endothelial cells is however a late occurrence in the development of diabetic retinopathy, usually associated with capillary non-perfusion [8], whereas selective loss of ECM-embedded pericytes, together with the basement membrane thickening, represents a much earlier event [9]. Pericyte drop-out has great consequences on capillary remodelling and may cause the first abnormalities that are observed clinically, since pericytes are closely linked to endothelial cells and may regulate their proliferation, while receiving nourishment and growth factors from them [10]. Thickening of the basement membrane can interrupt this link and cause pericyte apoptosis and drop-out, whereas endothelium, deprived of proliferation control, can initiate proliferation [11]. Absence of pericytes may render capillaries vulnerable to formation of microaneurysms and these, in turn, are usually associated with focal endothelial hypercellularity [12]. Also at much later stages, in proliferative diabetic retinopathy, a correlation between absence of pericytes and retinal neovascularization suggests that the former may have a suppressive effect on capillary growth [11,13,14].

Thiamine and benfotiamine, a lipophilic analogue of thiamine monophosphate, are well-known protective agents against the metabolic damage induced by high glucose (HG) in vascular cells [15]. In previous experiments, we reported reduced adhesion, but no changes in apoptosis, in bovine retinal pericytes (BRP) cultured on ECM produced by human endothelial cells in high hexose concentrations, probably due to glycation of matrix proteins, and its correction by thiamine and aminoguanidine [16,17]. However, bovine pericytes have been shown to behave differently from that of humans, when cultured in experimental conditions that mimic the diabetic microenvironment [18,19].

The aim of this study was therefore to verify the behaviour of human retinal pericytes (HRP), both wild-type and immortalized, when cultured on ECM produced by endothelial cells in HG, and the possible effects of thiamine and benfotiamine in modulating such behaviour.

Materials and methods

Cell cultures

Human umbilical vein endothelial cells (HUVEC) were obtained from human umbilical cords, as previously described [20]. Pools of cells from three to five cords were grown in Medium 199 – Hepes Modification, added with 20% foetal calf serum (FCS), until confluent. In secondary cultures, HUVEC were kept in M199 + 20% FCS +50 μg/mL endothelial cell growth supplement. HUVEC were characterized by von Willebrand factor indirect immunofluorescence.

HRP were purchased from Cambrex Corporation, East Rutherford, NJ, USA. They were used as a wild-type model (WT-HRP) or immortalized (Bmi-HRP), as described earlier [21]. Both types of pericytes were characterized by immunofluorescence staining for specific markers: α-smooth muscle actin, desmin, platelet-derived growth factor-ß receptor and proteoglycan NG-2. They were grown in DMEM 5.6 mmol/L glucose with 20% FCS in primary cultures and 10% FCS in secondary cultures. Experiments with non-immortalized cells were performed at the third–fifth subculture.

All reagents for cell cultures were purchased from Sigma-Aldrich, St Louis, MO, USA, unless otherwise stated.

Matrix production

HUVEC in primary culture were detached by trypsin-EDTA and seeded in six-well plates. After adhesion, medium was removed and changed with media containing physiological (5.6 mmol/L, NG) or high (28 mmol/L, HG) D-glucose. After 7 days of culture, wells were washed with Phosphate Buffered Saline (PBS) and treated for 4 min with 0.2 mol/L NH_4OH, to detach cells. Wells were then washed four times with PBS. WT- and Bmi-HRP were seeded in the same wells, onto the ECM produced by HUVEC, and cultured for either 18 h or 7 days in normal glucose (NG) or HG, depending on the experiment (see below).

In a further series of experiments, matrices were produced by culturing HUVEC in NG, HG, HG plus 50 or 100 μmol/L thiamine (T) or benfotiamine (BT). Equimolar concentrations of mannitol were added to both HUVEC and HRP cultures, as an osmotic control.

Cell adhesion

Pericytes were seeded on HUVEC-conditioned ECM or directly on plastic as control, and cultured for 18 h in NG. HRP were then counted in Bürker chambers, after Trypan Blue staining, to exclude dead cells.

Cell proliferation – counts

Pericytes were seeded either on HUVEC-conditioned ECM or directly on plastic and grown for 7 days in either NG or HG. Cells were then counted, as described above.

Cell proliferation – DNA synthesis

Incorporation of the pyrimidine analogue 5-bromo-2′-deoxyuridine (BrdU) instead of thymidine into the DNA of proliferating cells was detected by immunoassay (*Cell Proliferation ELISA, BrdU*, Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions, after 7 days of culture of pericytes in NG or HG on

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conditioned ECM. Absorbances were read at 450 nm in an ELISA reader (Victor 3, PerkinElmer, Wellesley, MA, USA).

Apotosis – DNA fragmentation

Apoptosis was evaluated using the *Cell Death Detection ELISA^{PLUS}* (Roche), according to the manufacturer's instructions, after 7 days of culture of pericytes in NG or HG on conditioned ECM. Absorbances were read at 405 nm (reference wavelength 490 nm) in the Victor 3 ELISA reader and normalized by total DNA content measured at 260 nm.

Apoptosis – Bax, Bcl-2 and p53 mRNA expression

mRNA expression of Bax, p53 and Bcl-2 was analysed by relative quantitative multiplex reverse transcriptase–polymerase chain reaction (RT–PCR). Total RNA was isolated from pericytes, after 7 days of culture on conditioned ECM, by the *High Pure RNA Isolation kit* (Roche). Contaminating DNA was removed using DNase I, enclosed in the kit. The yield of each RNA sample was checked by spectrophotometrical measurement of absorbance at 260 nm.

RT–PCR was performed with 0.5 μg RNA using the Qiagen OneStep RT–PCR kit (Qiagen GmbH, Hilden, Germany). The assay was designed for multiplex RT–PCR, each reaction set containing primers for the gene of interest and for β-actin as an internal control, using the QuantumRNA β-actin Internal Standards (Ambion, Austin, Texas, USA). Amplification was performed using the following cycling parameters: Bax: hold at 50 °C for 30 min (RT step), hold at 95 °C for 15 min [hot start to PCR], 28 cycles of 95 °C (30 s)/55 °C (30 s)/72 °C (1 min), followed by a final hold at 72 °C for 10 min; p53: 50 °C for 30 min (RT step), 95 °C for 15 min (hot start to PCR), 30 cycles of 95 °C (30 s)/55 °C (30 s)/72 °C (1 min), final hold at 72 °C for 10 min; Bcl-2: 50 °C for 30 min (RT step), 95 °C for 15 min (hot start to PCR), 35 cycles of 95 °C (30 s)/58 °C (30 s)/72 °C (1 min), final hold at 72 °C for 10 min. The number of cycles for each of the reactions was determined by the linear range of amplification (data not shown). The RT–PCR products were visualized by electrophoresis in 2% agarose gels containing 1 μg/mL ethidium bromide and were quantified using an image analysis system (*Kodak Gel Logic Image Analysis System*, Kodak, Rochester, NY, USA). To determine the levels of mRNA expression, the ratio of *Bax* (or *Bcl-2* or *p53*)/β-actin was evaluated (according to QuantumRNA β-actin Internal Standards instruction manual).

Primers used for Bax were as follows: 5′ primer, CCAGCTCTGAGCAGATCATG; 3′ primer, CTGGAAGAAGATGGGCTGAGG, which generated a 541-bp product. p53: 5′, CCTCACCATCATCACACTGG; 3′, GACAGAAGGGCCTGACTCAGAC (427-bp). Bcl-2: 5′, GCCAGGACCTCGCCGCTGCAG; 3′, GCTTGCATCACCCTGGGTGCC (504-bp).

Apoptosis – Bax, Bcl-2 and p53 protein concentration

Bax, Bcl-2 and p53 protein concentrations were determined by ELISA, using commercially available kits (Bax EIA Kit, Assay Designs Inc, Ann Arbor, MI, USA; Human Bcl-2 ELISA Kit, Bender MedSystems GmbH, Vienna, Austria; Human p53 ELISA Kit, Bender MedSystems). Absorbances were read at 450 nm in the Victor 3 ELISA reader and normalized by total protein content measured at 595 nm after the Bradford reaction.

Statistical analysis

Results are mean ± SD of six experiments. Results of mRNA expression are given as percentages of the results obtained with positive control conditions (i.e. HRP grown in NG on NG-conditioned ECM) within each experiment. Statistical comparisons between groups were carried out by Student's *t*-test for paired data or Wilcoxon's Signed Ranks test, as appropriate. Results were considered significant if the *p*-value was 0.05 or less.

Results

Cell adhesion

Both types of pericytes adhered less on HG-conditioned ECM and plastic than NG-conditioned ECM. Culture on plastic was used to rule out the possibility that glucose attachment to plastic itself could be responsible for the results, independent of matrix formation. The number of WT-HRP 18 h after seeding was 19% lower on HG-conditioned ECM and 22% lower on plastic than control (NG-conditioned ECM, $p < 0.05$, both) (Figure 1a). As for Bmi-HRP, their number was 16% lower on HG-conditioned ECM and 20% lower on plastic than on NG-conditioned ECM ($p < 0.05$, both) (Figure 1b).

Cell proliferation

We found no difference in cell numbers 7 days after seeding on either pericyte type, when they were grown in either NG or HG on the three different substrates (data not shown).

DNA synthesis was impaired when HRP were grown in HG on the different substrates. In particular, WT-HRP BrdU content after 7 days of culture in HG concentrations was 1.01 ± 0.21 abs units on NG-conditioned ECM *versus* 1.13 ± 0.21 of control (cells grown in NG on NG-conditioned ECM, $p < 0.05$), 1.02 ± 0.14 on HG-conditioned ECM ($p < 0.05$ *versus* control) and $0.91 \pm$

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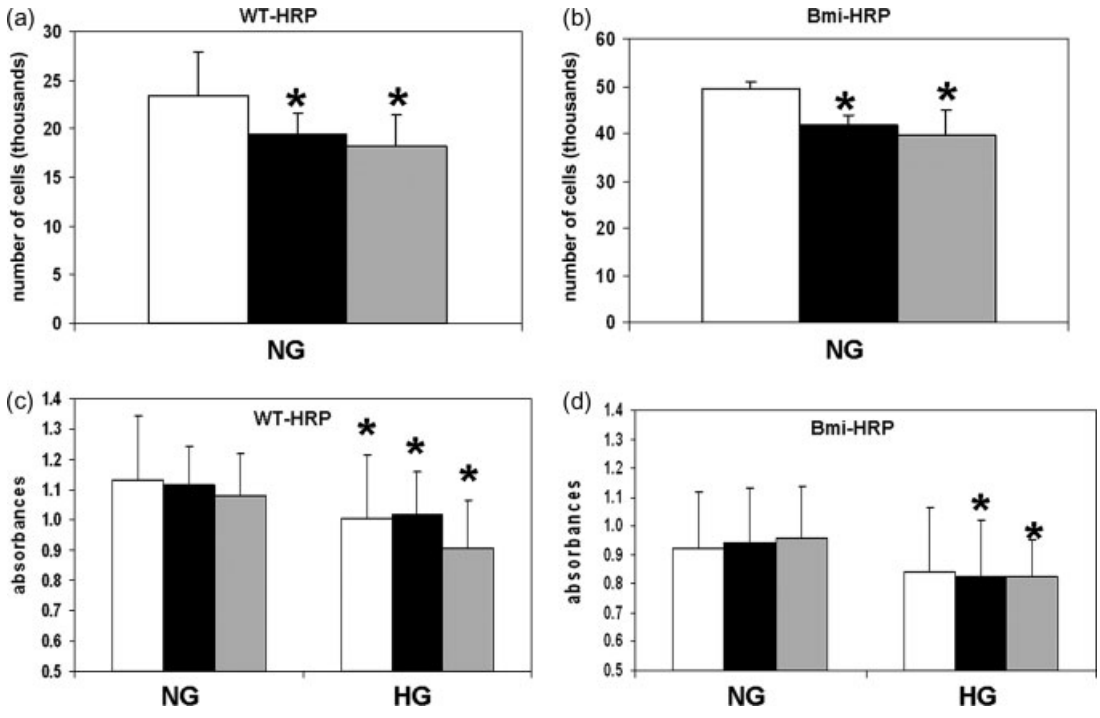

Figure 1. Adhesion of WT-HRP (a) and Bmi-HRP (b) and DNA synthesis of WT-HRP (c) and Bmi-HRP (d) on NG-conditioned extracellular matrix (ECM) (*white bar*), HG-conditioned ECM (*black bar*) or plastic alone (*grey bar*). *$p < 0.05$ *versus* control (NG-conditioned ECM)

0.16 on plastic ($p < 0.05$ *versus* control) (Figure 1c). Bmi-HRP BrdU content in HG cultures was 0.84 ± 0.23 on NG-conditioned ECM *versus* 0.93 ± 0.19 of control (cells grown in NG on NG-conditioned ECM, NS), 0.83 ± 0.19 on HG-conditioned ECM ($p < 0.05$ *versus* control) and 0.82 ± 0.13 on plastic ($p < 0.05$ *versus* control) (Figure 1d).

Apoptosis – DNA fragmentation

Apoptosis was greatly enhanced by HG-conditioned ECM, in both types of HRP, regardless of whether they had been cultured in normal or HG. In WT-HRP, absorbances normalized to DNA content were 1.20 ± 0.12 in cells grown in NG on HG-conditioned ECM *versus* 0.19 ± 0.14 of control ($p < 0.001$) and 1.03 ± 0.49 in cells grown in HG on HG-conditioned ECM ($p < 0.05$) (Figure 2a).

In Bmi-HRP, absorbances normalized to DNA content were 1.81 ± 0.41 in cells grown in NG on HG-conditioned ECM and 1.93 ± 0.67 in cells grown in HG on HG-conditioned ECM *versus* 0.06 ± 0.05 of control ($p = 0.000$ in both cases) (Figure 2b). Culture on plastic had no effect on apoptotic rate in either cell type, in NG or HG.

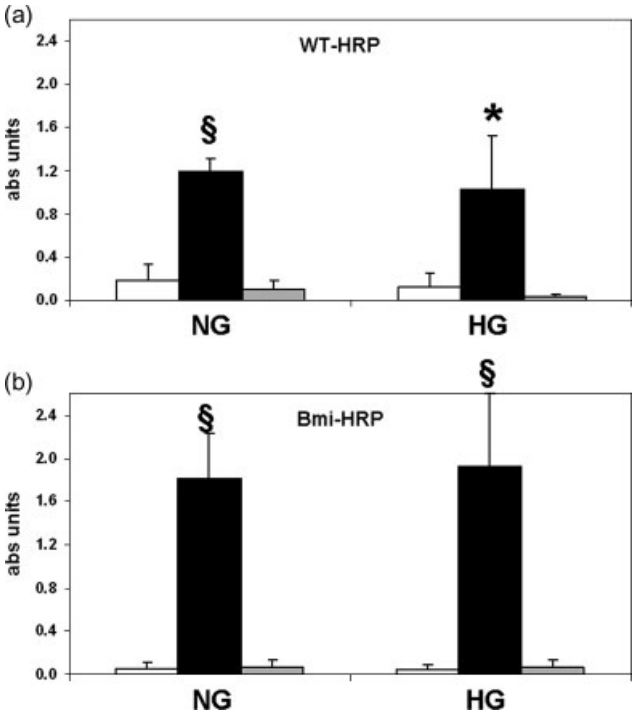

Figure 2. Apoptosis (DNA fragmentation) of HRP in NG or HG on NG-conditioned extracellular matrix (ECM) (*white bar*), HG-conditioned ECM (*black bar*) or plastic alone (*grey bar*). (a) WT-HRP; (b) Bmi-HRP. *$p < 0.05$ *versus* control (NG-conditioned ECM), $^{\S}p < 0.001$ *versus* control

Apoptosis – Bax, Bcl-2 and p53 mRNA expression

The results obtained, measuring DNA fragmentation, were confirmed by the study of mRNA expression of p53, Bcl-2 and Bax. p53 mRNA expression in WT- and Bmi-HRP was increased on HG-conditioned ECM after 7 days of culture in physiological and HG. In particular, p53 mRNA expression in WT-HRP was $120.2 \pm 8.1\%$ *versus* 100.0% of control ($p < 0.001$) for cells grown in NG

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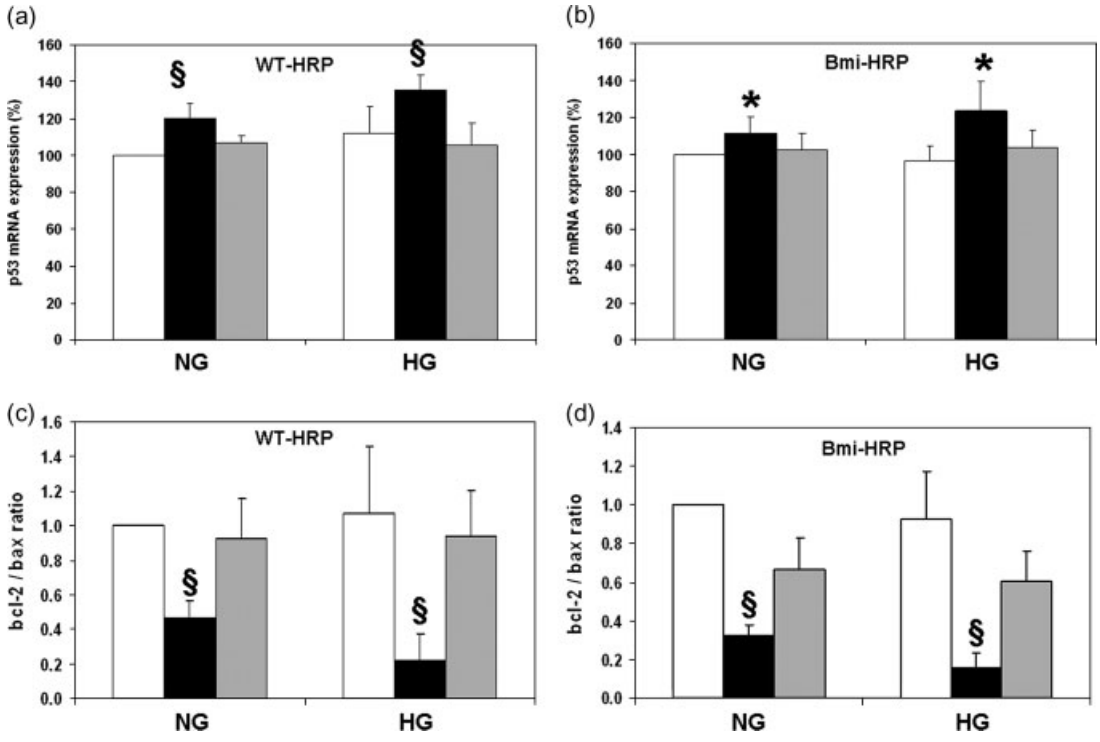

Figure 3. p53 and Bcl-2/Bax ratio (mRNA expression) in HRP in NG or HG on NG-conditioned extracellular matrix (ECM) (*white bar*), HG-conditioned ECM (*black bar*) or plastic alone (*grey bar*). (a) p53 on WT-HRP; (b) p53 on Bmi-HRP; (c) Bcl-2/Bax on WT-HRP; (d) Bcl-2/Bax on Bmi-HRP; *$p < 0.05$ *versus* control (NG-conditioned ECM), §$p < 0.001$ *versus* control

on HG-conditioned ECM and $135.5 \pm 8.2\%$ of control ($p < 0.001$) for cells grown in HG on HG-conditioned ECM (Figure 3a). In Bmi-HRP, p53 mRNA expression was $111.3 \pm 9.4\%$ *versus* 100.0% of control ($p < 0.05$) for cells cultured in NG on HG-conditioned ECM and $123.5 \pm 15.6\%$ of control ($p < 0.05$) for cells cultured in HG on HG-conditioned ECM (Figure 3b). Bcl-2 mRNA expression in WT and Bmi-HRP was decreased on all types of matrix while, concurrently, Bax mRNA expression increased on HG-conditioned ECM (data not shown). We also evaluated Bcl-2/Bax ratio, and found it drastically decreased on HG-conditioned ECM, this being a marker of enhanced apoptosis. In particular, Bcl-2/Bax mRNA expression in WT-HRP was reduced to 0.469 ± 0.092 (control = 1, $p < 0.001$) for cells grown in NG on HG-conditioned ECM and to 0.219 ± 0.154 ($p < 0.001$) for cells grown in HG on HG-conditioned ECM (Figure 3c). In Bmi-HRP, Bcl-2/Bax mRNA expression was reduced to 0.323 ± 0.054 (control = 1, $p < 0.001$) for cells cultured in NG on HG-conditioned ECM and to 0.157 ± 0.074 of control ($p < 0.001$) for cells cultured in HG on HG-conditioned ECM (Figure 3d).

Apoptosis – Bax, Bcl-2 and p53 protein concentration

p53 concentrations in cell lysates showed no modification in either cell type (Figure 4 a and b). Bcl-2 concentrations in WT-HRP and Bmi-HRP were again decreased in all types of matrixes while Bax concentrations increased on HG-conditioned ECM (data not shown). Bcl-2/Bax ratio was confirmed to be decreased on HG-conditioned ECM, consistently with mRNA expression results. In particular, Bcl-2/Bax concentration in WT-HRP was 0.06 ± 0.03 *versus* 0.14 ± 0.06 of control ($p < 0.05$) for cells grown in NG on HG-conditioned ECM and 0.09 ± 0.05 ($p < 0.05$) for cells grown in HG on HG-conditioned ECM (Figure 4c). In Bmi-HRP, Bcl-2/Bax concentration was 0.90 ± 0.08 *versus* 1.27 ± 0.18 of control ($p = 0.002$) for cells cultured in NG on HG-conditioned ECM and 0.76 ± 0.11 of control ($p = 0.002$) for cells cultured in HG on HG-conditioned ECM (Figure 4d).

Effects of thiamine and benfotiamine on pericyte apoptosis

In a further series of experiments, T and BT were added to HG medium on HUVEC while they were producing matrixes. Initial thiamine concentration in the culture medium was due to FCS only (12 nmol/L at the final 20% dilution, as determined by high-performance liquid chromatography), since M199 is a thiamine-deficient medium (20 pmol/L). This a concentration comparable to that reported in diabetic patients [22].

HRP were then cultured onto such conditioned-ECM only in the presence of physiological glucose, since we had ruled out a direct role for HG in the previous experiments.

While DNA fragmentation was confirmed to be greatly increased when pericytes were grown on HG-conditioned matrixes (WT-HRP: 1.27 ± 0.48 *versus* 0.06 ± 0.02 of

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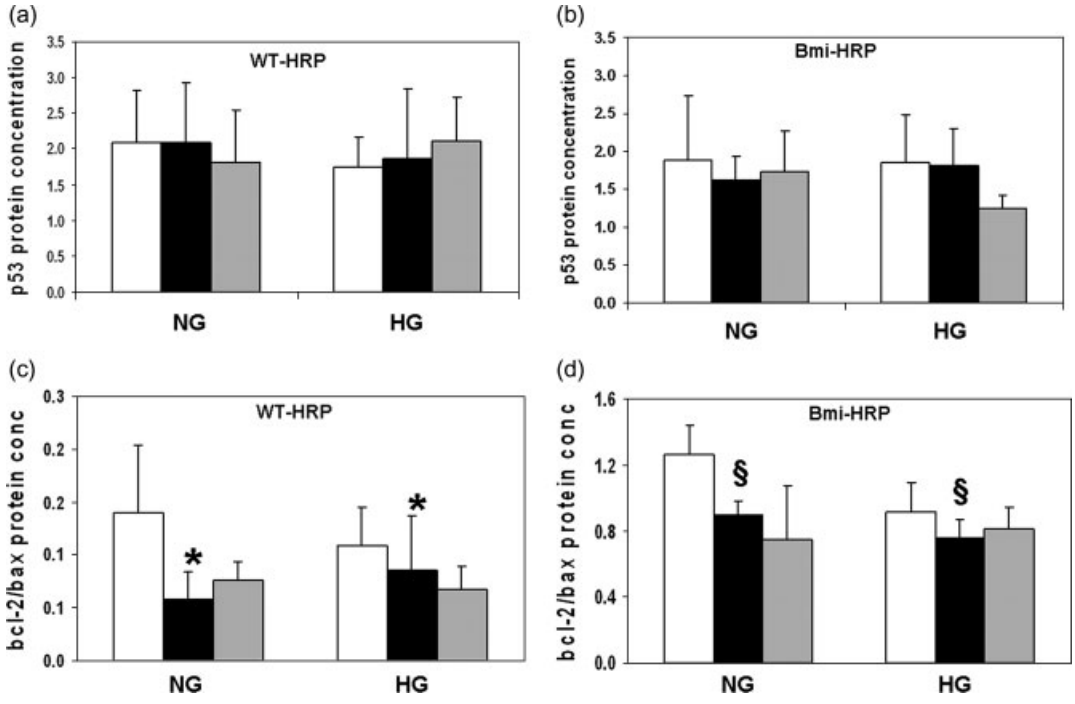

Figure 4. p53 and Bcl-2/Bax ratio (protein concentration) in HRP in NG or HG on NG-conditioned extracellular matrix (ECM) (*white bar*), HG-conditioned ECM (*black bar*) or plastic alone (*grey bar*). (a) p53 on WT-HRP; (b) p53 on Bmi-HRP; (c) Bcl-2/Bax on WT-HRP; (d) Bcl-2/Bax on Bmi-HRP. *$p < 0.05$ *versus* control (NG-conditioned ECM), $^{\S}p = 0.002$ *versus* control

NG-matrixes, $p = 0.002$; Bmi-HRP: 2.12 ± 0.24 *versus* 0.05 ± 0.01 of NG-matrixes, $p < 0.001$), T and BT, at both concentrations, were able to reverse the apoptosis-enhancing properties of ECM produced by endothelial cells in HG in both HRP types (Figure 5a and b).

The same positive effect of T and BT was found evaluating the Bcl-2/Bax ratio, as regards both mRNA expression (Figure 6a and b) and protein concentration (Figure 6c and d).

Osmotic controls

Measurements of glucose concentrations and pH in the media at the time of exchanging them and at the end of the experiments did not show any relevant modification from the starting conditions (data not shown). Osmotic pressure after the addition of HG was 334 *versus* 311 mosmol/L of NG; addition of T/BT had no relevant effect on osmotic pressure.

Equimolar addition of mannitol instead of glucose to the culture media had no effect (data not shown), ruling out a role for osmotic changes in the media in the modification of ECM.

Discussion

Pericytes live embedded in the capillary basement membrane and have functions similar to those of smooth muscle cells in larger vessels. In the retina, they play an important role in the maintenance of vascular tone

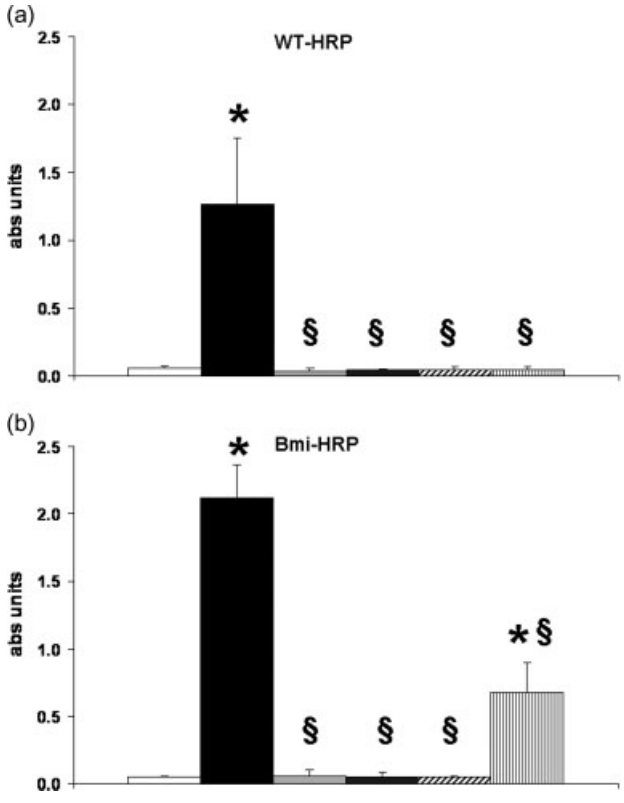

Figure 5. Apoptosis (DNA fragmentation) of HRP on NG-conditioned extracellular matrix (ECM) (*white bar*), HG-conditioned ECM (*black bar*), HG +50 μmol/L thiamine (*light grey bar*), HG +100 μmol/L thiamine (*dark grey bar*), HG +50 μmol/L benfotiamine (*diagonal lines*), HG +100 μmol/L benfotiamine (*vertical lines*). (a) WT-HRP; (b) Bmi-HRP. *$p < 0.002$ *versus* control (NG-conditioned ECM); $^{\S}p < 0.001$ *versus* HG-conditioned ECM

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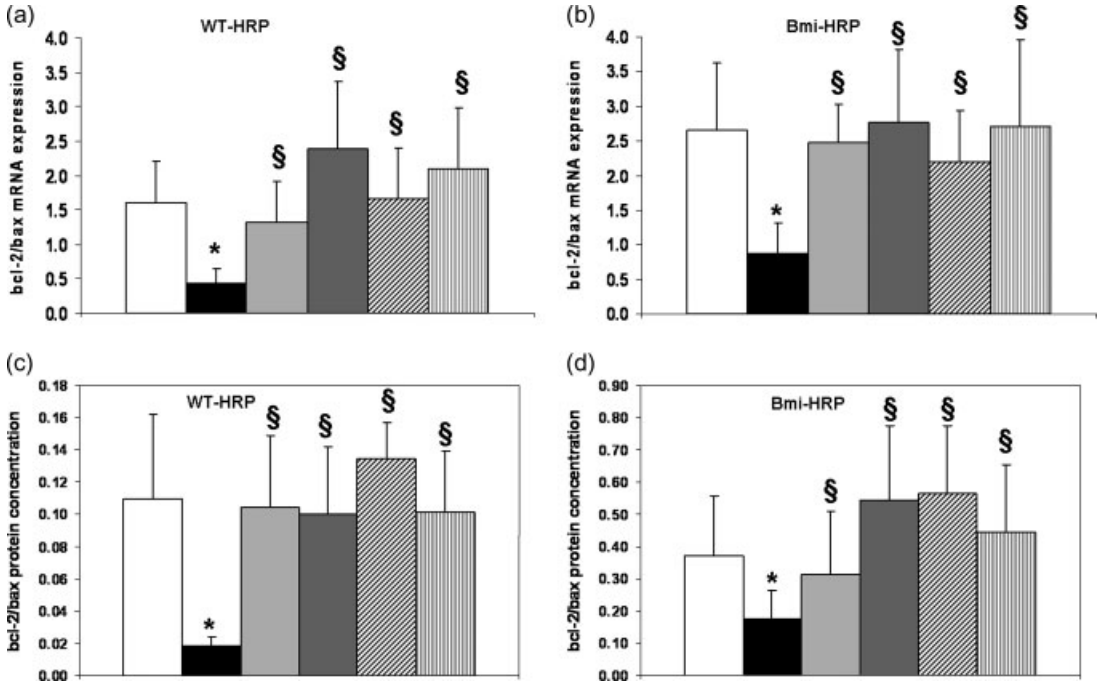

Figure 6. Bcl-2/Bax ratio: mRNA expression (a and b) and protein concentration (c and d) of HRP on NG-conditioned extracellular matrix (ECM) (*white bar*), HG-conditioned ECM (*black bar*), HG +50 μmol/L thiamine (*light grey bar*), HG +100 μmol/L thiamine (*dark grey bar*), HG +50 μmol/L benfotiamine (*diagonal lines*), HG +100 μmol/L benfotiamine (*vertical lines*). (a and c) WT-HRP and (b and d) Bmi-HRP. *$p < 0.05$ *versus* control (NG-conditioned ECM); §$p < 0.001$ *versus* HG-conditioned ECM

and in preventing endothelial cell proliferation. Early pericyte apoptosis and drop-out, together with thickening of the basement membrane, are characteristic features of diabetic retinopathy which have been implicated in the pathogenesis of endothelial cell proliferation and neoangiogenesis, as seen in proliferative diabetic retinopathy [13]. Thus, delineating the mechanisms that regulate pericyte proliferation and survival is pivotal to our understanding of the pathogenesis of this microvascular complication.

Bovine or rat pericytes have been mostly used so far as a model for the study of diabetic retinopathy. However, we have recently demonstrated that bovine cells behave differently from that of humans in experimental conditions designed to mimic the diabetic milieu [18,21]. Hence, we have developed an immortalized human retinal pericyte line (Bmi-HRP) [21] and compared its behaviour with that of the wild-type pericytes from which it was derived [18], demonstrating not only that the two human pericyte types are very similar in their behaviour but also that human pericytes behave quite differently from bovine ones, underlining the importance of establishing a reliable human pericyte model for the *in vitro* study of diabetic retinopathy.

The results of the present study show again that data obtained using our immortalized line are consistent with those obtained with wild-type cells. Moreover, this is the first study, to the best of our knowledge, to investigate *in vitro* the interactions between human pericytes and ECM produced by human endothelial cells.

We had previously shown that BRP adhere less to ECMs obtained after culturing human endothelial cells in high hexose concentrations [16,17], and that the addition of thiamine and aminoguanidine was able to revert this effect. However, we could not find any difference in their proliferation nor in their apoptotic rates. In this study, we demonstrate that HRP show a rather different behaviour, when seeded on ECM obtained after culturing HUVEC in normal or HG conditions. HRP adhere less to HG-conditioned ECM and plastic than physiological glucose-conditioned matrix, which is consistent with bovine cell experiments. Pericyte adhesion was measured by counting them 18 h after seeding. Since these cells have a long duplication time (72 h) in our experimental conditions, consistent with their prolonged estimated turnover *in vivo* [23], this time interval allowed them to adhere to the test substrate but not to start dividing. Such impaired adhesion could be due to glycation of matrix proteins or to abnormal expression of adhesion molecules [16]. Cross-link formation induced by AGE may cause stiffness of the basement membrane and impair tissue remodelling [24], since structural components of the ECM, such as collagen, are the prime targets of advanced glycation processes [25]. Moreover, a role for matrix metalloproteinases, the major matrix degrading enzymes, can also be hypothesized, as these proteins have been described to alter the balance between ECM synthesis and degradation in diabetes [26–28].

Impaired replication of endothelium on ECM obtained from other endothelial cells cultured in HG concentrations was described previously [29,30], and a role for excess exogenous fibronectin, altering cell–ECM interactions through increased adhesion and cytoskeletal rearrangements, was hypothesized [6]. HG was shown to increase

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type IV collagen and fibronectin mRNA synthesis [31] and to upregulate the transcription of their genes [29] in cultured human endothelial cells. Increased synthesis of basement membrane collagen in the retina [32] and of collagen [33,34], laminin [35] and fibronectin [36] in kidney glomeruli were demonstrated in diabetic rats, whereas fibronectin immunostaining is increased in the retina of diabetic subjects with background retinopathy [36]. It was also shown that increased modification of vascular basement membrane type IV collagen by methylglyoxal, a dicarbonyl-glycating agent which is increased in hyperglycaemia, formed arginine-derived hydroimidazolone residues at integrin-binding sites of collagen, causing endothelial cell detachment, anoikis and inhibition of angiogenesis [37].

However, in our previous work [16], we could not find any difference in BRP replication either on high hexose-conditioned ECM or on plates coated with fibronectin, laminin or collagen type IV. Our present results show again no difference in HRP numbers after 7 days of culture on the three different substrates, probably due to the long replication time of these cells, whereas DNA synthesis begins to be impaired at day 7 in HRP kept in HG conditions on the three different substrates.

The most interesting results, however, are those obtained after studying apoptosis. There was a dramatic increase in DNA fragmentation when wild-type and immortalized human pericytes were cultured on HG-conditioned ECM, regardless of whether they were kept in physiological or HG. This is consistent with the report by Miller *et al*. [19] who found no increase in apoptosis in HRP after a 7-day incubation in HG, while it is rather different from bovine cell behaviour. Our previous findings demonstrated no susceptibility of BRP to culture on different matrixes, at least in terms of induction of apoptosis [17], whereas they are highly sensitive to direct exposure to HG concentrations [38–40].

To better understand the mechanisms involved in increased apoptosis of human cells cultured on HG-conditioned ECM, we have evaluated mRNA expression and protein concentration of three molecules, p53, Bax and Bcl-2, which are involved in glucose-mediated apoptosis [41]. Members of the Bcl-2 family have been widely described as inducers and integrators of survival and death signals. Pro-survival proteins (like Bcl-2) are able to inhibit apoptosis induced by several cytotoxic mechanisms while pro-apoptotic members (like Bax) antagonize their effects by creating heterodimers or by killing the cell directly, through damaging of the organelles [40,42]. In fact, it has been observed that HG induces a Bax-mediated apoptotic program in the retinal vessels, through the activation of NF-κB in retinal pericytes, but not in endothelial cells; HG-induced NF-κB is pro-apoptotic and activates Bax [43]. It was also shown that Bax expression was increased in BRP cultured for up to 5 weeks in HG [43]. A role for p53 in HG-mediated apoptosis and a relationship between p53 and Bax-induced apoptosis have also been described [44,45].

Our results on p53, Bcl-2 and Bax mRNA expression are consistent with those obtained for measuring DNA fragmentation. p53 mRNA levels were increased by an average of 20% on HG-conditioned ECM, regardless of whether the cells grew in normal or HG. Bcl-2 mRNA levels were down-regulated by about 20% on all types of ECM in comparison with control and by about 60%, for both cell types, when cultured in HG on HG-conditioned ECM. Bax levels were dramatically increased (up to 180% for wild-type cells and 250% for immortalized ones) on HG-conditioned ECM. Consequently, the Bcl-2/Bax ratio for mRNA levels showed a clear decrease in pericytes grown on HG-conditioned ECM, which goes in the direction of increased apoptosis.

Consistent with these results were those on protein concentrations. There were no significant differences in p53, probably due to low sensitivity of the assay, but Bcl-2 and Bax followed the same trend of mRNA levels and again there was a clear down-regulation of their ratio when HRP were cultured on HG-conditioned ECM, in normal as well as HG.

It is well established that thiamine and benfotiamine act as protective agents against the metabolic damage induced by HG in vascular cells [15], by reducing excess reactive oxygen species production through the normalization of the four branches of the 'unifying mechanism' proposed to be at the basis of the pathogenesis of diabetic vascular complications [46,47]. High-dose thiamine (70 mg/kg) therapy has been shown to normalize food intake and prevent diabetic-induced increases in plasma cholesterol and triglycerides in streptozotocin-induced diabetic rats, thus counteracting dyslipidaemia [48]. This was due to the prevention of thiamine depletion and decrease of transketolase activity in rat liver, with a concomitant decrease in UDP-*N*-acetylglucosamine and fatty acid synthase activity. However, lower doses of thiamine (7 mg/kg), and benfotiamine at both concentrations, were ineffective [48], probably because exogenous thiamine is able to increase hepatic thiamine levels more effectively than benfotiamine in the 90-min post-prandial period [49]. In a recent pilot study, high-dose thiamine therapy produced regression of urinary albumin excretion in type 2 diabetic patients with microalbuminuria, showing that thiamine supplementation at high dose may provide improved therapy for early stage diabetic nephropathy [50].

We showed previously that addition of thiamine to HG in the media of HUVEC-producing matrixes is able to prevent decreased adhesion of BRP [16] and hypothesized that this could be due to its anti-glycating properties because aminoguanidine, which is known to inhibit the progression of labile adducts to permanent Advanced Glycation End-products (AGE) [51,52], had a similar effect. In addition, we observed that thiamine and aminoguanidine normalized BRP adhesion to matrix obtained in high D-glucose and D-galactose but not high L-glucose (an isoform which is internalized by the cell, but not metabolized), suggesting that excess glycation resulting from overproduction of intermediate

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metabolites was involved in this phenomenon. This interpretation is consistent with previous reports that the key intermediate of AGE formation, methylglyoxal, interferes with crucial cell–matrix interactions by reacting with the arginine residues of collagen [25]. Indeed, methylglyoxal-derived hydroimidazolone AGE is the major AGE formed on integrin-binding sites in collagen and fibronectin [37]. In this study, we demonstrate that addition of thiamine and benfotiamine is able, at both concentrations used, to reverse the apoptosis-enhancing properties of ECM produced by endothelial cells in HG, both in WT and Bmi-HRP, possibly via the same mechanism. It is worthwhile to stress once again that, while vitamin B1 exerts the same effect of possible prevention of matrix glycation, bovine and human cells behave differently, the former being affected in adhesion, the latter in apoptosis.

We can conclude from our present data that apoptosis of human pericytes is strongly affected by ECM produced in HG, that this is mediated by the impairment of the Bcl-2 to Bax ratio and that thiamine and benfotiamine reverse this effect, probably by acting on enhanced matrix glycation. The behaviour shown by human pericytes is rather different from that observed with bovine pericytes, underlining the importance of establishing a species-specific cell model for the study of diabetic retinopathy, a complication that affects humans but does not fully develop in animal models.

These *in vitro* results suggest, moreover, that alterations of the ECM may play a role in the early and selective disappearance of pericytes from retinal capillaries in diabetes. The rate of apoptosis *in vitro* is presumably higher than *in vivo*, as the network of mechanisms interacting on pericytes is probably less complex. In any event, further experiments with human cells may help in dissecting such mechanisms in well-controlled experimental conditions.

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Conflict of interest

The authors have no conflicts of interest.

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