Cell-age and cell type-dependent behavior of human vascular cells on micro-structured or soft polymer substrates

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Abstract

Increasing number of studies are focused on how adherent cells respond, *in vitro*, to different properties of a material. Typical properties are the surface chemistry, topographical cues (at the nano- and micro-scale) of the surface, and the substrate stiffness. Cell response studies are of importance for designing new biomaterials with applications in cell culture technologies, regenerative medicine, or for medical implants. However, only very few studies take the cell age factor, respectively the donor age, into account. In this work, we tested two types of human vascular cells (smooth muscle and endothelial cells) from old and young donors on (a) micro-structured surfaces made of poly(dimethylsiloxane) or on (b) flat polyacrylamide hydrogels with varying stiffnesses. These experiments reveal age-dependent and cell type-dependent differences in the cell response to the topography and stiffness, and may establish the basis for further studies focusing on cell age-dependent responses.

1 Introduction

Many studies focusing on cell aging are aiming to better understand molecular mechanisms characterizing the cell ageing phenomenon. These mechanisms are only partially understood, but there is a lack of knowledge about how aging affects complex cell functions such as cell-substrate interactions [1]. It is well known that adherent cells respond, *in vivo* and *in vitro*, to certain physical and chemical signals of the substrate [2,3]. These signals are e.g., the surface chemistry, surface topography at the micro- and nano-scale, and the substrate stiffness. Studying how cell aging affects the way cells respond to surface cues, may help to improve the understanding of some age-linked diseases such as cardiovascular clinical pictures. In addition, it will establish the base for the development of new cell age-adapted biomaterials with applications to medical implants as well as to regenerative medicine [4]. However, most studies focused on cell-substrate interactions soley and do not take the cell age factor into account. Only few studies, show age-dependent differences in the cellular response to different physical stimuli. Kaufmann *et al.* investigated how human fibroblasts, derived from skin samples of different old donors, respond to micro-sized grooves on poly(dimethylsiloxane) (PDMS) [5]. They found that cells from old donors adapted their morphology to a less degree than cells from young donors. In another work carried out by Zahn *et al.*, the intracellular stiffness of foreskin human fibroblasts from old and young donors was tested [6]. The authors observed that the cell body of older cells was softer than that of younger cells. Accordingly, they demonstrated that older cells had a lower quantity of actin than younger ones. Moreover, they tested cell behavior under cyclical uniaxial stretching of PDMS substrates and showed that old cells aligned faster perpendicular to the stretching direction than young cells.

In this present work, we investigated if cell responses to the substrate rigidity vary with cell age. Two types of human vascular cells derived from the coronary artery were used in this study: endothelial cells (ECs) and smooth muscle cells (SMCs)[7]. ECs and SMCs from young and old donors were assessed on (1) surfaces with a specific topographical pattern, consisting of micro-sized grooves and on (2) flat surfaces with varying stiffnesses. Cell morphology adaptation and cell-substrate adhesion complexes were examined. We used PDMS to fabricate the micro-structured surfaces and polyacrylamide (PAA) substrates to fabricate hydrogels of different stiffness. By means of soft lithography we micro-structured PDMS surfaces with parallel grooves. PAA hydrogels with different stiffnesses were obtained by varying the ratio of acrylamide with bis-acrylamide. We applied the micro-structured PDMS to study the degree of parallel cell orientation with respect to the grooves. Cell spreading, as well as cell-substrate adhesion complexes area, were analyzed on PAA hydrogels. The experiments could establish the base for further systematic studies about cell age-dependent cell responses to different substrate properties.

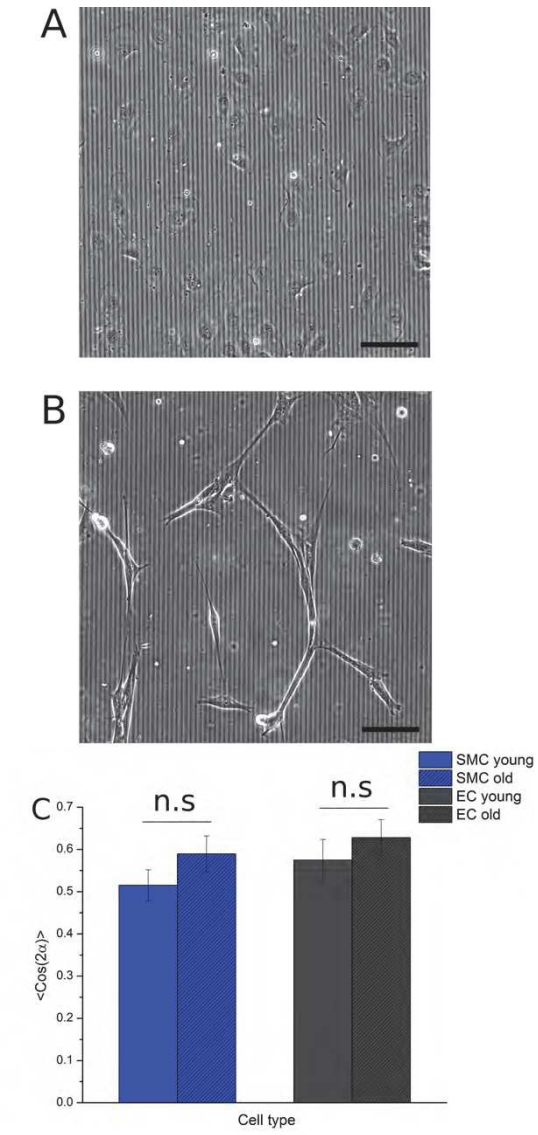

Figure 1. Human vascular cells seeded on 200nm deep and 4x4µm wide grooves and ridges on poly(dimethylsiloxane) (PDMS). (A) Phase contrast images of young endothelial cells (ECs) and (B) old smooth muscle cells (SMCs). (C) The average orientation parameter is given for each cell type. An orientation parameter of 1 means a perfect parallel cell orientation with respect to the micro-structure while 0 means a random cell orientation. Error bars represents the standart error of the mean. Scale bars: 100µm. "n.s" means statistically no-significant different (t test, p value > 0.05).

2 Materials and methods

2.1 Photolithography and physical vapor deposition

In order to obtain an array of micro-sized grooves, photolithography and physical vapor depositon (PVD). A previously described photolithography procedure was followed [8]. Briefly, silicon wafers were spin-coated with positive photoresist (ma-P1210 photoresist, MicroResist Technology, Germany), and cured and afterwards, illuminted through a photomask, with the desired pattern (ML & C, Jena, Germany). After developing the exposed photoresist, chromium was deposited by using PVD. Finally, the remaining photoresist was removed.

2.2 Soft lithography and functionalization

Poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning, USA) was mixed in a ratio of 1:10 (curing agent to elastomer), degased, casted on the previously micro-structured silicon wafer, and cured at 65ºC for 24h. PDMS substrates with the desired micropattern, was prepared for cell experiments. Firstly, it was shortly desinfected with ethanol 70%, followed by rinses with sterile PBS. 40µg/ml of poly-L-lysine (PLL) (Sigma-Aldrich) was incubated onto the substrate for 30min to enhance fibronectin adhesion. After some rinses with PBS, 10µg/ml of human fibronectin was incubated for 30min. Then, the substrate was washed again with PBS, and incubated for some minutes with the appropiate cell media, before cell seeding.

2.3 Polyacrylamide gels fabrication and functionalization

Previously cleaned and oxidized glass coverslips were functionalized with 3-amminopropylsilane (Sigma-Aldrich), letting it incubate for 5min at room temperature. After rinses with distilled water, they were properly dried and incubated with 0.5% of glutaraldehyde (Sigma-Aldrich) for 30min at room temperature. Coverslips were dried, and incubated with a previously fresh prepared PAA mixture (Bio-Rad). By varying the proportions between acrylamide and bis-acrylamide different stiffnesses were achieved. After PAA was fully polymerized, sulfo-SANPAH (Pierce) was pipetted on the samples and exposed for 8min under UV light. Then, after sterilizing them with 70% ethanol, and rinsing with sterile PBS, 10µg/ml of human fibronectin was incubated for 30min, to be covalently linked. Finally, PAA substrates were washed several times with PBS, and incubated with cell media before cell seeding.

2.4 Cell culture and microscopy

Human Coronary Artery Smooth Muscle Cells (HCASMC) and Endothelial Cells (HCAEC) (Promocell) from old donors (>50 years) and young donors (<30 years) were used in this study. Cells were cultured in Endothelial Cell Growth Medium and Smooth Muscle Cell Growth Medium 2 (Promocell), respectively, supplemented with 1% penicillin-streptomycin. Cells were incubated at 37ºC in a humidified atmosphere with 5% CO_2. Cell media was changed every second day. Cells were seeded on the substrates at a cell density of 50cells/mm^2. Cells used in this work were no more than passage 6. Phase contrast microscopy imag-

es from cells on PAA hydrogels were taken 24h after seeding, and after 48h from cells on PDMS substrates. Phase contrast pictures were obtained with an inverted microscope, and fluorescent images with an upright microscope (both from Carl Zeiss, Germany).

2.5 Immunocytochemistry

In order to immunostain the samples, cells were firstly fixed with 4% paraformaldehyde for 15min in the incubator. Afterwards, samples were rinsed several times with PBS and permeabilized by incubating 0.1%w/v Triton X-100 (Fluka) for 3min. After washing several times with PBS, samples were incubated with 1%w/v bovine serum albumina (BSA) (Serva) for 30min, to block unspecific interactions. The first antibody (anti-Paxillin from rabbit) (Abcam) was incubated in a 1:300 dilution for 1h in a wet environment and room temperature. After rinsing it properly several times, the secondary antibody (goat-anti-rabbit) (Invitrogen) with a conjugated red fluorescent molecule (Alexa fluor 568), was incubated for 30min in wet conditions and protected from the light. After this latter incubation, samples were properly rinsed with PBS, and kept in the same solution for visualization or storage.

2.6 Data analysis

Microscopy images were analyzed by using ImageJ (http://rsbweb.nih.gov/ij/index.html). At least, 20 cells and paxillin-positive cell-matrix adhesions (so called focal adhesions) for each hydrogel stiffness, and 50 cells on micro-structured surface, were analyzed. Cell and cell-matrix ahdesion contours were marked manually.

Cell orientation was calculated with the non-polar orientation parameter $\langle\cos(2\phi)\rangle$ [9]. For -1 cells are oriented perpendicular to the microstructure direction, for 0 cells are randomly oriented, and for 1 cells are totally parallel to the microstructures.

For significance analysis, a paired sample t-test was used. Probability values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 Alignement response to micro-structured surfaces is not cell age-dependent

Firstly, we examined whether there is an age-dependent cell response to microtopography. Thus, old and young SMCs and ECs were seeded on PDMS substrates containing 200nm depth microgrooves, with 4µm width and 4µm separation between them. Both cell types aligned paralell to microgrooves. To quantify their alignment the non-polar orientation parameter ($\langle\cos(2\phi)\rangle$) was calculated. In figure 1 C, orientation parameters for both cell types and ages are plotted. Values of $\langle\cos(2\phi)\rangle$ for SMCs are around 0.55, while they are slightly higher for ECs ($\langle\cos(2\phi)\rangle \approx 0.6$). In both cell types, the order parameter was slightly higher for old cells than for young cells. However, the differences were not significantly different for both cell types (old vs. young) and between the two cell types.

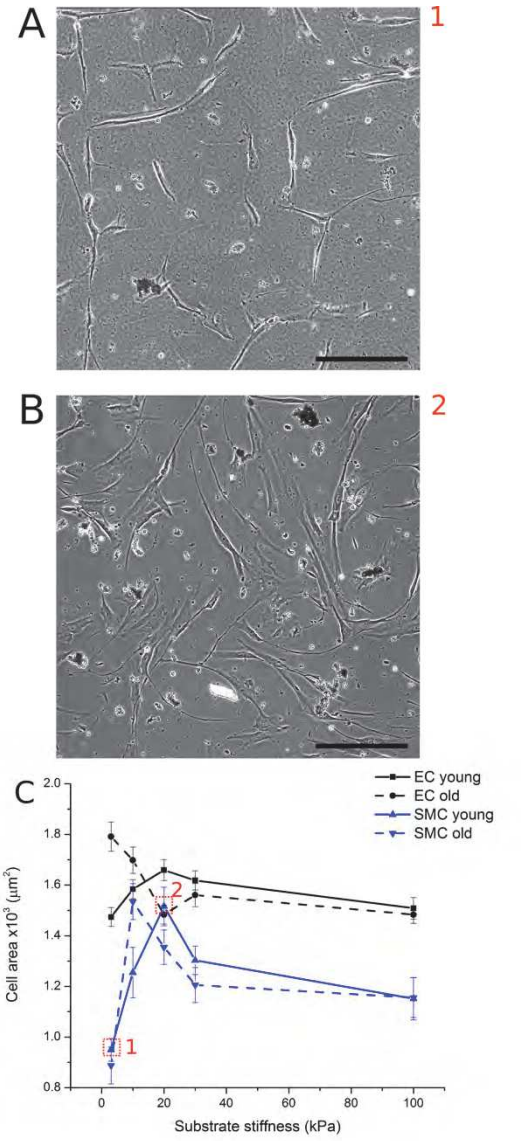

Figure 2. Phase contrast images of young smooth muscle cells (SMCs) on (A) 3kPa and (B) 20kPa polyacrylamide (PAA) hydrogels. (C) The average cell area is plotted versus different hydrogel stiffnesses for each cell type (SMC and endothelial cells (EC)) and age. Error bars represents the standart error of the mean. Scale bars: 100µm.

3.2 Maximum cell spreading depends on substrate stiffness and donor's age

In order to determine whether cell aging affects cells response to substrate stiffness, EC and SMC from young and old donors were cultured on PAA substrates with varying stiffnesses. The cell area was analyzed for each hydrogel stiffness (flat surface) and cell type. Figure 2 C shows averages of cell area for each stiffness. As it can be observed, generally ECs have a big-

ger cell area than SMCs. With exception of old ECs, all cell types had their minimum average area on 3kPa substrates. Old ECs, instead, had their maximum cell area on 3kPa substrates. As substrate stiffness increases, average cell area increases too, reaching its maximum at different stiffness depending on the age. Young SMCs and ECs have their maximum area at 20kPa, having an average area of ~1500µm^2 and ~1650µm^2, respectively. In contrast, old SMCs reached their maximum size at 10kPa instead.

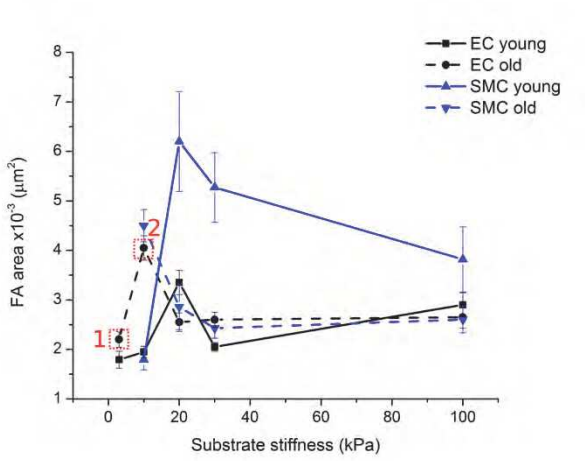

Figure 3. Plot of the average FA areas for all cell types (ECs and smooth muscle cells (SMCs)), over different substrate stiffnesses. Error bars represents the standart error of the mean. Scale bars: 30µm.

3.3 Cell-matrix adhesion area varies upon substrate stiffness and donor's age

We investigated whether there are also age-dependent differences regarding the area of cell-matrix protein complexes correlating with the results for the cell area. Thus we measured the size focal adhesions (FAs). Cells from the previous experiment were stained for paxillin as a marker of focal adhesions and their area was analyzed. Figure 3 C depicts average areas of focal adhesions for different substrate stiffnesses. ECs from both ages, had the smallest FAs on 3kPa (~0.002µm^2). For SMCs, FAs were not possible to analyze for that stiffness since the FAs were blurried and diffuse and could not be clearly detected. On 10kPa substrates, both old cells reached their maximum area, being 0.004µm^2 for old ECs, and 0.0045µm^2 for SMCs. Young cells, instead, reached their maximum FA area on 20kPa, being ~0.0062µm^2 for SMCs, and ~0.0033µm^2 for ECs. After old and young cells reached their respective FA maximum areas, the area decreased as stiffness increased. Similarly to cell area, a shift between young and old cells' FA curves can be appreciated.

4 Conclusions

In this *in vitro* study, we tested whether cell age affects the reaction of human vascular cells (SMC and EC) to surface topography and to the stiffnesses of a substrate. While the alignment of the cells along micrometer-sized grooves was independent of cell type and donor age, we found for both cell types an age-dependent behavior on flat PAA hydrogels with varying stiffness. Cells had a maximum in cell area at a particular substrate stiffness. Maximum FA area coincided to that observation. The age-dependent reponse to substrate stiffness may result from an altered cell stiffness due to changes in the cytoskeleton as observed previously [6]. Our observations could pave the way to deepen more into biophysical studies of cell aging and altered adhesion and may help at the development of age-matched biomaterials.

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5 References

[1] Starodubtseva MN. Ageing Research Reviews. Ageing Research Reviews 2011;10:16–25.

[2] Li Y, Huang G, Zhang X, Wang L, Du Y, Lu TJ, et al. Biotechnology Advances. Biotechnology Advances 2014;32:347–65.

[3] Lara Rodriguez L, Schneider IC. Directed cell migration in multi-cue environments. Integr Biol 2013;5:1306.

[4] Auger FA, Gibot L, Lacroix D. The Pivotal Role of Vascularization in Tissue Engineering. Annu Rev Biomed Eng 2013;15:177–200.

[5] Kaufmann D, Su Y, Biela S, Jungbauer S, Spatz JP, Kemkemer R. Soft lithography detects partial mechano-sensoric blindness to micrometre topography in cultured aged and diseased cells. International Journal of Materials Research 2012:1–7.

[6] Zahn JT, Louban I, Jungbauer S, Bissinger M, Kaufmann D, Kemkemer R, et al. Age-Dependent Changes in Microscale Stiffness and Mechanoresponses of Cells. Small 2011;7:1480–7.

[7] Biela SA, Su Y, Spatz JP, Kemkemer R. Different sensitivity of human endothelial cells, smooth muscle cells and fibroblasts to topography in the nano–micro range. Acta Biomaterialia 2009;5:2460–6.

[8] Jungbauer S, Kemkemer R, Gruler H, Kaufmann D, Spatz JP. Cell Shape Normalization, Dendrite Orientation, and Melanin Production of Normal and Genetically Altered (Haploinsufficient NF1)-Melanocytes by Microstructured Substrate Interactions. ChemPhysChem 2004;5:85–92.

[9] Kemkemer R, Jungbauer S, Kaufmann D, Gruler H. Cell Orientation by a Microgrooved Substrate Can Be Predicted by Automatic Control Theory. Biophysical Journal 2006;90:4701–11.