Research Paper

The extracellular matrix modulates $\text{H}_2\text{O}_2$ degradation and redox signaling in endothelial cells

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ABSTRACT

The molecular processes that are crucial for cell function, such as proliferation, migration and survival, are regulated by hydrogen peroxide ($\text{H}_2\text{O}_2$). Although environmental cues, such as growth factors, regulate redox signaling, it was still unknown whether the ECM, a component of the cell microenvironment, had a function in this process.

Here, we showed that the extracellular matrix (ECM) differently regulated $\text{H}_2\text{O}_2$ consumption by endothelial cells and that this effect was not general for all types of cells. The analysis of biophysical properties of the endothelial cell membrane suggested that this modification in $\text{H}_2\text{O}_2$ consumption rates was not due to altered membrane permeability. Instead, we found that the ECM regulated GPx activity, a known $\text{H}_2\text{O}_2$ scavenger. Finally, we showed that the extent of PTEN oxidation was dependent on the ECM, indicating that the ECM was able to modulate $\text{H}_2\text{O}_2$-dependent protein oxidation.

Thus, our results unraveled a new mechanism by which the ECM regulates endothelial cell function by altering redox balance. These results pinpoint the ECM as an important component of redox-signaling.

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1. Introduction

Extracellular matrix (ECM) regulation is key for normal vessel function. In resting vessels, most of the endothelial cell surface is in contact with a specialized ECM structure called basement membrane, which is composed of collagen type IV isoforms, laminin isoforms, heparan sulfate proteoglycans (perlecan or agrin) and nidogen (1 and 2). The ECM is not only a physical barrier but a source of signaling cues that are essential for the regulation of endothelial cell phenotype [1]. ECM signaling is received by cells through $\alpha\beta$ heterodimeric ECM receptors called integrins. Different $\alpha\beta$ subunit combinations are responsible for ECM binding specificity. After binding to the ECM, integrin clustering occurs, generating intracellular signaling. The signals produced by the vessel basement membrane are necessary to maintain quiescence and resting phenotype of endothelial cells [2,3]. New blood vessel growth, or angiogenesis, involves a switch from a quiescent to a proliferative and migratory phenotype which is concomitant with alterations in the ECM composition. Angiogenesis is a local process that can be induced by growth factors such as VEGF, as well as by ECM molecules such as fibronectin, newly produced by activated endothelial cells [4]. In addition, the angiogenic process also involves modification of integrin expression in endothelial cells, leading to alterations in the interactions between cells and the surrounding ECM [5]. Therefore, the regulation of the ECM is a dynamic process that is crucial for the maintenance of endothelial cell function. The disruption of ECM dynamics in blood vessels leads to pathological situations such as blood vessel fibrosis observed in aging hypertension [6], restenosis [7], atherosclerosis [8] and diabetes [9]. It is, therefore, crucial to understand the mechanisms regulating ECM–blood vessels interaction and how they contribute to endothelial cell function.

Reactive oxygen species (ROS), particularly $\text{H}_2\text{O}_2$, are also important regulators of endothelial cell homeostasis. $\text{H}_2\text{O}_2$ modulates molecular processes that are crucial for endothelial cell function such as proliferation, migration, survival and vasorelaxation [10]. Many of these molecular processes include regulation of intracellular signaling effectors, particularly those related with phosphorylation/dephosphorylation activation processes [11]. $\text{H}_2\text{O}_2$ is mainly produced by superoxide ($\text{O}_2^{-}\bullet$) dismutation, which can occur spontaneously or be catalyzed by a family of enzymes called superoxide dismutases (SOD). $\text{O}_2^{-}\bullet$ is produced by several enzymes such as NADPH oxidases (NOX and DUOX), lipoxygenase
and by the electron transport chain in mitochondria. In contrast, antioxidant enzymes such as glutathione peroxidases (GPx), peroxiredoxins (Prdx) and catalase are scavengers of H₂O₂ [12]. The counteracting activity of these enzymes is crucial for the regulation of cell signaling since it controls the amount of H₂O₂ available in specific locations within the cell and eventually the oxidation level of redox sensors.

In this paper, we show that the ECM regulates H₂O₂ degradation and protein oxidation, unveiling a novel mechanism through which the cell microenvironment regulates endothelial cell function.

2. Materials and methods

2.1. Cell lines

Human umbilical vein endothelial cells (HUVEC) were kindly provided by Sérgio Dias (Instituto de Medicina Molecular, Av. Prof. Egas Moniz, 1649-028, Lisbon, Portugal) and cultured in EBM2-supplemented medium (Lonza) with 5% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), penicillin (100 U/mL)-streptomycin (100 µg/mL) solution (Hyclone, Thermo Scientific) and 2 mM L-glutamine (Hyclone, Thermo Scientific), on 0.2% (w/v) gelatin – (G1890, Sigma-Aldrich), 10 µg/mL laminin – (L2020, Sigma-Aldrich) or 10 µg/mL fibronectin – (F0895, Sigma-Aldrich) coated plates. Plates used were between 3 and 12.

HeLa cervical carcinoma cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Hyclone, Thermo Scientific) supplemented with 10% (v/v) FBS, penicillin (100 U/mL)-streptomycin (100 µg/mL) solution (Hyclone, Thermo Scientific) and 2 mM L-glutamine (Hyclone, Thermo Scientific), on uncoated or 0.2% (w/v) gelatin – (G1890, Sigma-Aldrich), 10 µg/mL laminin – (L2020, Sigma-Aldrich), 10 µg/mL fibronectin – (F0895, Sigma-Aldrich) or 120 µg/mL collagen I – (#3440-100-01, R&D Systems) coated plates.

2.2. H₂O₂ consumption rate

Determination of H₂O₂ consumption rate by HUVEC or HeLa intact cells plated on different extracellular matrix-coated dishes (no EMC, gelatin, laminin, fibronectin, and collagen) was performed as previously described [13]. Briefly, 5 × 10⁵ HUVEC or 2.5 × 10⁵ HeLa cells were plated on 6-well plates 24 h before the assay. The culture medium was replaced by 6 mL (reaction volume) of RPMI (Lonza) one hour before H₂O₂ was added to cells. H₂O₂ consumption rate was determined by adding 50 µM of H₂O₂ to cells and following the decrease of H₂O₂ concentration [H₂O₂] over time. [H₂O₂] was quantified indirectly by measuring O₂ formation after H₂O₂ degradation by catalase. The obtained measurements were corrected for culture medium volume and compared to a previously generated calibration curve of H₂O₂. After [H₂O₂] determination, ln [H₂O₂] versus time was plotted and the slope was used to calculate H₂O₂ consumption rate (slope*reaction volume)/(number of plated cells) (3–8 independent experiments for HUVEC and 4–9 independent experiments for HeLa cells).

2.3. Quantification of cell number

For determination of the relative cell number of HUVEC after culturing in different ECMs, 5 × 10⁵ cells were plated on a 96-well plate either coated with gelatin or laminin (3 wells/condition), and incubated for 48 h. Then, cells were fixated with 4% PFA for 15 min, stained with 0.5% (w/v) crystal violet (Sigma-Aldrich) for 10 min, and incubated with 2% (w/v) SDS for 20 min to solubilize. Finally, absorbance was read at 550 nm (Sigma-Aldrich) (3 independent experiments).

2.4. Fluorescence spectroscopy

Membrane cell labeling for fluorescence spectroscopy measurements was performed as previously described, with minor adaptations [14]. Briefly, 1 × 10⁵ HUVEC were plated 24 h before the assay on gelatin- or laminin-coated 12-well plates. On the day of the assay, cells were trypsinized, resuspended in PBS with 10% (v/v) FBS, and incubated for 5 min with 2 µM of the fluorescent probe trans-Parinaric acid (t-PnA) or 3 µM of 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) (Invitrogen). The blank samples (negative control) were prepared by adding the same volume of ethanol or methanol, which was always below 1% of the cell suspension volume.

All fluorescence measurements were performed with a Horiba Jobin-Yvon Spex Fluorolog model Tau-3.22. For time-resolved measurements, the pulsed light sources used were nanoLEDs from Horiba-Jobin Yvon, with a wavelength of 315 nm for excitation of t-PnA and 370 nm for TMA-DPH, with an additional band pass filter UGI-370 for the latter. The emission wavelengths were 404 nm and 450 nm, and the band widths 15 nm and 5 nm, for t-PnA and TMA-DPH, respectively. Ludox (colloidal silica diluted to 50% (v/v) in water (Sigma-Aldrich)) was used as the scatterer to obtain the instrumental response function. For the analysis of the experimental polarized fluorescence decays, the program TRFA data processor version 1.4 (Minsk, Belarus) was used. Semi-micro quartz Suprazil® cuvettes (Hellma) were used, with the 1.00 cm optical path in the excitation direction.

The fluorescence intensity decays at the magic angle were retrieved as mentioned in the Supplementary methods and analyzed using the same iterative reconvolution method and program as for the polarized components of the decays. The fluorescence decays at the magic angle were described by multi-exponential functions of the type

\[
I(t) = \sum_{i=1}^{n} a_i \exp(-t/\tau_i)
\]

In this expression, \(a_i\) and \(\tau_i\) are the normalized amplitude and lifetime of component \(i\), respectively. The intensity-weighted mean fluorescence lifetime is given by

\[
\tau = \sum a_i \tau_i^2 \sum a_i \tau_i
\]

and the amplitude-weighted average fluorescence lifetime is defined as

\[
\tau_m = \sum a_i \tau_i
\]

The parameters describing the fluorescence anisotropy decay (rotational correlation time, \(\phi\), and residual anisotropy, \(r_m\)) were determined using a non-linear least squares global analysis method fitting simultaneously the vertically and horizontally polarized emission components as detailed in [15] and in the Supplementary methods.

The steady-state fluorescence anisotropy \(r\) values shown were obtained from the integration of the fluorescence intensity and fluorescence anisotropy decays, as described under Supplementary methods. Three to five independent experiments were performed.

2.5. Determination of catalase, GPx and Prdx expression by Western blotting

To determine the expression of enzymes responsible for H₂O₂ consumption in different ECMs, 5 × 10⁵ HUVEC or 2.5 × 10⁵ HeLa cells were plated on gelatin- or laminin-coated 6-well plates and grown for 24 h. Total proteins were then extracted by lysing cells.
with RIPA buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% (v/v) NP-40, 0.25% (w/v) Na-deoxycholate and 0.2% (w/v) SDS. After electrophoresis (SDS-PAGE), Western blotting was performed, using the following primary antibodies: catalase (H-300) (1:200, sc-50508, Santa Cruz Biotechnology), GPx-1/2 (E-7) (1:300, sc-74498, Santa Cruz Biotechnology), PRX (C-6) (1:300, sc-271047, Santa Cruz Biotechnology) and α-tubulin (1:2000, T9026, Sigma-Aldrich). Images were obtained with Image Quant LAS500 (GE Healthcare Life Sciences) (3 independent experiments).

2.6. Determination of catalase and GPx activity

Determination of catalase and GPx activity was performed as previously described [16]. Briefly, 6 × 10^5 HUVEC were plated 24 h before the assay on gelatin- or laminin-coated 6 Well plates. On the day of the assay, native proteins were extracted by sonication on phosphate buffer and quantified. For the different conditions, similar protein amounts were separated by native polyacrylamide gel electrophoresis and in-gel assays were performed to allow determining catalase or GPx activity levels. For catalase, the gel was incubated with 0.003% (v/v) H_2O_2 for 10 min, followed by washing with water and incubation with the same volume of 2% (w/v) ferric chloride and 2% (w/v) potassium ferricyanide until development occurred. The reaction was stopped by washing with water. For GPx, the gel was washed several times with 2 mM GSH, incubated with 0.008% (v/v) cumene hydroperoxide for 10 min, washed with water, and incubated with the same volume of 1% (w/v) ferric chloride and 1% (w/v) potassium ferricyanide containing GSH until development occurred. The reaction was stopped by washing with water. Differences in catalase or GPx activity levels between samples were determined by observation of achromatic clearing on the gel. For this, gels were scanned and quantification was performed using Fiji [17] (5 independent experiments).

2.7. PTEN oxidation levels

To determine PTEN oxidation levels, 4 × 10^5 HUVEC were plated 48 h before the assay on gelatin- or laminin-coated P60 culture dishes. On the day of the assay, the medium was replaced by RPMI (Lonza) supplemented with penicillin (100 U/mL)-streptomycin (100 µg/mL) solution (Hyclone, Thermo Scientific) and 2 mM L-glutamine (Hyclone, Thermo Scientific). One hour later, cells were incubated for 15 min with 50 µM H_2O_2 that was added by bolus addition. Cells incubated with H_2O_2 and control cells were scrapped on ice with a non-reducing buffer containing PBS and 40 mM N-ethylmaleimide (NEM). Proteins were then extracted by lysing cells in a non-reducing buffer containing 100 mM Tris (pH 6.8), 2% SDS (w/v) and 40 mM NEM. Proteins were loaded onto a polyacrylamide gel using a non-reducing loading buffer and without heating samples. After electrophoresis, a Western blotting was performed, using an anti-PTEN antibody (1:1000, #9552, Cell Signaling Technology) that detects the protein on both its oxidized and reduced states. Images were obtained with Image Quant LAS500 (GE Healthcare Life Sciences) and the intensity of bands corresponding to these states was quantified with Fiji [17]. Differences in PTEN oxidation in the presence of H_2O_2 between the two ECMs were obtained by dividing the percentage of oxidized/reduced PTEN in cells cultured in both ECMs by the percentage obtained in gelatin in each experiment (7 independent experiments).

2.8. Statistical analysis

Statistical analysis was undertaken using a two-tailed Student’s t test or a Mann–Whitney U test for comparison between means of two different groups. For comparison of more than two different groups, analysis was undertaken using a one way analysis of variance (ANOVA), followed by a Tukey test. The results were considered to be statistically significant when p < 0.05. p values were classified as * for p < 0.05, ** for p < 0.01 and *** for p < 0.001.

3. Results

3.1. The extracellular matrix regulates H2O2 consumption by endothelial cells

Alterations in the ECM of blood vessels have been observed in many diseases which are associated with alterations in cellular redox homeostasis [6–8]. In order to test whether the ECM modulates redox metabolism in endothelial cells, we investigated whether H_2O_2 consumption rate varied when HUVEC were exposed to different extracellular matrix proteins (gelatin, fibronectin and laminin). H_2O_2 consumption rate was determined by measuring H_2O_2 extracellular concentration at different time points after addition of 50 µM of H_2O_2 to cells. The obtained H_2O_2 consumption rates of HUVEC cultured in gelatin (0.65 ± 0.06 min⁻¹ mL⁻1/10^6 cells) and laminin (0.37 ± 0.03 min⁻¹ mL⁻1/10^6 cells) were significantly different (Fig. 1). The different consumption rates observed were not due to H_2O_2 reaction with the different ECM proteins, since the consumption rates of coated plates (without cells) were negligible (data not shown). They were also not due to different cell growth or viability, since the relative number of cells in the plates was similar (Supplementary Fig. 1). Interestingly, H_2O_2 consumption rate dependency on ECM did not occur in HeLa cells, which presented similar rate values between all conditions analyzed (Supplementary Fig. 2). These results indicate that the ECM alters H_2O_2 metabolism of endothelial cells.

3.2. Global biophysical properties of the endothelial cell membrane are not dependent on the extracellular matrix

Variation in H_2O_2 consumption rates might be due to alterations in cell membrane passive permeability, which is related to lipid organization and composition. To assess the influence of the ECM on lipid organization of the plasma membrane, we compared the biophysical properties of the cellular membranes of HUVEC cultured in either gelatin or laminin. For this, we performed fluorescence spectroscopy using two fluorescence probes, DMA-DPH and T-rPnA, which reflect the biophysical behavior of the

![Fig. 1. H2O2 consumption rates of HUVEC in different ECMs. Comparison of H2O2 consumption rates (slope= reaction volume) / (number of plated cells) of HUVEC plated on dishes coated with gelatin, fibronectin or laminin, showed that consumption of H2O2 by these cells was lower in the presence of laminin when compared with gelatin (values show the mean and SEM; N=3–8; Tukey test, **p=0.005).](https://example.com/image)
lipids. TMA-DPH stays anchored by TMA on the membrane surface and reports on the global order of the membrane. In contrast, t-PnA partitions preferentially into ordered domains, which are typically found in signaling platforms called lipid rafts, and has its tetraene fluorophore buried in the hydrophobic core of the lipid palisade [18]. Accordingly, comparing the initial anisotropy of these probes ($r_0$) obtained from the anisotropy decays (Supplementary Fig. 3) with the reported fundamental anisotropy [19] allowed to infer the presence of a very fast rotational correlation time in the case of TMA-DPH and its absence for t-PnA. This confirms a preferential labeling of more fluid lipid regions in the case of TMA-DPH and of ordered membrane microdomains by t-PnA.

The fluorescence lifetime of DPH family probes, such as TMA-DPH, is very sensitive to membrane perturbations that increase membrane hydration, which causes a strong reduction of the probe fluorescence lifetime and quantum yield [20]. Since alterations in lipid organization and composition of cellular membranes reflect $H_2O_2$ permeability [21], we measured fluorescence lifetime values of TMA-DPH of HUVEC cultured in gelatin or laminin. Our results showed a remarkable similarity between all of the fluorescence lifetime values obtained for TMA-DPH, either amplitude-weighted ($\tau_{av}$) or intensity-weighted ($\langle \tau \rangle$) (2.96 ns $\tau_{av}$ and 4.79 ns $\langle \tau \rangle$ for gelatin; 3.01 ns $\tau_{av}$ and 4.77 ns $\langle \tau \rangle$ for laminin), a strong indication that there was no difference in membrane water penetration (Fig. 2A). The steady-state fluorescence anisotropy ($r$) is the most common parameter used to assess membrane fluidity. The values of the steady-state anisotropy for TMA-DPH (0.215 for gelatin and 0.216 for laminin) indicated a fluid lipid bilayer and that there were no significant differences on the global fluidity of the membrane surface induced by the ECM proteins (Fig. 2B). The fluorescence anisotropy decay allows to decompose ($r$) into several contributions, with a direct molecular meaning and nanosecond time-resolution. The rotational correlation time ($\phi$) and infinite anisotropy values ($r_{\infty}$) contain information on the variety, speed and hindering of rotational modes of the probe wobbling. Both of these parameters were also similar for cells cultured in the two ECMs analyzed (4.61 ns $\phi$ and 0.170 $r_{\infty}$ for gelatin; 4.22 ns $\phi$ and 0.169 $r_{\infty}$ for laminin) (Fig. 2C, D). Altogether, the results obtained with the TMA-DPH probe suggest that there were no differences in membrane water permeability between cells cultured in gelatin and laminin. Therefore, the observed differences in hydrogen peroxide consumption were probably not caused by differences in the rate of passive permeation.

We then performed fluorescence analysis with the t-PnA probe. Regarding the mean fluorescence lifetimes, either amplitude-weighted ($\tau_{av}$) or intensity-weighted ($\langle \tau \rangle$), no significant differences were detected between cells cultured in either ECM (1.46 ns $\tau_{av}$ and 3.56 ns $\langle \tau \rangle$ for gelatin; 1.50 ns $\tau_{av}$ and 3.71 ns $\langle \tau \rangle$ for laminin), indicating a similar average degree of compactness of the acyl chains in those ordered domains (Fig. 2E). Concomitantly, the steady-state anisotropy ($\langle \tau \rangle$) values obtained with t-PnA for cells cultured in laminin and gelatin were similar (0.334 and 0.340, respectively) (Fig. 2F), in agreement with the absence of differences observed with the TMA-DPH probe. However, the long lifetime component ($\tau_3$) (which reports compactness of the most ordered regions) was significantly shorter in the case of cells cultured in gelatin (9.07 ns) as compared to those cultured in laminin (10.14 ns), indicating a small difference in the packing of the acyl chains of some lipids in the hydrophobic core of the membrane (Fig. 2E), with the acyl chains of cells cultured in laminin being less packed than the ones cultured in gelatin. In addition, the rotational correlation time ($\phi$) was significantly longer in the case of cells cultured in gelatin (7.82 ns) as compared to those cultured in laminin (5.03 ns) (Fig. 2G) but with similar infinite anisotropy values ($r_{\infty}$) (0.225 for gelatin and 0.244 for laminin) (Fig. 2H). These results show that cells grown in gelatin had packed ordered lipid domains, preferentially surrounding proteins (lipid anulus), which rotated slower than lipids in cells cultured in laminin. Therefore, cells cultured in different ECMs presented alterations in lipid–protein interactions in ordered membrane microdomains.

Altogether, the results obtained with both the probes suggest that there are no differences in membrane $H_2O_2$ passive permeability between endothelial cells cultured in gelatin and laminin.

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**Fig. 2.** Biophysical properties of HUVEC cell membrane lipids. Analysis of TMA-DPH (A) amplitude-weighted ($\tau_{av}$) and intensity-weighted ($\langle \tau \rangle$) mean fluorescence lifetime, (B) steady-state fluorescence anisotropy ($r$), (C) rotational correlation time ($\phi$) and (D) infinite anisotropy ($r_{\infty}$) from HUVEC cultured in gelatin or laminin showed that the ECMs had no effect on water penetration and induced no changes on the global order and dynamics at the membrane surface. Analysis of t-PnA (E) long fluorescence lifetime component ($\tau_3$), amplitude ($\tau_{av}$) and intensity-weighted mean fluorescence lifetime, (F) steady-state fluorescence anisotropy ($r$), (G) rotational correlation time ($\phi$) and (H) infinite anisotropy ($r_{\infty}$) from HUVEC cultured in gelatin or laminin showed that the ECMs had no effect on water penetration but induced subtle changes in the packing and dynamics of some lipids in ordered membrane microdomains (values are the mean and SEM; $N$=3–5; two-tailed Student’s $t$ test, *$p<0.02$, **$p<0.00002$).
3.3. Cell adhesion to the extracellular matrix modulates GPx activity in endothelial cells

Our fluorescence spectroscopy results showed that endothelial cells presented similar membrane biophysical properties in the presence of different ECMs, with the observed different hydrogen peroxide consumptions probably not caused by differences in the rate of passive permeation. An alternative explanation for the variations observed in the H$_2$O$_2$ consumption rates of HUVEC in the different ECMs is the alteration of activity of enzymes responsible for H$_2$O$_2$ consumption. To test this hypothesis, we analyzed the expression of catalase, GPx-1 and -2, and Prdx-I, -II and -IV by Western blotting. Our results showed no differences in expression of these proteins (Supplementary Fig. 4). Differences in H$_2$O$_2$ consumption rates of HUVEC in the different ECMs could still be due to alterations in expression of other H$_2$O$_2$ scavengers or alterations in enzyme activity due to post-translational modifications. We, therefore, used in-gel activity assays to assess total catalase and GPx activity in HUVEC cultured in gelatin or lammin. Catalase activity was similar between the cells cultured in the two substrates (Fig. 3A). However, GPx activity of endothelial cells cultured in the presence of laminin was 56% lower than when cultured in the presence of gelatin (Fig. 3B). This is in the same proportion as the variation of the corresponding consumption rates (44%, Fig. 1), strongly suggesting that the alteration in GPx activity was the main responsible for the differences observed in the consumption rates of endothelial cells when cultured in gelatin or laminin.

3.4. The extent of PTEN oxidation is dependent on the extracellular matrix

H$_2$O$_2$, either from extracellular or intracellular sources, is able to alter the function of several proteins regulating cell signaling pathways in a process called redox signaling [22]. Phosphatase and tensin homolog (PTEN), a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (EC 3.1.3.67), is a redox-regulated protein that is reversibly inactivated by H$_2$O$_2$ through the formation of a disulfide between Cys71 and Cys124 [23]. In order to test whether the extracellular matrix is able to modulate redox-signaling, we quantified the oxidation level of PTEN in endothelial cells cultured in gelatin or laminin. Catalase oxidation levels in HUVEC on different ECMs showed that in the presence of H$_2$O$_2$, PTEN was mainly in the reduced form, in the presence of H$_2$O$_2$, PTEN was oxidized in an ECM-dependent manner, since endothelial cells cultured in laminin showed increased PTEN oxidation when compared with cells cultured in gelatin. Percentage of oxidized/reduced PTEN obtained for each ECM in each experiment was normalized by the percentage obtained for gelatin (values show the mean and SEM; N = 7; Mann–Whitney U test, *p < 0.03).

![Fig. 4. PTEN oxidation levels in HUVEC on different ECMs. Western blots for PTEN after non-reducing SDS-PAGE and subsequent quantification of PTEN oxidation levels in HUVEC on different ECMs showed that in the presence of H$_2$O$_2$, PTEN was oxidized in an ECM-dependent manner, since endothelial cells cultured in lammin showed increased PTEN oxidation when compared with cells cultured in gelatin. Percentage of oxidized/reduced PTEN obtained for each ECM in each experiment was normalized by the percentage obtained for gelatin (values show the mean and SEM; N = 7; Mann–Whitney U test, *p < 0.03).](image)

![Fig. 3. Catalase and GPx activity in HUVEC plated on different ECMs. In-gel assays to assess the activity of catalase and GPx (band intensity) in HUVEC cultured in gelatin or lammin showed that (A) catalase activity was similar between cells cultured in the two ECMs, whereas (B) GPx activity in cells cultured in the presence of lammin was lower than when cultured in the presence of gelatin (a.u., arbitrary units; values show the mean and SEM; N = 5; two-tailed Student’s t test, **p = 0.005).](image)

4. Discussion

In this paper, we have shown that the ECM modulates H$_2$O$_2$ consumption by HUVEC and the regulation of GPx activity. Adhesion to different matrices changes PTEN oxidation levels induced by extracellular H$_2$O$_2$, indicating that the ECM is able to contribute to redox signaling in endothelial cells. The balance between H$_2$O$_2$ production and degradation is essential for redox signaling activation, maintenance and cessation [24]. In an organ context, H$_2$O$_2$ consumption rate of a given cell potentially influences the redox signaling not only of that particular cell but also of other neighboring cells, since it modulates the concentration of extracellular H$_2$O$_2$. For example, during inflammation, H$_2$O$_2$ production is induced in cells not only from the immune system but also from the adjacent tissue [25]. This increase in H$_2$O$_2$ concentration induces leukocyte migration and activates signaling pathways such as NF-$\kappa$B, which are responsible for the inflammatory process [24,26]. Therefore, the alteration of the H$_2$O$_2$ degradation kinetics in endothelial cells by the ECM might be responsible for the inflammatory outcome.
4.1. The ECM does not alter membrane permeability but modulates the activity of the antioxidant enzymatic system

The consumption rate of extracellular H$_2$O$_2$ varies between cells and is a consequence of the activity of antioxidant enzymatic systems, the size of exposed surface membrane and its permeability [27]. Membrane exposed surface and permeability are responsible for the amount of H$_2$O$_2$ that reaches the intracellular space, where it is then degraded by the antioxidant enzymatic system [28].

Here, we have shown that the attachment to different matrices had no major implications in the biophysical properties of the membrane. These results suggested that differences in H$_2$O$_2$ consumption rate induced by the ECM in HUVEC were not related to alterations in H$_2$O$_2$ passive permeability. Instead, we have shown that there were alterations in the antioxidant enzymatic system, in particular in the activity of intracellular GPx, which was shown to be important for endothelial cell function [29,30]. However, it remains to be determined whether this regulation occurred at the GPx expression level (other than GPx-1 and -2) or by modulation of its activity by post-translational modifications. Importantly, the same alterations were not verified in the case of catalase, showing that this was not a general regulation of the antioxidant enzymatic system. Since cell medium was replaced before consumption rate determination, it is unlikely that alterations in the secretion of molecules with reducing potential could be responsible for the differences observed in HUVEC consumption rates. However, it is possible that the presence of gelatin or laminin modifies the secretion profile of cells, particularly of extracellular GPx, such as GPx-3 [31]. The ECM might also alter the activity of other antioxidant enzymes such as peroxiredoxins that contribute to a decrease in H$_2$O$_2$ consumption rate. However, the decrease observed in H$_2$O$_2$ consumption rate of cells cultured in laminin was of the same magnitude as the decrease in GPx activity in the same cells, indicating that GPx activity was the major factor altered by the two ECMs tested. Besides the activity of antioxidant enzymes, the activity of aquaporins might also influence H$_2$O$_2$ consumption rate in cells. Aquaporin-3 and -8 are able to transport H$_2$O$_2$ in addition to water across mammalian plasmatic membrane, increasing H$_2$O$_2$ intracellular concentration [32]. A possible role of aquaporin-3 and -8 in the regulation of H$_2$O$_2$ metabolism by ECM cannot be excluded, although the expression and function of these two aquaporins have not been reported in HUVEC. Since each ECM signals through specific integrins, our results indicate that HUVEC might express particular integrins that were responsible for the alteration of GPx activity observed in our assays.

4.2. The ECM alters membrane lipid–protein interactions

Although no differences in the global order of the membrane were observed, the lipid–protein organization of ordered microdomains presented variations between cells cultured in gelatin or laminin. The ordered microdomains sensed by T-PnA constitute the majority of lipid rafts and in the case of laminin presented more acyl chain packing and faster lipid rotation than in cells cultured in gelatin. If only lipid–lipid interactions were contributing to these results, tighter packing would lead to slower rotation. Therefore, lipid–protein interactions are playing a critical role in this behavior. Integrins are present and preferentially activated in these specialized membrane microdomains called lipid rafts [33,34]. In vitro experiments using raft-mimicking models have shown that each integrin organizes differently in cellular membranes, promoting particular lipid–protein interactions [35]. Therefore, the differences between the ordered microdomains of cells cultured in gelatin vs. laminin are probably due to the recruitment and/or clustering of specific integrins at the cell membrane. Our results, therefore, suggest that lipid raft organization is dependent on the type of integrins activated by different ECMs.

4.3. The ECM modulates redox signaling

PTEN antagonizes the PI3K pathway, which is activated during angiogenesis by several pro-angiogenic factors including VEGF [36]. Therefore, PTEN inactivation, both by transcriptional regulation and post-translational modifications (such as oxidation), promotes endothelial activation and proliferation [37]. Our results have shown that, in the case of endothelial cells, PTEN oxidation upon H$_2$O$_2$ exposure was higher in cells cultured in laminin when compared with those cultured in gelatin, most likely by the regulation of GPx activity. This might be a mechanism by which cells regulate the angiogenic process in particular pathogenic situations in which ECM composition is modified. Indeed, laminin upregulation was observed in endothelial cells in both in vivo and in vitro models of inflammation that induces endothelial cell activation and capillary sprouting [38,39]. This alteration in the ECM composition is also important for wound healing because it increases angiogenesis and T cell extravasation [40–42]. Notably, the use of laminin to promote wound healing by increasing angiogenesis has already been proposed [40]. The mechanism of action of these potential therapeutic agents remains largely unexplored and might be dependent on a still neglected redox-regulated process. Although only PTEN was used as a read-out for alterations in redox signaling, other redox-regulated proteins are probably similarly affected by the ECM to which cells adhere.

5. Conclusions

The microenvironment surrounding a particular cell influences cell fate through the activation of particular signaling pathways. Our results show that the ECM is an important microenvironment component that modifies the kinetics of H$_2$O$_2$ consumption and the redox signaling outcome. The ability of the ECM to modulate H$_2$O$_2$ consumption might be important for endothelial cell function during angiogenesis, for endothelial cell migration and tube formation, and in the quiescent state, for endothelial cell survival and homeostasis regulation. Our results, therefore, demonstrate the existence of a crosstalk between ECM-dependent signaling and redox signaling in order to direct endothelial cell behavior.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2015.09.006.
