A comparative study unraveling the effects of TNF-α stimulation on endothelial cells between 2D and 3D culture

Bo Wang, Ruomeng Chen, Hongqian Gao, Xiaohan Lv, Lifang Chen, Weirong Wang, Yaxiong Liu, Nanbo Zheng and Rong Lin

1 Department of Pharmacology, School of Basic Medical Sciences, Xi’an Jiaotong University Health Science Center, Xi’an 710061, Shaanxi, People’s Republic of China
2 Mechanical and Electrical Engineering Department, Tangshan University, Tang Shan 063000, Hebei, People’s Republic of China
3 Department of Medical Laboratory Animal Science, School of Basic Medical Sciences, Xi’an Jiaotong University Health Science Center, Xi’an, People’s Republic of China
4 State Key Laboratory for Manufacturing Systems Engineering, Xi’an Jiaotong University, Xi’an 710061, Shaanxi, People’s Republic of China
5 Rong Lin and Nanbo Zheng contributed equally to this work.

E-mail: linrong63@aliyun.com and znbtougao@aliyun.com

Keywords: endothelial dysfunction, 3D culture, 2D-TCPs, bioinformatics, ACE, CD40

Abstract
Endothelial cell (EC) dysfunction is an important predictor of and contributor to the pathobiology of cardiovascular diseases. However, most in vitro studies are performed using monolayer cultures of ECs on 2D tissue polystyrene plates (TCPs), which cannot reflect the physiological characteristics of cells in vivo. Here, we used 2D TCPs and a 3D culture model to investigate the effects of dimensionality and cardiovascular risk factors in regulating endothelial dysfunction. Cell morphology, oxidative stress, inflammatory cytokines and endothelial function were investigated in human umbilical vein endothelial cells (HUVECs) cultured in 2D/3D. The differentially expressed genes in 2D/3D-cultured HUVECs were analysed using Enrichr, Cytoscape and STRING services. Finally, we validated the proteins of interest and confirmed their relevance to TNF-α and the culture microenvironment. Compared with 2D TCPs, 3D culture increased TNF-α-stimulated oxidative stress and the inflammatory response and changed the mediators secreted by ECs. In addition, the functional characteristics, important pathways and key proteins were determined by bioinformatics analysis. Furthermore, we found that some key proteins, notably ACE, CD40, Sirt1 and Sirt6, represent a critical link between endothelial dysfunction and dimensionality, and these proteins were screened by bioinformatics analysis and verified by western blotting. Our observations provide insight into the interdependence between endothelial dysfunction and the complex microenvironment, which enhances our understanding of endothelial biology or provides a therapeutic strategy for cardiovascular-related diseases.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ECs          | endothelial cells |
| TCPs         | tissue polystyrene plates |
| ET-1         | endothelin-1 |
| NO           | nitric oxide |
| PGI₂         | prostacyclin 2 |
| 2D           | two-dimensional |
| 3D           | three-dimensional |
| ECM          | extracellular matrix |
| PDMS         | polydimethylsiloxane |
| GO           | Gene Ontology |
| KEGG         | Kyoto Encyclopedia of Genes and Genomes |
| PPI          | protein–protein interaction |
| ACE2         | angiotensin-converting enzyme 2 |
| TNFR         | tumor necrosis factor receptor |

1. Introduction
Cardiovascular disease is a complex process involving many steps and the interplay of systemic and local factors. Endothelial dysfunction is an early predictor...
of and important contributor to the pathobiology of atherosclerosis and future cardiovascular events [1]. The endothelium acts as a barrier to control the exchange of nutrients and messengers with surrounding tissues and controls vascular tone by releasing vasoconstrictors such as ET-1 and vasodilators such as NO and PGI2 [2]. However, when challenged with classic cardiovascular risk factors such as proinflammatory cytokine TNF-α, low shear stress and hypercholesterolemia (e.g. oxidatively modified lipoproteins), ECs undergo a coordinated program of gene activation and alter many of these vital functions [3]. EC dysfunction encompasses various nonadapptive alterations in the normal EC phenotype, with important implications for the regulation of haemostasis and thrombosis, local vascular tone and redox balance, and the orchestration of chronic and acute inflammation [4].

The vast majority of current research on endothelial dysfunction is typically performed using monolayer cultures of ECs performed on 2D TCPs [5]. However, 2D cell cultures are not capable of reflecting the physiological characteristics of the endothelium inside vascularized tissue. In the body, the vascular endothelium is a single layer of cells that line the internal surface of the vasculature and grow on a collagen and proteoglycan ECM in a 3D microenvironment (figure 1(A)). The 3D character of the cell microenvironment serves as not only structural support but also a source of biochemical and biophysical cues that trigger and regulate cell behavior [6]. For example, matrix composition and stiffness have significant impacts on endothelial attachment, proliferation and functions, and matrix stiffening serves as a potent destructive factor for the endothelium [7]. Zhang et al. analysed the global gene expression of ECs in 2D or 3D culture to explore the influence of dimensional culture conditions in altering EC proliferative phenotypes [8]. Some studies used hollow 3D PDMS tubes with ECs on the interior surfaces to emulate human blood vessels [9, 10]. However, the hydrophobic PDMS surface directly affects cell adhesion and does not provide the physical resistance of the ECM, ECs cultured in 3D PDMS models may not be ideal tools to recapitulate the physiological characteristics of the endothelium inside vascularized tissue. Hydrogels have been utilized to recapitulate the features of native tissues and provide diverse biochemical (e.g. cell adhesion sites) and biophysical (e.g. structural features, mechanical stiffness, and degradation) cues to regulate EC behaviour [11]. On this basis, a shift in EC culture from 2D TCPs towards 3D hydrogel models resembling an environment similar to the physiological environment may provide a more reliable tool for basic research or drug testing [12].

The advantages of using 3D culture over 2D monolayers have already been widely demonstrated in studies of cancer progression, signal transduction, and drug toxicity [6]. Recently, it has become apparent that the responses of various cells to multiple stimuli are significantly influenced by the 2D/3D culture microenvironment, and stimulation of cells in 3D culture models can yield a more in vivo-like response than that of conventional 2D monolayer cell culture [13–15]. For example, the interdependence between culture dimensionality and the response to hypoxia in tumor cells was revealed, and interleukin-8 emerged as a major player in the microenvironmental regulation of the hypoxia response [16]. Teresa et al demonstrated that treatment with Shiga toxins resulted in elevated secretion of kidney injury marker 1 and cytokines in 3D cultured renal proximal tubule cells compared with that of 2D cultures [17]. However, how the interactions between cardiovascular risk factors (i.e. TNF-α) and culture dimensionality regulate endothelial function are poorly understood, and it is critical to separate the effects of TNF-α and culture dimensionality in 2D monolayer and 3D culture models.

Here, we used 2D TCPs and a 3D culture model of human umbilical vein endothelial cells (HUVECs) to distinguish the independent and dependent effects of the microenvironment and TNF-α in regulating endothelial dysfunction. 3D culture models were fabricated by seeding HUVECs into a gelatin-based engineered carotid artery (3D-Gel) according to our previous works (figure 1(B)) [18]. Cell morphology, oxidative stress, inflammatory cytokines and endothelial function were investigated in HUVECs cultured in 2D TCPs and 3D models. Differentially expressed genes in 2D/3D-cultured ECs were analysed for functional characteristics, important pathways and key proteins using Enrichr, Cytoscape and STRING services. Finally, we validated the proteins of interest and confirmed their relevance to TNF-α and different culture microenvironments. Insights gained from this study may advance our understanding of endothelial dysfunction and identify routes towards more effective therapy (figure 1(C)).

2. Materials and methods

2.1. Reagents

The microbial transglutaminate (mTG, Activa-TI, Ajinomoto Inc. activity of approximately 100 U g−1) was provided by Ajinomoto Inc. The gelatin (G2500; Sigma) was provided by Sigma-Aldrich (Saint Louis, MO, USA). TNF-α was obtained from PeproTech (Rocky Hill, NJ, USA). LDH kit was obtained from R&D (San Diego, CA, USA), Detection kits for MDA and the activities of SOD, Human ET-1 and PGI2 ELISA kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). p-Akt,
Figure 1. A schematic diagram of the systematic strategies for unraveling the effects of TNF-α on HUVECs cultured in 2D and 3D environment. (A) In the body, the vascular endothelium is a single layer of cells that line the luminal surface of the vasculature, grow on a collagen and proteoglycan ECM. (B) We designed the geometry of human common carotid artery and fabricated the mold using stereolithography, obtained the gelatin-based carotid artery model. Then HUVECs suspension (2.0 × 10^5 cells ml^{-1}) was pipetted into the carotid artery model and incubated. (C) Experimental design to compare response of HUVECs to TNF-α in 2D versus 3D culture. Experimental design comprised culturing HUVECs on 2D conventional tissue culture 6 polystyrene (TCP) or cultured in the engineered carotid arteries (3D-Gel) treated with TNF-α for 24 h. Subsequently, the shape index, release of LDH, oxidative stress, inflammatory response and endothelial function were detected. Then the protein of HUVECs was isolated and detected by western blotting. Besides, the RNA-seq analysis was performed to investigate the altered biological function of the differentially expressed genes using Enrichr, ClueGO and STRING.

TNRF1, ACE2, CD40 and Sirt6 primary antibodies were purchased from ABclonal Biotech Co., Ltd. (College Park, MD, USA). Akt, ERK, p-ERK, ACE, and HRP-labeled anti-mouse and -rabbit IgG secondary antibodies were obtained from Beyotime Biotech Co., Ltd (Shanghai, China). eNOS, p-eNOS, Sirt1 and β-actin primary antibodies were ordered from Cell Signaling Technology (Boston, MA, USA). All other chemicals were of high purity and were obtained from commercial sources.

2.2. Cell culture in 2D TCPs and 3D culture models
HUVECs were grown in low glucose medium supplemented with 10% foetal bovine serum (FBS). To investigate HUVECs cultured in a 3D microenvironment, we created a gelatin-patterned, endothelialized carotid artery system (figure 1(A)). Briefly, we analyzed the geometry of the human common carotid artery and fabricated a mold using stereolithography, obtaining the gelatin-based carotid artery model as shown in figure 1(B). Then, the HUVECs suspension (2.0 × 10^5 cells ml^{-1}) was pipetted into the carotid artery model and incubated at 37 °C for 3 min. The devices were rotated 90° from the horizontal axis, refilled with suspended HUVECs, and incubated until the HUVECs evenly coated the channel walls as previously reported. The endothelialized devices were incubated at 37 °C for 4 h while rotating about the horizontal axis at 8 rpm using a HulaMixer (Life Technologies, Carlsbad, CA). The distribution of HUVECs inside the engineered carotid artery was observed by microscopy.

2.3. Experimental protocols
The endothelialized engineered carotid arteries were randomly divided into seven groups, each of
which contained 5 endothelialized engineered carotid arteries; the control group and different concentrations of TNF-α (1.56, 3.125, 6.25, 12.5, 25, 50 and 100 ng ml⁻¹) groups. Briefly, HUVECs were seeded in the 3D culture model and treated with different concentrations of TNF-α for 24 h. Then, the culture supernatants were collected to measure oxidative stress and inflammation markers, and the cells were digested by trypsin, washed by ice-cold PBS three times. In the 2D TCP groups, HUVECs were cultured in 6-well or 96-well plates and incubated with the corresponding concentrations of TNF-α for 24 h.

2.4. Shape index of HUVECs
The single image was taken of each model piece, with fifty representative HUVECs analyzed from each image. To quantify cell elongation, we used the shape index (SI) Where A = area and P = perimeter.

\[ SI = \frac{4\pi A}{P^2} \]

Images were analyzed using Image J and Graph-Pad Prism 7 to analyze the shape index (SI), a metric that ranges from 0 to 1, describing how rounded the cell is; the closer the SI is to 1, the rounder the cell. The code recognizes the boundaries of the chosen representative cell based upon pixel intensity to determine the area, perimeter, and shape index of the EC nucleus, routinely used to quantify cell morphology.

2.5. Levels of oxidative stress, inflammatory cytokines and endothelial function in culture medium
After the cells were treated with TNF-α and vehicles, the culture supernatant was collected. Cytotoxicity was quantified by measuring lactate dehydrogenate (LDH) release in the medium according to the manufacturers’ instructions. The MDA and total SOD activity was determined by the thioabiturbic acid reaction using a commercial kit. Optical density levels were measured at 450 nm using a microplate reader (Multiskan MK3, Thermo, USA). The levels of IL-1β, IL-6, PGI2 and ET-1 were detected using the appropriate ELISA kits. NO content present in the medium was evaluated by measuring nitrite according to the Griess methods using Total Nitric Oxide Assay Kit. The values of the samples were calculated through the corresponding comparison of the absorbance values with prediluted standards (R&D Systems, Inc.).

2.6. GO analysis
RNA-seq processing for EB Seq analysis and normalized gene expression for HUVEC samples used a previously deposited dataset (GEO: GSE93511) [8]. To find the relevant functions linked with a given gene list, the differentially expressed genes showing statistically significant difference between the 2D and 3D culture HUVECs were evaluated by GO. The genes were enriched according to the GO information including biological process (BP), molecular function (MF) and cellular component (CC). The genes were submitted to analyze the GO enrichment using the Cytoscape plugin ClueGO app. Cytoscape v3.6.1 is an open source software project for integrating biomolecular interaction networks with high-throughput expression data and other molecular states into a unified conceptual framework. ClueGO is a Cytoscape plug-in that visualizes the non-redundant biological terms for large clusters of genes in a functionally grouped network. The P-value < 0.05 was considered statistically significant.

2.7. KEGG and PPI network analysis
To analyze the differentially expressed genes at the functional level, the Enrichr server was used to provide functional interpretation of the KEGG pathway analysis. The KEGG pathway analysis were used to acquire related pathway information. In addition, the gene names were submitted to Search Tool for the Retrieval of Interacting Genes/Proteins 10.0 (STRING 10.0), to provide a biological functional interpretation of the genes. The STRING database is an open-access tool designed to evaluate the PPI information. To assess the interactive relationships, the identified differentially expressed genes were mapped via STRING. Then the results were loaded and visualized by Cytoscape Network Analyzer to construct the PPI networks between the differentially expressed genes. The node colors were mapped to degree and the edge color were mapped to combined score.

2.8. Western blot analysis
HUVECs were separated by trypsin digestion and dissociated by Cell Lysis Buffer. Protein concentration was quantified and an equal amount of protein was loaded, subjected to electrophoresis and electrophoretically transferred to polyvinylidine difluoride filter (PVDF) membrane. The membrane was blocked with 5% nonfat milk for 2 h, followed by an overnight incubation at 4 °C with specific primary antibodies. Thereafter the membrane was incubated with peroxidase-conjugated secondary anti-mouse or rabbit antibodies for 1 h. Bands were detected by ECL (GE Healthcare, Buckinghamshire, UK) as described previously.

2.9. Statistical analysis
All values were expressed as mean ± standard error (SEM) and analyzed using SPSS software (SPSS for windows version 16.0, USA). All experiments were repeated at least five times to obtain the data. Differences between mean values of normally distributed data were evaluated by one-way ANOVA followed by
Figure 2. (A) Morphology of HUVECs cultured in the engineered carotid arteries (3D-gel) under the influence of TNF-\(\alpha\). Morphological images were obtained using an inverted microscope connected to a digital camera. ECs imaged at \(\times\) 100 magnification. All images are of ECs that have been stimulated with 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 ng ml\(^{-1}\) TNF-\(\alpha\). Scale bars, 100 \(\mu\)m. (B) The stimulation of TNF-\(\alpha\) caused a significant decrease in the EC shape index when compared to control group. (C) HUVECs were incubated with TNF-\(\alpha\) (1.56, 3.125, 6.25, 12.5, 25, 50 and 100 ng ml\(^{-1}\)) for 24 h cultured in 2D-TCP or 3D-Gel. The release of LDH was detected by commercial kits. \(*P<0.01\) vs. 2D-TCP control group; \(##P<0.01\) vs. 3D-Gel control group. Tukey’s post hoc test. \(P<0.05\) was considered to be statistically significant between groups.

3. Results

3.1. Effect of TNF-\(\alpha\) on the morphology of HUVECs cultured in a 3D model

TNF-\(\alpha\) is a highly pleiotropic cytokine that induces biological effects such as inflammatory responses, apoptosis and necrosis. To validate our 3D culture model for the study of TNF-\(\alpha\)-induced endothelial dysfunction, we established a TNF-\(\alpha\)-stimulated EC damage model by incubating HUVECs with different concentrations of TNF-\(\alpha\) (1.56, 3.125, 6.25, 12.5, 25, 50 and 100 ng ml\(^{-1}\)) for 24 h. Cell phenotypes and functions \(\textit{in vivo}\) are driven by a wide variety of factors that are mainly related to the extracellular environment. Therefore, we observed the morphology and investigated the SI of HUVECs in 3D culture. Visually, HUVECs appeared elongated compared with that of cells in the control group (figure 2(A)). HUVECs exposed to TNF-\(\alpha\) had a significantly lower SI than that of the HUVECs in control group (figure 2(B)).

3.2. Effect of TNF-\(\alpha\) on LDH activity in HUVECs in 2D and 3D models

Cell viability was detected at 24 h post-treatment initiation using LDH assays and is expressed as a percentage of the corresponding 2D or 3D control (figure 2(C)). Specifically, treatment with 6.25 ng ml\(^{-1}\) TNF-\(\alpha\) increased the release of LDH in the cell culture supernatants of HUVECs cultured in 3D but did not affect the release of LDH in cells cultured in 2D-TCPs. At increasing concentrations (i.e. 12.5, 25 ng ml\(^{-1}\) and higher concentrations), TNF-\(\alpha\) treatment resulted in additional concentration-dependent decreases in cell viability in treated cells compared with that of control cells. We concluded that TNF-\(\alpha\) at medium and high concentrations triggers cytotoxicity in HUVECs similarly in both 2D and
Figure 3. Dimensionality regulates the levels of oxidative stress and inflammatory response in HUVECs. HUVECs were treated with TNF-α (1.56, 3.125, 6.25, 12.5, 25, 50 and 100 ng ml$^{-1}$) for 24 h. The level of MDA and the activity of SOD in the media samples were collected and detected by respective assay kits (A) and (B). The levels of IL-1β and IL-6 were measured by ELISA (C) and (D). Data were expressed as the mean ± SEM of five independent experiments. *∗$P<0.01$ vs. 2D-TCP control group; ##$P<0.01$ vs. 3D-Gel control group.

3.3. Dimensionality regulates the levels of oxidative stress in HUVECs

Because increased levels of oxidative stress are associated with endothelial dysfunction, we investigated the integrated effects of culture dimensionality and TNF-α on the level of MDA and the activity of SOD. As shown in figures 3(A) and (B), 1.56 and 3.125 ng ml$^{-1}$ TNF-α did not affect MDA or SOD in either 2D TCPs or 3D cultures. Interestingly, 6.25 ng ml$^{-1}$ TNF-α increased the level of MDA in 3D cultures and inhibited the activity of SOD, whereas 2D cultures were unaffected. In addition, after treatment with 12.5, 25, 50 and 100 ng ml$^{-1}$ TNF-α, the levels of MDA in both 2D and 3D cultures were markedly increased and the level of SOD was decreased compared with those of the corresponding controls. The above results indicate that the 3D culture microenvironment exacerbates TNF-α-induced HUVEC oxidative stress.

3.4. TNF-α induces inflammatory cytokine secretion in 3D and 2D cultures

Since inflammatory cytokines play important roles in TNF-α-induced endothelial dysfunction, we examined differences in the secretion of inflammatory cytokines in 3D and 2D cultures. In both 3D and 2D cultures, the secretion of IL-1β and IL-6 was not induced 24 h after exposure to 1.65 and 3.125 ng ml$^{-1}$ TNF-α (figures 3(C) and (D)). Statistically significant changes in the expression of IL-1β and IL-6 were observed in response to 6.25 ng ml$^{-1}$ TNF-α in 3D cultures. However, in 2D cultures, the secretion of IL-1β and IL-6 was increased by TNF-α at a concentration of 12.5 ng ml$^{-1}$. The secretion of IL-1β and IL-6 in both 3D and 2D cultures was enhanced by TNF-α at concentrations of 12.5, 25, 50 and 100 ng ml$^{-1}$, but the increase was less pronounced in 2D cultures. This increased secretion of cytokines in 3D versus 2D culture indicates that a property of the 3D culture system upregulates both sensitivity and inflammatory response in HUVECs, which is similar to the effects on LDH release and oxidative stress.
Figure 4. Endothelial functional factor secretion of HUVECs changes with culture platform. HUVECs were treated with TNF-α (1.56, 3.125, 6.25, 12.5, 25, 50 and 100 ng ml\(^{-1}\)) for 24 h in 2D-TCP or 3D-Gel. Media samples were collected to measure NO release using Greiss Reagent (A). The levels of PGI\(_2\) and ET-1 were detected by ELISA (B) and (C). Data were expressed as the mean ± SEM of five independent experiments. **P < 0.01 vs. 2D-TCP control group; ***P < 0.01 vs. 3D-Gel control group.

3.5. The culture platform changes endothelial functions of HUVECs
Vasodilators and vasoconstrictors produced by ECs, such as NO, PGI\(_2\) and ET-1, play crucial roles in the regulation of vascular homeostasis and prevent the initiation and progression of endothelial dysfunction. In the present study, we compared systemic NO, ET-1 and PGI\(_2\) levels in HUVECs affected by TNF-α in 2D and 3D cultures (figures 4(A)–(C)). In 3D cultures, TNF-α at the concentrations 6.25, 12.5, 25, 50 and 100 ng ml\(^{-1}\) inhibited the production of NO and PGI\(_2\) and increased the levels of ET-1. ET-1 secretion was induced 4.2-fold by 100 ng ml\(^{-1}\) TNF-α in 3D cultures, which was higher than that induced in 2D cultures by the same concentration of TNF-α. In addition, the secretion of PGI\(_2\) and ET-1 was markedly lower in 3D cultures than in 2D-TCPs.

3.6. GO analysis insight into the distinct roles of dimensionality
Considering the differences in cell behaviours between 3D-cultured HUVECs and 2D TCP-cultured HUVECs, we next analysed the gene expression profiles of HUVECs in 2D and 3D microenvironments. To investigate the altered biological functions of differentially expressed genes, the annotation clusters of GO terms associated with the changes in gene expression between the 3D and 2D cultures were identified. The results shown in figure 5(A) represent the annotation clusters of CC terms, which showed that most of the differentially expressed genes that were upregulated in the 3D culture microenvironment were located at or near the plasma membrane. As was predicted, there was enrichment in the BP categories, such as vasculature development, inflammatory response, cardiovascular system development and cell surface receptor signalling pathway (figure 5(B)). Then, we performed MF analysis and found that the genes were associated with functional characteristics such as integrin binding, histone deacetylase binding, collagen binding involved in cell-matrix adhesion, phosphatidylinositol 3-kinase binding, and ECM binding (figure 5(C)). Interestingly, histone deacetylase binding and phosphatidylinositol 3-kinase binding were highly ranked among the top functions.

3.7. KEGG and PPI insight into the distinct roles of the 2D/3D culture microenvironment
To identify the signalling pathways associated with culture dimensionality-induced changes in HUVECs, we performed KEGG pathway enrichment analyses, and the top ten enriched pathways are listed by their statistical significance in figure 6(A), providing insight into the potential pathways affected by the 3D culture environment. Interestingly, the PI3K-Akt signalling pathway was highly ranked with 23 differentially expressed genes. In addition, to explore the possible PPIs among these genes, the gene list was submitted to STRING. The PPI network is shown in figure 6(B), and we observed that ACE, CD40, BCL2, CXCR4, CCL2 and ICAM-1, emerged as key node proteins. Taken together, the bioinformatics analysis suggests that PI3K-Akt signaling is one of the top functions linked to the culture dimension, and ACE and CD40 are important proteins linked to the changes in ECs in the 3D and 2D culture.

3.8. Impact of 3D culture on the phosphorylation of Akt, ERK and eNOS
Based on our combined results of GO MF (figure 5(D)) and KEGG pathway analysis (figure 6(A)), and PI3K-Akt signalling suggested to have important effects on blood vessel morphogenesis and endothelial dysfunction, we hypothesized that the 3D culture environment might affect HUVECs by regulating PI3K-Akt signalling. To test this hypothesis, we investigated the activation of Akt, ERK and eNOS (figure 7(A)). HUVECs cultured on 2D TCPs were characterized by increased phosphorylation of ERK1/2 compared with that of cells cultured in 3D hydrogels. Expression of phosphorylated Akt and eNOS in 3D-cultured HUVECs was increased compared with that of cells cultured in 2D-TCPs.
Figure 5. GO analysis of the differentially expressed genes that were upregulated in the 3D culture microenvironment. To find the relevant functions linked with a given gene list, the differentially expressed genes showing statistically significant difference between the 2D and 3D culture ECs were evaluated by GO analysis. (A) The top cellular component (CC) terms that have significant differentially expressed genes. Biological process (B) and molecular function (C) terms sorted by $P$ value < 0.05 were listed.
Figure 6. (A) KEGG pathways analysis was used to evaluate functional networks from the gene expression data. Top ten ranked pathways were identified based on the z-score in the Enrichr server. (B) PPI of the differentially expressed genes in 2D and 3D HUVECs. To better visualize the network and separate genes from different sources, the list of individual interactions with scores was generated with STRING and subsequently analysed by Cytoscape. The colors of node were changed with the degree, and the colors of edge were changed with the combined score.

Consistent with the bioinformatics analyses, these results suggest that the activities of Akt, ERK and eNOS are associated with regulating the EC phenotype in 2D TCPs and 3D cultures.

3.9. Influence of 3D culture and TNF-α on endothelial ACE and ACE2 expression

Because ACE is prominently regulated by culture dimensionality at the individual gene (figure 5(A)) and PPI levels (figure 6(C)), and ACE plays an important role in maintaining the balance between the vasodilatation and the renin angiotensin system, we chose this protein as a representative candidate for further investigation. As shown in figure 7(B), the expression of ACE was significantly higher in HUVECs grown in 3D culture than in HUVECs grown on 2D TCPs, which is consistent with the RNA-seq analysis. Specifically, TNF-α treatment resulted in significantly increased ACE expression compared with the respective expression under 2D and 3D control conditions. Moreover, ACE expression was markedly upregulated in the 3D-TNF group compared with that in 2D-TNF group.
Figure 7. The expression of key proteins in HUVECs treated with or without TNF-α in 2D-TCP or in 3D environment. (A) Total and phosphorylated Akt, ERK and eNOS for HUVECs cultured on 2D TCP surfaces (2D) or in 3D Gel environment (3D). (B) Expression of ACE and ACE2 in HUVECs treated with TNF-α (12.5 ng ml⁻¹) for 24 h were compared to those from standard 2D TCP cultures. (C) The expression of CD40 and TNRF1 were detected using western blotting. (D) Interaction between TNF-α and dimensionality-associated changes in Sirt1 and Sirt6 expression were validated at the protein expression levels. Data were expressed as the mean ± SEM of five independent experiments. **P < 0.01.

ACE2 is the first known homologue of human ACE, functions as a negative regulator of the renin angiotensin system in cardiovascular system, and has been shown to participate in the regulation of vascular functions and vascular dysfunction. Therefore, we also detected the expression of ACE2 under different conditions. ACE2 was downregulated by TNF-α in both 2D and 3D cultures but was not affected by the dimensionality of the different culture environments. These results suggest that the 3D microenvironment increased the basal levels and TNF-α-induced expression of ACE, and the differences in 2D- and 3D-
cultured HUVECs might be related to ACE but not ACE2.

3.10. Dimensionality and TNF-α interdependently regulate the expression of CD40

Interestingly, bioinformatics analysis suggested that CD40, which is a member of the TNFR family and has pleiotropic effects through the regulation of inflammation, is one of the important node genes affected by culture dimensionality (figure 5). We hypothesized that this protein may be a representative candidate for further investigation. As shown in figure 7(C), CD40 expression was significantly higher in 3D cultures than in 2D cultures, independent of treatment with TNF-α (12.5 ng ml⁻¹). When administered TNF-α both in 2D TCP and 3D culture, CD40 expression was upregulated compared with that of the corresponding control groups. Moreover, CD40 was markedly upregulated in the 3D-TNF group compared with that in the 2D-TNF group.

Because TNF-α exerts its biological effects by binding to its cognate membrane receptors, we also detected the expression of TNFR1, which is a member of the TNF receptor superfamily and plays critical role in transmitting the death signal from the cell surface to the intracellular signalling pathways. The expression of TNFR1 was upregulated by treatment with TNF-α in both 2D and 3D culture and was not affected by culture microenvironment. These results indicate that 3D culture conditions upregulate CD40, and the differences in HUVECs in 2D and 3D culture might be related to CD40.

3.11. Expression of Sirt1 and Sirt6 in HUVECs cultured in 2D TCPs and 3D cultures

GO analysis showed that the MF term ‘histone deacetylase binding’ was enriched (figure 5(D)), and the histone deacetylases Sirt1–7 are emerging as important key regulatory players in cardiovascular disease. Therefore, we next analysed the expression of Sirt1 and Sirt6 in 2D and 3D cultures with or without TNF-α. As shown in figure 7(D), treatment with TNF-α downregulated Sirt1 and Sirt6 expression in both 2D and 3D cultures. Notably, treatment of 3D cultures with TNF-α further inhibited the expression of Sirt1 and Sirt6. Taken together, these results confirmed that 3D culture does not affect the basal expression of Sirt1 or Sirt6 but further inhibits the expression of Sirt1 and Sirt6 in HUVECs treated with TNF-α.

4. Discussion

In the last few years, it has become increasingly apparent that the morphology and function of cells strongly depend on the cell culture environment. Although 2D culture systems are useful models for quickly discovering crucial molecular pathways, they have been proven to have some limits because of the lack of ECM and spatial arrangement [19]. Recently, a growing body of evidence has suggested that 3D cell culture systems more accurately represent the physiological microenvironment in humans than classical 2D culture systems [20–22]. Whether HUVECs in 2D-TCPs and 3D culture models differ is the subject of our study, as is defining the relative contribution of the 2D/3D culture environment to TNF-α-induced endothelial dysfunction.

In the present study, we investigated the influence of the 3D culture microenvironment on endothelial dysfunction by analysing HUVECs treated with TNF-α in 2D TCPs and 3D culture conditions. As cell morphology is a key indicator of cellular health, we observed the morphology and investigated the SI of HUVECs in 3D culture. Compared with the control group, HUVECs appeared elongated as the concentration of TNF-α increased and had a significantly reduced SI. In addition, we concluded that TNF-α at medium and high concentrations triggers cytotoxicity in ECs similarly in both 2D and 3D cultures, and ECs in 3D cultures are more sensitive to TNF-α at low concentration (6.25 ng ml⁻¹) than those in 2D cultures. Oxidative stress and inflammation are both involved in the development of endothelial dysfunction [3], and so we examined whether 3D culture elicits a different response in the level of oxidative stress and secretion of inflammatory cytokines compared with that of 2D TCPs. ECs grown in the 3D model displayed a significantly higher level of oxidative stress upon TNF-α treatment than HUVECs grown on 2D TCPs. Our data demonstrated that 3D cultures produced statistically increased amounts of IL-1 and IL-6 in response to TNF-α. This is consistent with a study showing that TNF-α induced higher secretion of the cytokines GM-CSF and IL-6 in 3D HUVEC spheroids embedded in poly-2-hydroxyethyl methacrylate than in cells cultured in 2D TCPs [23]. NO, PGL₂ and ET-1 play important roles in regulating the diameter of blood vessels and maintaining an antiproliferative and anti-inflammatory environment in the vessel wall [1]. Endothelial dysfunction is thought to arise due to changes in the secretion of endothelial-derived autocrine and paracrine mediators [3]. Our results demonstrated that HUVECs in 3D culture were more sensitive to TNF-α and more seriously injured than HUVECs in 2D TCPs. It has become apparent that matrix composition and stiffness have significant impacts on endothelial functions, and the elastic modulus of the subendothelial matrix increases from less than 5 kPa in healthy blood vessels to 6–891 kPa when cardiovascular diseases occur [24]. In our study, the Young's modulus of gelatin hydrogel was 400 kPa, which is similar to the stiffness of ECM in cardiovascular diseases. Consistent with our results, a recent report demonstrated that matrix stiffening promotes an atherosclerosis-prone EC phenotype by inducing increased endothelial.
permeability and leukocyte transmigration, both of which are hallmarks of atherosclerosis progression [7]. Thus, our combined results demonstrate that the 3D culture model, which is more physiologically relevant, enables a more detailed analysis when investigating the endothelial dysfunction induced by TNF-α in HUVECs compared with that of cells under 2D conditions.

The spatial and physical aspects of 3D cultures affect signal transduction from the outside to the inside of cells, and ultimately influence gene expression, cell-cell signalling and epigenetic gene regulation. Previous studies have revealed the advantages of 3D cell models over 2D cultures due to differences in gene expression and cellular behaviours [25]. Zhang et al. analysed the RNA sequencing profile to understand the influence of 2D and 3D cell culture platforms on global gene expression programmes in ECs that regulate vascular function [8]. Zanotelli also investigated the genome-wide gene expression of ECs embedded in 3D hydrogel and in 2D culture conditions, and found that 3D-ECs had increased expression of genes related to vasculature development, ECM and glycolysis, while 2D-ECs had increased expression of genes related to cell proliferation [26]. In our study, we analysed the differentially expressed genes by identifying GO terms using the Enrichr server. Genes that were upregulated in 3D ECs relative to those of ECs cultured in 2D TCPs were enriched within the GO BP categories vasculature development, cardiovascular system development, and regulation of cell proliferation. In addition, the GO BP terms ‘the integrin-mediated signalling pathway’ and ‘cell surface receptor signalling pathway’, and the GO MF terms ‘integrin binding’, ‘collagen binding involved in cell-matrix adhesion’ and ‘ECM binding’ were enriched, which was proven in a recent study showing that 3D matrix-embedded ECs exhibit altered integrin expression and express significantly reduced phosphorylated focal adhesion kinase compared with those of ECs cultured on 2D TCPs [27]. Zhang et al. also reported that ECs cultured on TCPs adopt a proliferative phenotype compared with ECs cultured on top of or encapsulated in PEG hydrogels and gelled Matrigel, which was related to the BP term ‘regulation of cell proliferation’ that was enriched in our results [8]. These data provide preliminary evidence for validating GO terms using a bioinformatics approach.

Interestingly, the GO MF term ‘phosphatidylinositol 3-kinase binding’ was highly ranked in the top functions, and KEGG pathway analysis also revealed that the ‘PI3K-Akt signalling pathway’ participates in the regulation of differences in HUVECs in 3D and 2D culture. PI3K-Akt signalling has been proven to have important effects on blood vessel morphogenesis and endothelial dysfunction. Akt is a member of the AGC kinases, and phosphorylation at Ser615 and Ser633 plays a crucial role in multiple cellular and physiological effects including proliferation, migration, cell growth and metabolism [28]. Akt can directly phosphorylate eNOS, thus potentiating the enzyme’s catalytic activity in response to a variety of stimuli [29]. eNOS plays a predominant role in the production of NO, which is an important regulator of endothelial function [3]. Therefore, we detected the activation of Akt and eNOS and found that expression of phosphorylated Akt and eNOS expression in HUVECs cultured on 2D TCPs was decreased compared with that in cells in 3D culture. In addition, activation of Akt can modulate ERK1/2, which plays a detrimental role in the process of oxidative stress, inflammation, remodelling and apoptosis in cardiovascular diseases [30]. Therefore, we detected the phosphorylation level of ERK and found that HUVECs cultured on 2D TCPs were characterized by increased phosphorylation of ERK1/2 compared with that of cells cultured in 3D hydrogels, which was consistent with a previous study that showed that HUVECs cultured on 2D TCPs were characterized by increased phosphorylation of ERK1/2 compared with that of cells cultured in 3D PEG hydrogels [8].

Furthermore, to identify the functional connections between the proteins that were identified as differentially expressed genes, PPI analysis was conducted. The key node proteins that were upregulated in ECs in 3D culture relative to those in 2D culture included CD40, ACE, CD34, BCL2, CXCR4, and ICAM-1. Among these proteins, CD40, ACE, CXCR4 and ICAM-1 are transmembrane proteins that participate in various processes, such as inflammation, angiogenesis and proliferation. Previously, Abraham found that the expression of CD34 in 2D-ECs was reduced compared with that of ECs embedded in the 3D matrices [31]. Sivarapatna found that ECs in a 3D biomimetic flow bioreactor had significantly upregulated expression of CXCR4 [32]. Gapizov found that the expression of ICAM-1 in 3D EC spheroids cultured in poly-2-hydroxyethyl methacrylate was upregulated compared with that of cells cultured in 2D TCPs [23]. Previous studies on the molecular mechanisms underlying 3D/2D culture-mediated influences have already confirmed several important proteins that were identified in our bioinformatics analysis.

Interestingly, ACE and CD40 emerged among the key proteins in the PPI network. ACE is a central enzyme localized on the surface of vascular ECs and produces angiotensin II, which induces vasoconstriction and raises blood pressure, and its biochemistry and implications in hypertensive and cardiovascular diseases have been studied and reviewed extensively over the last several decades [33]. In our study, differences in ACE were observed between endothelial 2D and 3D cultures exposed to TNF-α. Our results indicate that the expression of ACE was significantly higher in ECs grown in 3D culture than in ECs grown on 2D TCPs with or without TNF-α treatment. ACE2, which
is highly expressed in the vascular endothelium, was the first known homologue of human ACE and functions as a negative regulator of the renin angiotensin system in the cardiovascular system. ACE2 is a key enzyme that plays a crucial physiological role as a vasodilator, and the balance between ACE and ACE2 is the key factor in regulating angiotensin levels [34]. In our studies, ACE2 was downregulated by TNF-α in both 2D and 3D cultures but was not affected by the different culture environments. Our results suggest that both 3D culture and treatment with TNF-α can upregulate the expression of ACE, and the differences in ECs in 2D and 3D culture might be related to ACE but not ACE2.

CD40, which is a member of the TNFR family, has pleiotropic effects through regulating inflammation [35]. Our previous study and others have shown that TNF-α upregulates the expression of CD40 and leads to endothelial dysfunction [36]. In this study, we found that CD40 was expressed at significantly higher levels in 3D-cultures than in 2D-cultures independent of treatment with TNF-α, and CD40 was markedly upregulated in 3D cultures compared with that of cells cultured in 2D TCPs when treated with TNF-α. As TNF-α exerts its biological effects by binding to its cognate membrane receptor TNFR1, we also detected the expression of TNFR1. TNFR1 is ubiquitously expressed membrane receptor that binds TNF-α on the surface of target cells and mediates the association of distinct adaptor proteins [37]. TNF-α interacts with its receptor TNFR1, thereby regulating a number of inflammatory changes. In our study, we found that the expression of TNFR1 was not affected by the dimensionality of different culture environments with or without TNF-α treatment. These results indicate that the 3D microenvironmental condition increased basal levels and TNF-α-induced upregulation of ACE and CD40 compared with that of the 2D culture.

Histone modifications have been proven to be involved in the process of endothelial dysfunction [38]. In our GO analysis of the differentially expressed genes in 2D/3D HUVECs showed enrichment of the MF term ‘histone deacetylase binding’. Sirtuins (Sirt1–7) are a family of histone deacetylases that catalyse deacetylation of both histone and non-histone lysine residues [39]. Among all seven sirtuin isoforms, Sirt1 and Sirt6 are now emerging as important regulatory key players in cardiovascular disease [39]. Studies have demonstrated that Sirt1 exerts atheroprotective effects by activating eNOS or by diminishing NF-kB activity in ECs [40]. Moreover, pharmacological Sirt1 activation protected ECs from endothelial dysfunction induced by TNF-α and LPS and disturbed flow. Our previous studies have also shown that activation of Sirt1 inhibits CD40 expression and inflammation in HUVECs. In addition, accumulating evidence suggests that Sirt6 shares some of the Sirt1-mediated protective effects, such as repressing inflammation and slowing the ageing process, two of which are causative factors of endothelial dysfunction [41]. In addition, our previous study indicated that Sirt1 positively regulated the expression of Sirt6, and both Sirt1 and Sirt6 inhibit TNF-α-induced inflammation [42]. In the present study, the 3D culture microenvironment further attenuated the expression of Sirt1 and Sirt6 in the presence of TNF-α. Taken together, these results confirmed that 3D culture further inhibited the expression of Sirt1 and Sirt6 in TNF-α treated HUVECs. Our combined results seem to be consistent with the expression of CD40 found by the same groups, as Sirt1 exhibits anti-inflammatory effects by decreasing the expression of CD40 [36].

Using 3D culture models and 2D TCPs to evaluate endothelial dysfunction by controlling TNF-α treatment and the culture microenvironment, we have demonstrated that the TNF-α-induced injury to HUVECs is more severe in the 3D models compared with that under 2D-TCP conditions. Then we analysed the global gene expression patterns in 2D/3D HUVECs and noted the influence of the culture environment on global expression of genes that regulate endothelial function. Specifically, we found that some key proteins, notably ACE, CD40, Sirt1 and Sirt6 represent a critical link between endothelial dysfunction and dimensionality. These observations provide insight into the interdependence between endothelial dysfunction and the complex microenvironment, which enhances our understanding of endothelial biology and provides a therapeutic strategy for cardiovascular-related diseases.

Acknowledgments

This work was supported by funding from the National Natural Science Foundation of China (Grant Nos. 81770459, 81970369 and 81873520).

Conflicts of interest disclosure

The authors declare no conflicts of interest.

Statement of contribution

Rong Lin and Nanbo Zheng designed the research methods and revised the manuscript. Bo Wang and Ruomeng Chen performed the experiments and analyzed the data. Bo Wang, Weirong Wang and Yaxiong Liu drafted the manuscript. Hongqian Gao and Xiaohan Lv participated in bioinformatic analysis and data collection. Bo Wang and Lifang Chen drew the pictures. All authors have read and approved the final manuscript.
Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID iD

Rong Lin  https://orcid.org/0000-0002-7513-6733

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