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Impaired angiogenic differentiation of dental pulp stem cells during exposure to the resinous monomer triethylene glycol dimethacrylate

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ABSTRACT

Objective. Dental pulp stem cells (DPSCs) can differentiate into tissue specific lineages to support dental pulp regeneration after injuries. Triethylene glycol dimethacrylate (TEGDMA) is a widely used co-monomer in restorative dentistry with adverse effects on cellular metabolism. Aim of this study was to analyze the impact of TEGDMA on the angiogenic differentiation potential of DPSCs.

Methods. DPSCs were characterized by flow cytometry. Short-term (max. 72 h) cytotoxicity of TEGDMA was assessed by MTT assay. To evaluate TEGDMA effects on angiogenic differentiation, DPSCs were cultivated in angiogenic differentiation medium (ADM) in the presence or absence of short-term non-toxic TEGDMA concentrations (0.1 mM and 0.25 mM). Subsequently, angiogenic differentiation was analyzed by qRT-PCR analysis of mRNA markers and *in vitro* spheroid sprouting assays.

Results. DPSCs treated with 0.25 mM TEGDMA revealed downregulation of angiogenesis-related marker genes *PECAM1* (max. 3.8-fold), *VEGF-A* (max. 2.4-fold) and *FLT1* (max. 2.9-fold) compared to respective untreated control. In addition, a reduction of the sprouting potential of DPSCs cultured in the presence of 0.25 mM TEGDMA was detectable. Larger spheroidal structures were detectable in the untreated control in comparison to cells treated with 0.25 mM TEGDMA. In contrast, TEGDMA at 0.1 mM was not affecting angiogenic potential in the investigated time period (up to 28 days).

Significance. The results of the present study show that TEGDMA concentration dependently impair the angiogenic differentiation potential of DPSCs and may affect wound healing and the formation of granulation tissue.

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1. Introduction

The dental pulp consists of several different cell types *e.g.* odontoblasts, neurons, cells of the immune system, blood cells as well as adult mesenchymal stroma cells, also known as dental pulp stem cells (DPSCs). DPSCs were initially characterized by Gronthos et al. [1]. They have the ability for self-renewal and can differentiate into different cell types. It was shown that DPSCs can differentiate into muscle cells, neurons and cartilage [2]. Gronthos et al. showed the odontogenic potential of these cells forming dentine-like structures [3,1]. In this way, DPSCs can support regeneration processes after injuries of the dental pulp. In comparison to the osteogenic/odontogenic differentiation potential of DPSCs, little is known about their angiogenic differentiation potential. It was shown that stem cells from human exfoliated deciduous teeth (SHED) can turn into endothelial-like cells [4]. Similar to SHED, DPSCs [5–8] and stem cells from the apical papilla (SCAP) [9] have the potential to differentiate into endothelial-like cells. Beside the capability of angiogenic differentiation, it is known that DPSCs are able to secret several proangiogenic factors leading to a paracrine mediated angiogenesis [10,11].

Historically, the term angiogenesis denoted the sprouting of new capillaries from pre-existing blood vessels. More recently, the term angiogenesis was used to describe the general process of remodeling the network of post-capillary venules from a primitive to a complex network [12]. The sprouting of new capillaries from pre-existing structures is a well-coordinated process of high complexity. Certain stimuli, such as hypoxia and inflammation cause an enhanced permeability of vessels for angiogenic growth factors and chemokines, which have positive effects on endothelial proliferation and migration. Vascular endothelial growth factor A (*VEGF-A*) is the most important pro-angiogenic factor. It belongs to the family of vascular endothelial growth factors including VEGF-B, VEGF-C, VEGF-D and placental growth factor (PGF). Several isoforms of *VEGF-A* can be generated through alternative splicing [13]. A single endothelial tip cell can be found on the tip of vascular sprouts [14]. This 'tip cell' expresses the vascular endothelial growth factor receptor 2 (VEGFR-2) allowing the tip cell to detect a *VEGF-A* gradient and to guide the growing sprout through the extracellular matrix (ECM) into the direction of the *VEGF-A* source. Hereby, long cellular processes on the tip cell, called filopodia secret ECM-degrading enzymes. The ECM degradation provides space for the developing sprouts. Stalk-like cells next to the tip cell proliferate and elongate into the direction of the tip cell, resulting in an elongation of the sprouting vessel [15].

In contrast to angiogenesis, the process of angioblasts differentiating into endothelial cells and the assembling of these endothelial cells to a capillary network is known as vasculogenesis [15].

The angiogenic differentiation potential of DPSCs is until today only roughly understood. Accordingly, no studies exist to our knowledge, which addressed the effects of dental biomaterials on the angiogenic potential of these cells. Composite materials are widely used in restorative dentistry. Triethylene glycol dimethacrylate (TEGDMA) is the most commonly used co-monomer in dental composites. It has been well documented that unpolymerized TEGDMA can elute into the oral environment [16,17]. Subsequently, DPSCs may get in contact with TEGDMA after a composite restoration has been placed, primarily through dentine tubules [18]. A plethora of studies showed cytotoxic effects of TEGDMA monomers on various cell types [19,20]. TEGDMA mediated cytotoxicity causes a depletion of the intracellular glutathione pool [20,21]. The reduced capability of cells maintaining redox homeostasis leads to an enhanced level of reactive oxygen species (ROS) [22]. Ultimately, this may result in DNA strand breakage [23,24]. It has been already shown that TEGDMA impairs the odontogenic differentiation potential of dental stem cells [25,26]. Several clinical experimental studies have been published, which addressed future pulp regeneration therapies for patients with irreversible pulp diseases [27,28]. Nakashima et al. transplanted autologous DPSCs in five patients. These stem cells were grown from pulps of extracted teeth and expanded *in vitro* [27]. It was necessary on some patients, which revealed deep caries lesions, to restore a missing wall with composite resin before transplantation of DPSCs. This resulted in a direct contact of DPSCs with leached components, *e.g.* TEGDMA, of the composite resin.

Prerequisite of successful pulp regeneration, also with the use of tissue engineering is that the newly formed tissue is supplied with sufficient nutrients and oxygen. Thus, a deep understanding of angiogenesis and vasculogenesis is of outmost importance for the regeneration of oral tissues.

Therefore the aim of this study was to analyze the angiogenic differentiation potential of DPSCs in the presence of clinically relevant, concentrations of the co-monomer TEGDMA. DPSCs from third molars were isolated using an enzymatic digestion method. These cells were subsequently characterized by flow cytometry. Short-term cytotoxicity assays were carried out in order to determine the TEGDMA concentrations, which were applied for long-term differentiation experiments. Subsequently, we carried out long-term angiogenic differentiation experiments with DPSCs in the presence of 0.1 mM and 0.25 mM TEGDMA. These concentrations may be eluted also *in vivo* over time [29,30]. The potential of DPSCs to differentiate into the angiogenic lineage was analyzed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments and spheroid sprouting assays.

2. Materials and methods

2.1. *Isolation and cultivation of dental pulp stem cells (DPSCs)*

Human DPSCs were derived from a healthy 19 year old female person after signing a written consent form prior to the study. For cell isolation, pulp tissue from two extracted third molars were cut into small pieces before enzymatic digestion with 3 mg/ml collagenase type I (Gibco, Grand Island, NY, USA) and 4 mg/ml dispase II (Sigma-Aldrich, Steinheim, Germany) for 1 h in α-minimal essential medium (α-MEM; Gibco) at 37 °C. Afterwards, cells were passed through a 70 μm cell strainer (EASYstrainer, Greiner bio-one, Frickenhausen, Germany). The isolated DPSCs were expanded in

complete culture medium (CCM) containing α-MEM supplemented with 15% fetal bovine serum (Biochrom, Berlin, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin (both from Biochrom), 2.5 μg/ml Amphotericin B (Capricorn Scientific Ebsdorfergrund, Germany) and 100 μM L-ascorbic acid phosphate (Sigma-Aldrich). Incubation took place in a humidified atmosphere at 37 °C and 5% CO_2. For all experiments, cells up to passage (p.) 4 were used. Cell cultures were tested routinely for mycoplasma contamination with a mycoplasma detection kit (Venor GeM, Minerva Biolabs, Berlin, Germany). The present study was approved by the Ethical Committee of the Hannover Medical School (No. 1096).

2.2. Characterization of DPSCs by flow cytometry

The 'stemness' of DPSCs was initially characterized by flow cytometry. The cells were grown in a 75 cm^2 flask until almost reaching confluency. Subsequently, cells were detached by trypsinization with 0.25% Trypsin — 1 mM EDTA (Merck, Darmstadt, Germany). Cells were re-suspended in staining buffer, consisted of PBS supplemented with 1% (w/v) bovine serum albumin (BSA) and 0.1% sodium azide (NaN_3). Afterwards, cell concentration was adjusted to 1×10^6 cells/100 μl. Cells were blocked with 1 μg of human IgG (Sigma-Aldrich) for 15 min on ice in order to prevent unspecific Fc receptor binding. After Fc receptor blocking, fluorochrome-conjugated antibody staining was carried out for 25 min at 4 °C in the dark. For detection of typical dental mesenchymal stromal cell markers, the following mouse anti-human antibodies were used: CD105-APC (allophycocyanin), CD73-FITC (fluorescein isothiocyanate), CD90-FITC, CD146-PE (phycoerythrin), STRO1-FITC, SSEA-4-FITC, CD45-PE, CD34-APC (all from BioLegend, Fell, Germany). Afterwards, cells were washed twice with staining buffer and centrifuged for 5 min at 120 g and 4 °C. Flow cytometry was carried out using a BD LSR II Flow Cytometer (BD Biosciences, Heidelberg, Germany). For each sample, 1×10^5 events were recorded. Data were analyzed using the BD FACSDiva software (BD Biosciences, Heidelberg, Germany). Experiments were performed as three independent biological replicates.

2.3. MTT cell viability assay

Cytotoxicity was evaluated using the methyl thiazolyl tetrazolium (MTT) method. The MTT assay is based on the measurement of NAD(P)H-dependent oxidoreductases, which reflects the cell viability. 1×10^4 cells per well were seeded in a 96-well plate and allowed to adhere for 24 h. Subsequently, cells were treated with TEGDMA (0.05–5 mM) for 24 h, 48 h and 72 h. TEGDMA (VOCO, Cuxhaven, Germany) stock solutions were prepared in absolute ethanol (Baker Griesheim, Germany). The stock solutions were always freshly diluted in culture medium for each experiment. The final concentration of ethanol was 0.25% (v/v) for each treatment. Cells treated with 0.25% ethanol were used as solvent controls. After treatments, medium was discarded and 100 μl of a MTT solution consisting of 0.5 mg/ml MTT dissolved in medium was added to each well. Subsequently, cells were incubated for 2 h at 37 °C and 5% CO_2. Afterwards, the precipitated insoluble formazan was dissolved in 100 μl DMSO under permanent agitation for 30 min. The light absorbance was measured at 550 nm in a microplate reader (Spectra Max 250, MWG Biotech, Ebersberg, Germany). The experiment was performed at least three times with six technical replicates each.

2.4. Induction of angiogenic differentiation and treatment with TEGDMA

For angiogenic differentiation experiments, DPSCs were seeded in collagen coated 6-well plates. Thin coating was carried out according to the manufacturer's instructions (Corning, New York, USA). Briefly, collagen I derived from rat tail was diluted to 50 μg/ml using 0.02 N acetic acid. 1 ml per well was transferred and incubated for 1 h at room temperature. Afterwards, remaining solution was carefully aspirated and wells were rinsed using PBS to remove the acid. Plates were stored for up to one week at 4 °C before they were used.

At the beginning of the differentiation experiments, 3×10^5 DPSCs per well were seeded in CCM. After 24 h of adhesion, medium was discarded and replaced by CCM containing 0.1 mM or 0.25 mM TEGDMA. Control cells were treated with 0.25% ethanol. Another 48 h later, angiogenic differentiation was initiated by exposure to angiogenic differentiation medium (ADM) containing M199 medium (Gibco) supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 2.5 μg/ml amphotericin B (Gibco), 50 μg/ml heparin (Sigma-Aldrich), 1 μg/ml hydrocortisone (Sigma-Aldrich), 60 μg/ml endothelial cell growth supplement (ECGS, Sigma-Aldrich), 10 ng/ml epidermal growth factor (EGF, Biochrom), 25 ng/ml recombinant human basic fibroblast growth factor (rh-bFGF, Biochrom) and 50 ng/ml vascular endothelial growth factor (VEGF-165, BioLegend). Medium was exchanged every 2–3 days. For mRNA expression analysis, cells were cultivated for a maximum of 28 days after the initiation of differentiation. For spheroid sprouting assays, cells were precultured for 14 days in CCM or ADM. Cells grown in CCM served as controls in order to assess the effects of ADM. Angiogenic differentiation was evaluated by qRT-PCR measurements of endothelial cell marker gene expression as well as by spheroid sprouting assays.

2.5. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Gene expression was analyzed using qRT-PCR. Firstly, total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). During RNA isolation, an additional on-column DNase digestion was carried out. Subsequently, 1 μg of isolated RNA was used for reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative RT-PCR was performed on a Rotor-Gene Q cycler (Qiagen, Hilden, Germany). The QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) was used for target amplification and quantification of the following endothelial cell marker gene expression: FMS-related tyrosine kinase 1 (*FLT1*), kinase insert domain receptor (*KDR*), vascular endothelial growth factor A (*VEGF-A*) and platelet endothelial cell adhesion molecule 1 (*PECAM1*). The primers are shown in Table 1. Amplification conditions were as fol-

Table 1 – QuantiTect Primer Assays (Qiagen) used for determining angiogenic differentiation by qRT-PCR analysis. Qiagen Primer Assay numbers as well as detected transcripts are given.

Gene	Abbreviation	QuantiTect Primer Assay	Detected transcript(s)
18S ribosomal RNA	18sRNA	QT00199367	X03205
Actin, beta	*ACTB*	QT00095431	NM_001101
Beta-2-microglobulin	*B2M*	QT00088935	NM_004048 XM_005254549 XM_006725182
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	QT01192646	NM_002046 NM_001289745 NM_004168
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	*SDHA2*	QT01668919	NM_001294332 XM_005248329 XM_005248331
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	*YWHAZ*	QT02321522	NM_001135699 NM_001135700 NM_001135701 NM_001135702 NM_003406 NM_145690 XM_005251060 XM_005251061 XM_005251062 XM_005251063 NM_000442
Platelet and endothelial cell adhesion molecule 1	*PECAM1*	QT00081172	XM_005276880 XM_005276881 XM_005276882 XM_005276883 XM_006721944 XM_006721945
Fms-related tyrosine kinase 1 or vascular endothelial growth factor receptor 1	*FLT1*	QT00073640	NM_002019
Kinase insert domain receptor or vascular endothelial growth factor receptor 2 (VEGFR-2)	*KDR*	QT00069818	NM_002253
Vascular endothelial growth factor A	*VEGF-A*	QT01010184	NM_001025366 NM_001025367 NM_001025368 NM_001033756 NM_001171623 NM_001171624 NM_001171625 NM_001171626 NM_001171629 NM_003376 NM_001287044

lows: initial incubation step of 5 min at 95 °C to activate HotStarTaq DNA polymerase and 40 cycles of denaturation (at 95 °C for 5 s) and combined annealing/extension (at 60 °C for 10 s). Afterwards, a melting curve was recorded. Exported raw data were analyzed. Baseline correction was carried out and mean amplification efficiencies per amplicon as well as CT values for each sample were calculated using LinRegPCR [31]. Expression of six candidate reference genes was measured, including beta actin (*ACTB*), beta-2-microglobulin (*B2M*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA (*RRN18S*), succinate dehydrogenase flavoprotein subunit (*SDHA2*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*). The two most stable genes were identified by geNorm [32]. These were used for normalization. Subsequently, fold changes in gene expression were calculated according to the efficiency adjusted delta delta-CT method [33].

2.6. *Spheroid sprouting assays*

Matrigel™ (Corning, New York, USA) was thawed on ice. 289 µl of a Matrigel stock solution (~10 mg/ml) was added to each well of a 24-well plate. Afterwards, plates were incubated at 37 °C for 30–60 min. After incubation, remaining liquid was carefully removed.

DPSCs were cultured on 6-well plates in either ADM or CCM for 14 days, with or without exposure to different TEGDMA concentrations. The medium was exchanged every 2–3 days (see above). After 14 days, cells were detached by trypsinization and transferred to a vial with fresh medium. After centrifugation for 5 min, cells were re-suspended in

ADM (composition see: Induction of angiogenic differentiation) supplemented with 0.1 mM or 0.25 mM TEGDMA. As solvent control, CCM and ADM were supplemented with 0.25% ethanol. As a positive control for sprouting human umbilical vein endothelial cells (HUVECs) were used. Cells were re-seeded into the Matrigel™ coated plates at 1.2×10^5 cells per well. Plates were incubated in a humidified atmosphere at 37 °C and 5% CO_2. After 21 h, images were taken using an inverted microscope (Olympus IMT-2, Hamburg, Germany), equipped with a digital camera (Olympus SC100). Connected spheroids were counted under the microscope to quantify morphological alterations induced by TEGDMA. Spheroids connected to each other were classified according to the number of connections for an easier evaluation. The classified groups were as follows 2–4, 5–7, 8–10 and 11+ spheroids connected to each other.

2.7. Statistical analysis

All MTT and spheroid sprouting assays were run in three independent biological experiments with six technical replicates each. Results are represented as mean values ± standard deviation (SD). One-way ANOVA, followed by Dunnett test was performed and statistical significance was set for $p < 0.05$. qRT-PCR experiments were run in duplicate and mean values of three independent biological replicates are shown. For statistical analysis, fold changes were log 2 transformed and one way ANOVA with Bonferronís multiple comparison test was used with $p < 0.05$ set as the level of statistical significance. All statistical calculations were carried out using GraphPad Prism 6.0 (GraphPad Software, Inc.; La Jolla, CA 92037 USA).

3. Results

3.1. DPSC characterization by flow cytometry

In order to characterize DPSCs, single stain flow cytometry experiments were carried out targeting typical mesenchymal (CD73, CD90, CD105, CD146, STRO-1), hematopoietic (CD34, CD45) and embryonic (SSEA-4) stem cell markers (Fig. 1). More than 98% of the cells were positive for CD73 as well as for CD90. 83.2% ± 0.2% of the DPSCs showed expression of CD146 and 96% ± 0.2% were positive for CD105. Only a small percentage of cells (2.4% ± 0.7%) showed an expression of STRO-1. In addition, hematopoietic marker expression was below 1.3% (CD34 and CD45) in the tested DPSCs. The stage specific embryonic antigen 4 (SSEA-4) was expressed on 45.8% ± 6.2% of the cells.

3.2. TEGDMA induced cytotoxicity

The results of the MTT assays are shown in Fig. 2. A time- and concentration-dependent cytotoxicity was found in TEGDMA-treated cultures. An early cytotoxic effect was observed at a concentration of 0.5 mM TEGDMA (85%). TEGDMA concentrations of 1 mM and 2 mM showed a clear cytotoxic effect, when the cells were incubated for 48 h (80% ± 5.8% and 63% ± 11.3%) or 72 h (64% ± 11.7% and 47% ± 15.0%). DPSCs incubated with these concentrations for 24 h did not show a statistically significant decrease in viability in comparison to the control. TEGDMA concentrations ≥3 mM showed cytotoxic effects after just 24 h exposure. However, when the highest concentration (5 mM) was applied for 72 h, almost no DPSCs survived (1.9% ± 2.2% viability). The EC_{50} values (concentration that reduces the total cell number to 50% of the untreated control cells) varied between 4 mM (24 h), 3 mM (48 h) and 2 mM (72 h). Since 0.1 mM and 0.25 mM TEGDMA treatments in short term MTT experiments for up to 72 h showed no significant reduction in cell viability (98.2% ± 1.3% and 97.7% ± 2.5%), these concentrations were selected for long-term angiogenic differentiation experiments.

3.3. Effect of TEGDMA on mRNA expression of angiogenic marker genes

The results of the qRT-PCR analysis after exposure of the DPSC to angiogenic conditions for up to 28 days are presented in Fig. 3.The three angiogenic marker genes *PECAM1*, *VEGF-A* and *KDR* showed at all time points a higher expression level in cells grown in ADM in comparison to control cells grown in CCM. The expression values increased to a maximum of 16-fold (± 23) for *PECAM1* (28 days), 4-fold (± 1) for *VEGF-A* (28 days) and 19-fold (± 9) for *KDR* (28 days). In contrast, DPSCs grown in CCM exhibited a higher expression of *FLT1* (max. 5-fold (± 1) at 28 days) compared to DSPCs grown in ADM (max. 2-fold (± 0.2) at 28 days). This was found throughout the experiments. DPSCs treated with 0.1 mM TEGDMA showed similar expression profiles for *PECAM1*, *VEGF-A*, *KDR* and *FLT1* compared to non-treated control cells. This was documented for both DPSCs cultivated in ADM, as well as in CCM. Nevertheless, the higher TEGDMA concentration (0.25 mM) showed a significant suppressing effect on the expression of *PECAM1* and *VEGF-A* in DPSCs, which were grown in ADM compared to the non-treated ADM control. First significant effects of 0.25 mM TEGDMA were measured for *PECAM1* already after 7 days and for *VEGF-A* as well as for *FLT1* after 14 days. The expression level of *PECAM1* in cells treated with 0.25 mM TEGDMA and grown in ADM was always similar that of cells grown in CCM. In contrast to *PECAM1*, *KDR* and *VEGF-A*, only *FLT1* showed a higher expression over time in cells grown in CCM compared to cells grown in ADM. Furthermore, DPSCs grown in CCM in the presence of 0.25 mM TEGDMA showed a significant decreased expression of *FLT1* after 21 days and 28 days in comparison to the non-treated CCM control.

3.4. Effect of TEGDMA on spheroid sprouting

The results of the *in vitro* sprouting assays after exposure of DPSCs to angiogenic conditions are presented in Fig. 4(A, B) . All DPSCs tended to form spheroid-like structures, when seeded on Matrigel™. DPSCs pre-cultured in ADM showed larger spheroid structures than those grown in CCM. In addition, the ADM cultivated cells were able to develop capillary-like sprouts. These capillary-like sprouts connected some of the spheroids with each other. The DPSCs pre-cultured in CCM showed a clearly different morphology. Only very scarce capillary-like sprouts were found on the spheroids. These capillary sprouts were much shorter in comparison to those produced by DPSCs pre-cultured in ADM. Furthermore, no connections between spheroids could be documented in cells pre-cultured in CCM. Cells grown in ADM and treated

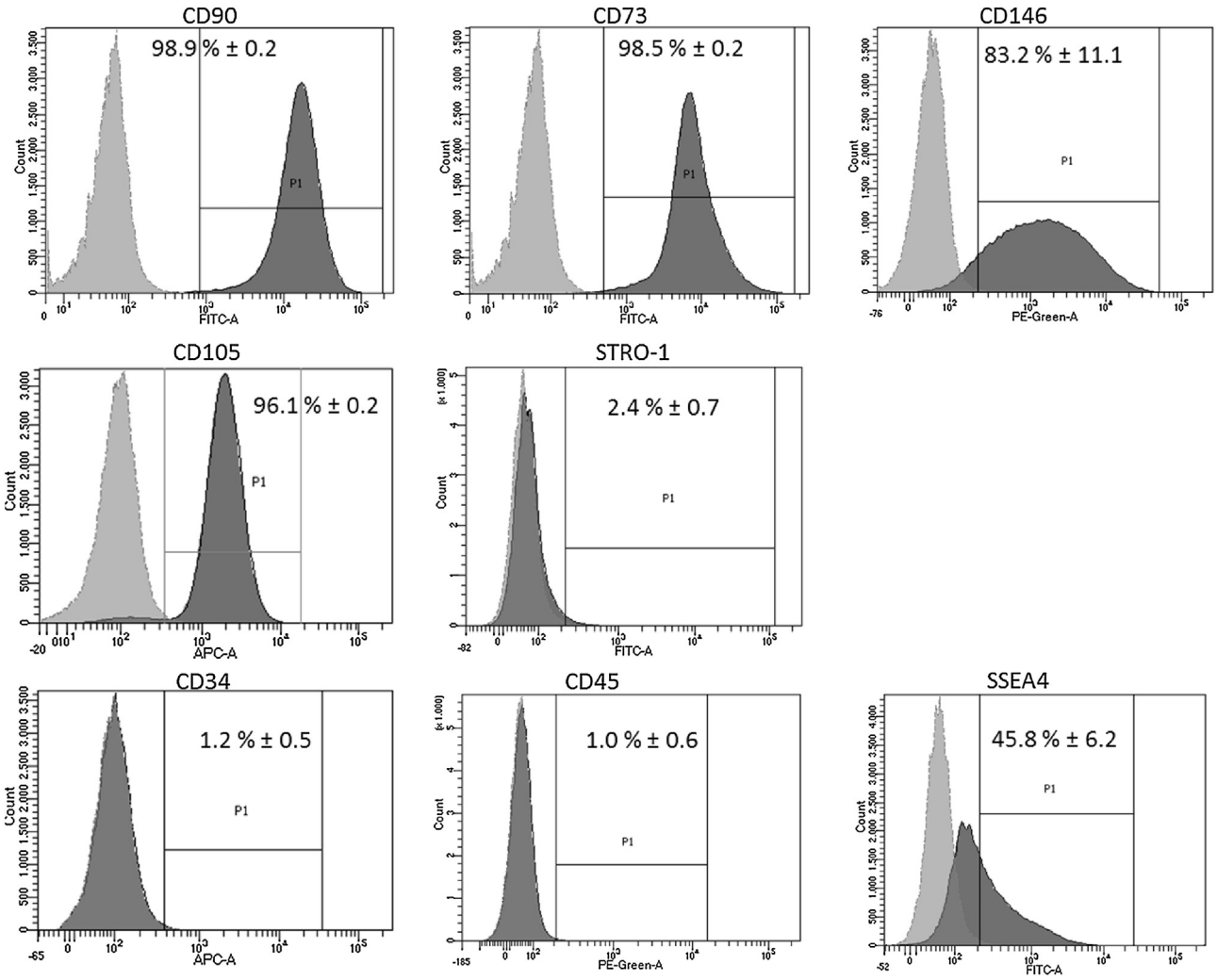

Fig. 1 – Immune-phenotypic characterization of DPSCs using flow cytometry. Histograms show expression of mesenchymal (CD73, CD90, CD105, CD146, STRO-1), hematopoietic (CD34, CD45) and embryonic (SSEA-4) stem cell markers. Grey: unstained control; Black: stained cells. Regions are set for positive cells and percentages of positive cells are shown (mean value from three independent experiments ± SD).

with 0.25 mM TEGDMA showed a significantly higher number of 2–4 spheroids connected to each other in comparison to the ethanol control (Fig. 4B). More than 80% of spheroids belonged to this group when treated with 0.25 mM TEGDMA, but only approximately 50% of the non-treated control cells. However, larger cell structures consisting of more spheroids connected to each other (8–10 and 11+) were significantly more frequent in the non-treated control in comparison to the cells treated with 0.25 mM TEGDMA. Only about 2% cells treated with 0.25 mM TEGDMA showed 8 to 10 connected spheroids. Almost no structures of more than 11 spheroids connected to each other were found. In contrast, more than 20% of spheroids of the non-treated control belonged to the groups of 8 to 10 and 11+ connected spheroids.

4. Discussion

Objective of our study was to analyze the angiogenic potential of DPSCs and to evaluate the impact of TEGDMA on the cellular angiogenic response. TEGDMA is a very important comonomer in most dental composite resins. Our results show that DPSCs differentiate into the angiogenic lineage. TEGDMA at low, non-cytotoxic concentrations affects the angiogenic response of DPSCs *in vitro* regarding mRNA-expression and cell morphology. Like all mesenchymal stromal cells (MSCs), DPSCs can be considered as a 'heterogeneous' cell population. Until now, no single surface marker is known to identify DPSCs or other MSCs unequivocally. In this respect, different functional or phenotypic assays are used to characterize cell populations containing adult stem cells. The International Society for Cell Therapy published in 2006 'minimal criteria' for defining MSCs [34]. One of these criteria is plastic adhesion of MSCs. Additionally, some marker molecules are requested to be expressed on MSCs and some to be absent on MSCs. According to these criteria ($CD73^{pos}$, $CD90^{pos}$, $CD105^{pos}$, $CD34^{neg}$, $CD45^{neg}$) we carried out flow cytometry experiments in order to characterize our cell population (Fig. 1). However, some of these markers are known to be expressed on further cell types besides being expressed on MSCs. For exam-

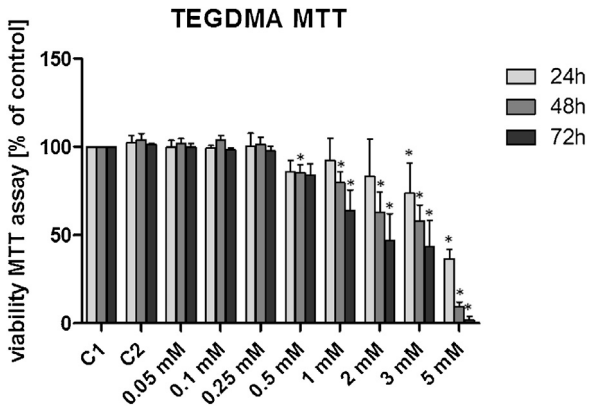

Fig. 2 – MTT viability assay of TEGDMA treated DPSCs. Viability of cells after short-term exposure (24 h, 48 h or 72 h) to different TEGDMA (0.05 mM–5 mM) concentrations was measured by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. C1: cells grown in CCM without TEGDMA. C2: cells grown in CCM with 0.25% ethanol. C1 is set to 100%. Viability is shown in% in comparison to C1. Error bars represent SD of three independent experiments. Asterisks show statistically significant differences to the ethanol control (one-way ANOVA, followed by Dunnetťs test, $p < 0.05$).

ple, fibroblasts, positive for CD73, CD90, CD105 and CD44 were identified [35]. Therefore, we included the additional markers CD146, Stro-1 and SSEA-4 in our flow cytometry analysis. We found an expression of more than 96% for CD73, CD90 and CD105 in the cell population used in this study. Although CD105 (endoglin) expression is often used for identification of MSCs, it was shown that endothelial cells highly express CD105 as well [36]. Furthermore, CD105 expression rises in MSCs derived from adipose tissue with increased culture passages [37]. A high percentage of DPSCs were positive for CD146 (83.2%). It was shown that CD146 is a key marker for bone marrow MSCs [38]. But it can be also found on endothelial cells, ganglion cells and activated T lymphocytes [39,40]. STRO-1 was reported to be a marker of freshly isolated MSCs [41]. Several other studies showed that STRO-1 is progressively lost in cultured cells over time and that it is not essential for the differentiation potential of MSCs [42,43]. Accordingly, only 2.4% of cells in our DPSC biopsy were positive for STRO-1. SSEA-4 is an embryonic stem cell-associated marker. In addition, it was shown that this marker can be also expressed on DPSCs. It has been reported that SSEA-4 positive DPSCs showed multilineage differentiation potential toward osteoblasts, chondrocytes and neurons [44]. Therefore, we also analyzed the expression of SSEA-4 in our DPSCs. Approximately 46% of the cells showed a positive signal. CD34 as well as CD45 expression was analyzed in order to differentiate between DPSCs and hematopoietic stem cells. Positive expression of CD34 and CD45 was found on 1.2% and 1% of the cells, respectively. This result confirms that our analyzed DPSC cultures contained only a negligible share of hematopoietic stem cells. Taken together, our flow cytometry analysis is proving the presence of mesenchymal stem cells.

The evaluation of TEGDMA cytotoxicity using MTT viability assays revealed a time and concentration-dependent reduction of cell viability (Fig. 2). Our results are in accordance with data of MTT viability assays using deciduous teeth stem cells treated with TEGDMA [25]. The initial reduction of cell viability started at a concentration of 0.5 mM TEGDMA. TEGDMA concentrations higher than 1 mM caused a significant reduction of viability of cultures incubated for 48 h or 72 h. We found cytotoxic effects only at higher concentrations of TEGDMA in comparison to previous studies with deciduous teeth stem cells [25], which might be attributed to the difference in biological properties of different types of dental MSCs. TEGDMA can leach from composites in high amounts within the first days after initial polymerization. Furche et al. found that the highest amount of TEGDMA leached from dental fissure sealants was detectable after seven days [29]. But lower concentrations can leach for a significant longer period of time. Therefore, our experimental design simulated a long-term influence of TEGDMA on DPSCs and on their ability for angiogenic differentiation. Thus, we selected TEGDMA concentrations that did not cause cytotoxic effects analyzed by MTT assay within 72 h after application. Accordingly, we used TEGDMA concentrations of 0.1 mM and 0.25 mM in our *in vitro* model to treat DPSCs cultured in CCM or ADM for up to 28 days and performed qRT-PCR analyses to evaluate expression of typical angiogenic marker genes, such as *PECAM1*, *VEGF-A*, *KDR* and *FLT1* (Fig. 3). In addition, microscopic evaluation during 28 days also showed no indication for cytotoxic effects.

PECAM1 expression on endothelial cells is considered to play a decisive role in the regulation of the vascular barrier. It is needed for leukocyte transendothelial migration (TEM) under most inflammatory conditions [45]. Furthermore, *PECAM1* is also involved in angiogenesis by influencing cell migration, junctional development, capillary morphogenesis and development of cell-matrix-interactions [46–49]. We found that *PECAM1* expression increased significantly when cells were grown in ADM in comparison to CCM. This indicates a successful differentiation process of DPSCs cultured in ADM into the angiogenic lineage. In comparison to day 0, cells grown in CCM for a longer time (min. 21 days) showed a slightly constant increased *PECAM1* expression as well. One can speculate that this slight increase might represent a small number of cells, which spontaneously differentiated toward the angiogenic lineage. A clearly impaired expression of *PECAM1* was documented, when cells were grown in ADM and treated with 0.25 mM TEGDMA in comparison to cells treated only with 0.1 mM TEGDMA or not treated cells. This observation showed that even short-term non-toxic concentrations of TEGDMA monomers are able to influence the expression of *PECAM1* and very likely the entire angiogenic differentiation process significantly, at least when applied for longer periods of time.

Furthermore, we analyzed the expression of genes encoding for *VEGF-A*, VEGFR-1 and VEGFR-2. VEGFR-1 as well as VEGFR-2 are able to bind *VEGF-A*. Both receptors consist of a single transmembrane region and an intracellular tyrosine kinase domain [50]. When *VEGF-A* binds to VEGFR-2, an auto-phosphorylation of tyrosine residues of VEGFR-2 leads to the activation of several kinases that mediate cell migration and activation of MAPK signaling (phosphorylation of MEK/ERK). Subsequently, this stimulates endothelial

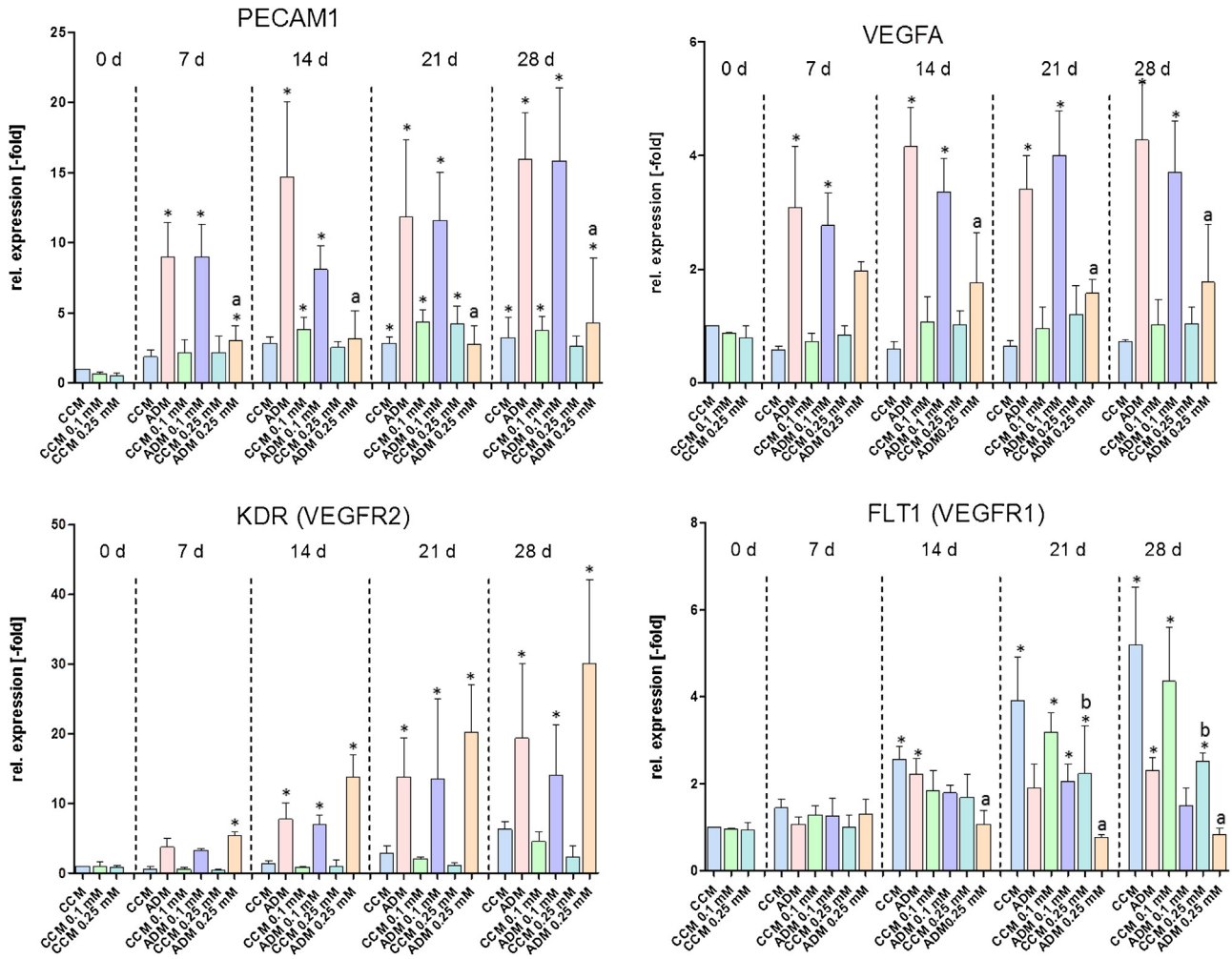

Fig. 3 – Effect of TEGDMA on angiogenic marker gene expression in DPSCs. Gene expression of angiogenic marker genes *PECAM1*, *VEGF-A*, *FLT1* and *KDR* was analyzed in DPSCs cultured for 7 days (7 d), 14 days (14 d), 21 days (21 d) or 28 days (28 d) in CCM or angiogenic differentiation medium (ADM). In addition cells were treated with 0.1 mM TEGDMA or 0.25 mM TEGDMA. Control cultures were grown in CCM or ADM supplemented with 0.25% ethanol. 0 d represents the time point, when angiogenic differentiation was started by medium exchange. Data is normalized to the geometric mean of 18S ribosomal RNA (*RRN18S*) and beta-2-microglobulin (*B2M*) expression (housekeeping genes). Mean values of three independent experiments are shown; error bars represent SD. Asterisks show significant difference to 0 d CCM control. a and b represent significant differences to the untreated cells grown in ADM (a) or cells grown in CCM (b) respectively for the corresponding time points (one-way ANOVA with Bonferroni's multiple comparison test was carried out using log2 transformed fold changes, $p < 0.05$).

proliferation [51]. Interestingly, *KDR*, which encodes VEGFR-2, showed the highest expression when cells were cultured in ADM and additionally treated with 0.25 mM TEGDMA. This increase was time-dependent and was only visible for cells cultured in ADM and treated with 0.25 mM TEGDMA. But, this 'triggering effect' of 0.25 mM TEGDMA on the expression of *KDR* was not statistically significant (ANOVA with Bonferroni's multiple comparison test). In contrast, the enhanced *KDR* expression in non-treated cells cultured in ADM in comparison to cells cultured in CCM was significant. This indicates an initiated angiogenic differentiation process. In addition, the high expression of *KDR* in 0.25 mM TEGDMA treated cells showed that DPSCs were still vital and were able to regulate gene expression. This suggests that the decreased mRNA-expression of the other investigated genes was not due to TEGDMA-induced cytotoxic effects. The different impact of 0.25 mM TEGDMA on *KDR* gene expression in comparison to the other evaluated genes remains elusive. VEGFR-1 exists in a membrane-bound and a soluble form. In contrast to VEGFR-2, which stimulates the formation of new blood vessels, the soluble form of VEGFR-1 may act as a decoy receptor [15]. The soluble VEGFR-1 lacks the transmembrane and signaling domains and thus cannot transduce pro-angiogenic signals. It can rather lower the amounts of free *VEGF-A* available to activate VEGFR-2 [51]. This might explain our observation that expression of *FLT1*, the gene encoding VEGFR-1, is more pronounced in CCM cultured cells compared to ADM cultured cells. Nevertheless, a weak increase in *FLT1* expression was

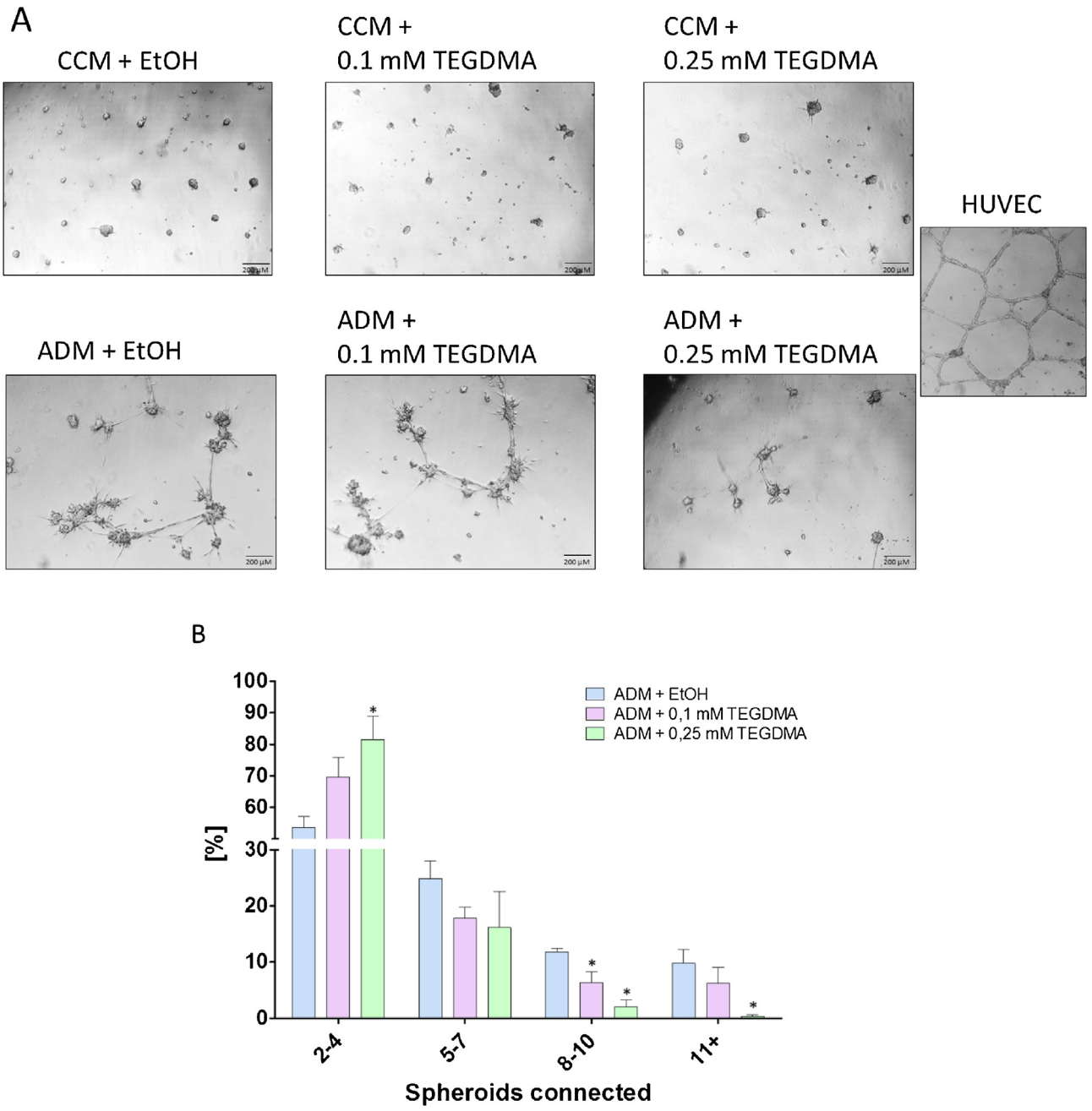

Fig. 4 – *In Vitro* Spheroid Sprouting Assay of DPSCs in the presence of TEGDMA. DPSCs were grown for 14 d in CCM or in ADM in the presence of 0.1 mM or 0.25 mM TEGDMA. Control cells were incubated with 0.25% ethanol (ADM + EtOH). After 14 d, cells were trypsinized and re-suspended in ADM. Subsequently, 1.2 × 10^5 cells per well were seeded on Matrigel™. As sprouting control HUVECs (Human umbilical vein endothelial cells) were seeded on Matrigel™ for 21 h. Plates were incubated in a humidified atmosphere at 37 °C and 5% CO_2. (A) After 21 h, images were taken. (Images are representative for three independent experiments). (B) Connected spheroids were quantified and grouped (2–4, 5–7, 8–10 and 11+) for each treatment. Percentages of each group are shown. Asterisks indicate statistically significant difference to the control (ADM + EtOH) of each group (one-way ANOVA, followed by Dunnett test, $p < 0.05$).

documented in ADM grown cells over time. *FLT1* expression was significantly lower, when cells were treated with 0.25 mM TEGDMA. This was true for cells grown in ADM as well as in CCM, when they were cultured for a minimum of 14 days or 21 days, respectively. This clearly indicates a marked influence of 0.25 mM TEGDMA on *FLT1* gene expression. Further analyses have to be carried out to investigate the meaning of enhanced *FLT1* expression in CCM cultured cells and of the decreasing effect of TEGDMA.

Besides two receptors of VEGF, we also documented the expression of *VEGF-A* itself. *VEGF-A* is the most important pro-angiogenic factor. Its expression is highly regulated. It

was demonstrated that *VEGF-A* expression is increased during hypoxia mediated through the transcription factor HIF-1 (hypoxia inducible transcription factor 1). *VEGF-A* mRNA can be stabilized involving unidentified factors binding to its 3′UTR [52]. Similar to *PECAM1* and VEGFR-2, the expression of *VEGF-A* is significantly induced when cells were cultured in ADM. A strong inhibition of *VEGF-A* expression was found in 0.25 mM TEGDMA treated cells.

Taken together, the results of our gene expression experiments confirmed the angiogenic differentiation potential of DPSCs cultured in ADM. The experiments showed that a concentration of 0.25 mM TEGDMA markedly reduces the expression of angiogenic related marker genes (*PECAM1, VEGF-A*), whereas other genes like *KDR* were not affected.

In addition to the aforementioned analysis, the present study aimed to correlate the observed TEGDMA-induced effects in the expression of certain angiogenesis-related markers to potential morphological changes attributed to *in vitro* angiogenic differentiation. Therefore, the angiogenic potential of DPSCs was visualized in cell culture by an *in vitro* sprouting assay (Fig. 4). It is known that endothelial cells are able of giving rise to capillary-like structures *in vitro* resembling the physiological arrangement of endothelial cells in vessels *in vivo* [53]. This phenomenon can be observed when endothelial cells are seeded on a gel containing an assortment of ECM proteins. Furthermore, it has been documented that MSCs can form spheroidal structures, when seeded on a 3D matrix [54,55]. Accordingly, it was demonstrated that DPSCs have the ability to form spheroids as well [56]. Our results showed that after seeding on Matrigel™ (a commercially available gelatinous protein mixture derived from mouse tumor cells) DPSCs rearranged from a monolayer to a prominent spheroid structure. It is well known, that MSCs are able to form spheroids [57]. In contrast to 'pre-cultured' cells for 14 days in CCM, the DPSCs, which were grown in a 'pre-culture' in ADM for 14 days, produced spheroidal structures connected to each other 21 h after seeding on Matrigel™. The structures resembled capillary sprouts formed by endothelial cells. Aksel and Huang reported similar observations with human and swine DPSCs [8]. This observation clearly supports our qRT-PCR results and confirms also on morphological level that anangiogenic differentiation took place *in vitro*. Treatment with 0.25 mM TEGDMA resulted in significantly less interconnecting spheroids. Based on our data we conclude that a concentration of 0.25 mM TGEDMA has an inhibitory effect on the angiogenic differentiation potential of DPSCs.

It has been demonstrated that dental methacrylates, TEGDMA as well as HEMA, can severely affect the odontogenic differentiation of pulp stem/progenitor cells, which may result in impaired pulp tissue homeostasis and repair [25]. It may be speculated that the negative effects of 0.25 mM TEGDMA on angiogenic differentiation also compromise pulp repair processes.

Taken together, our experiments indicate the ability of DPSCs to differentiate toward the angiogenic lineage when cultured in ADM. TEGDMA in sufficiently high concentrations diminished or inhibted this angiogenetic potential of DPSCs. This indicates potential inhibiting effects of TEGDMA, released from dental resins, on neo-angiogenesis processes occurring during pulp repair subsequently to restorative procedures. The analysis and comprehensive knowledge of effects of composite resins and other resinous oral biomaterials regarding the differentiation potential of DPSCs are important for restorative dentistry and tissue regeneration, for novel tissue engineering procedures, as well as for the development of biocompatible dental and medical materials.

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