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Cyclic strain disrupts endothelial network formation on Matrigel

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ABSTRACT

Most forms of tissue healing depend critically on revascularisation. In soft tissues and *in vitro*, mechanical stimuli have been shown to promote vessel-forming activity. However, in bone defects, increased interfragmentary motion impairs vascular regeneration. Because these effects seem contradictory, we aimed to determine whether a range of mechanical stimuli exists in which angiogenesis is favoured. A series of cyclic strain magnitudes were applied to a Matrigel-based "tube formation" assay and the total lengths of networks formed by human microvascular endothelial cells measured at 24 h. Network lengths were reduced at all strain levels, compared to unstretched controls. However, the levels of pro-angiogenic matrix metalloproteases-2 and -9 in the corresponding conditioned media were unchanged by strain, and vascular endothelial growth factor was uniformly elevated in stretched conditions. By repeating the assay with the addition of conditioned media from mesenchymal stem cells cultivated in similar conditions, paracrine stimuli were shown to increase network lengths, but not to alter the negative effect of cyclic stretching. Together, these results demonstrate that directly applied periodic strains can inhibit endothelial organisation *in vitro*, and suggest that this may be due to physical disruption rather than biochemical modulation. Most importantly, the results indicate that the straining of endothelial cells and their assembly into vascular-like structures must be studied simultaneously to adequately characterise the mechanical influence on vessel formation.

Introduction

Revascularisation is a critical process in most forms of tissue regeneration. In ovine bone healing, excessive interfragmentary movement inhibits or delays revascularisation, and results in the impaired restoration of the tissue's mechanical properties (Claes et al., 2002; Lienau et al., 2005, 2006). Conversely, increased blood flow and tissue stretching have been shown to stimulate angiogenesis (Brown and Hudlicka, 2003; Ichioka et al., 1997; Pietramaggiori et al., 2007), and rigid stabilisation diminishes the vascular response in fracture healing (Sarmiento et al., 1984). Therefore the mechanism and threshold of mechanical impairment during bone healing remain uncertain. Possible explanations include alterations in paracrine signalling on the one hand, and the physical disruption of newly formed vessels by excessive local stresses on the other hand (Carter et al., 1998; Rhinelander, 1974).

In vitro studies have shown that the formation of vessel-like structures by endothelial cells can be stimulated by prior mechanical conditioning (Morrow et al., 2007; Von Offenberg Sweeney et al., 2005). Additionally, angiogenic activity is enhanced by conditioned media from other cells cultured under strain (Kasper et al., 2007; Zheng et al., 2001). However, studies of the direct effects of stress or strain on vessel formation are scarce. No enhancement of endothelial sprouting is reported when cyclic strain is applied directly in gel-based assays; rather, a change in "vessel" morphology and/or orientation is observed (Joung et al., 2006; Matsumoto et al., 2007). In contrast, cyclic stretching of a confluent endothelial cell layer resulted in a strain-dependent contraction of the monolayer into a reticulated network (Shukla et al., 2004). While this suggests that increasing strain may enhance vessel formation, the mechanism of network formation differs fundamentally from those of routinely used angiogenesis/vasculogenesis assays (in which "vessel-like" structures either sprout from clusters or assemble from separated cells in relatively low-density cultures), making direct comparison difficult. To date, such a quantitative assessment of vessel-like network formation across a range of applied cyclic strain levels has not been made in a standard assay.

To address this issue, we used a commercially available mechanical strain system for cell cultures to apply a periodic stretch to the well-established "tube formation" assay (Kubota et al., 1988). Pro-angiogenic responses were quantified by the length of tube-like networks formed by endothelial cells on Matrigel. As an initial test for a

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mechano-regulation mechanism for the results, vascular endothelial growth factor (VEGF) and matrix metalloproteases-2 and -9 (MMP-2 and MMP-9—gelatinases)—all of which may stimulate and/or regulate angiogenic behaviour *in vitro* (Fang et al., 2000; Ferrara and Davis-Smyth, 1997; Jadhav et al., 2004; Schnaper et al., 1993; Zheng et al., 2001)—were quantified in the conditioned media from these cultures. Finally, because mesenchymal stem cells (MSCs) are able to enhance angiogenesis by mechanically regulated paracrine signalling (Kasper et al., 2007), we further investigated whether this could compensate for the negative effects of strain on the endothelial response.

Materials and methods

Cell culture

Immortalised human microvascular endothelial cells (HMEC-1) were kindly donated by Prof. G. Schönfelder (Institut für Klinische Pharmakologie und Toxikologie, Charité-Universitätsmedizin Berlin, Germany) and cultured in MCDB 131 (Gibco, Invitrogen, Karlsruhe, Germany) with 5% foetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 2 mM L-glutamine (Biochrom) and 100 IE/ml penicillin + 100 µg/ml streptomycin (Biochrom). During routine culture, 1 µg/ml hydrocortisone (Sigma-Aldrich Chemie GmbH, Munich, Germany) was added, but was omitted in assays.

MSCs were isolated from bone marrow aspirates from patients (with informed consent) and characterised as reported previously (Kasper et al., 2007). Investigations using these cells were approved by the Ethics Commission of the Charité-Universitätsmedizin Berlin (application number EA1-072-08). The MSCs were cultivated in either RPMI-1640 (Invitrogen, Karlsruhe, Germany) with 5 mM HEPES (Sigma-Aldrich, Steinheim, Germany), 2 mM L-glutamine (Biochrom) and 50 µM beta-mercaptoethanol (Sigma-Aldrich, Munich, Germany) or Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Karlsruhe, Germany), both supplemented with 10% FBS and penicillin/streptomycin as above.

"Tube Formation" assay with cyclic stretch

Each well of collagen-I-coated Bioflex 6-well culture plates (Flexcell, Dunn Labortechnik GmbH, Asbach, Germany) was coated with 500 µl ice-cold Matrigel (BD Biosciences, Heidelberg, Germany), diluted to 8 mg/ml with complete culture medium (to reduce viscosity). The solution was allowed to gel for 1 h at 37 °C. HMEC-1 cells were delivered onto the gels at 2×10^5/well (208 cells/mm^2) and allowed to attach for 1 h before commencing the stretching regime.

Cyclic stretching was applied using the Flexercell Tension Plus FX-4000T system (Flexcell International Corp., Hillsborough, NC, USA) with 25 mm BioFlex Loading Stations (Flexcell). Nominal strains ranging from 2.5% to 20% were employed at a frequency of 1 Hz. A further set of experiments at 2.5% nominal strain used UniFlex plates (collagen-coated; Flexcell) to apply a uniaxial strain to the cultures; due to the uneven surfaces of the UniFlex membranes, 600 µl of Matrigel solution was used for coatings. Experiments contained duplicate strained and unstrained samples, with additional cell-free controls. Each strain level was tested in three separate experiments.

After 24 h of stretching, endothelial networks were photographed in five fields per well with a 5× objective magnification. The lengths (in pixels) of cords of interconnecting cells were measured using ImageJ (version 1.39u, 2007, W.S. Rasband, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/) and totalled for each condition (± strain).

Analysis of conditioned media

At the conclusion of each experiment, a sample of conditioned medium (CM) was collected from each well and stored at −80 °C and subsequently assayed for MMPs involved in angiogenesis. CM samples from each strain level, and corresponding controls, were assayed for MMP-2 and MMP-9 using gelatin zymography. 10% Novex gels (Invitrogen) were used according to manufacturer's instructions, with 15 µl CM per lane. The resulting band densities were measured using ImageJ. A subset of CM samples were also assayed for cell viability using an LDH assay (LDH-Cytotoxicity Assay Kit II, BioCat, Heidelberg, Germany), according to the manufacturer's instructions.

An enzyme-linked immunosorbent assay (ELISA) was carried out to probe conditioned media from each experiment for VEGF. The assay was conducted on 200 µl samples according to the manufacturer's instructions (Quantikine® Human VEGF Immunoassay, R&D Systems, Minneapolis, USA).

Supplementation with conditioned media from mesenchymal stem cells

MSCs (passage 5, 81-year-old male patient) were cultured for 3 days under identical conditions to the "tube formation" assays, i.e. in Matrigel-coated BioFlex wells, with and without a nominal strain of 2.5%. Two millilitres of the conditioned medium (DMEM-based) from each sample was added to endothelial cells, seeded in 1 ml of the routine MCDB 131-based medium. Controls with both types of non-conditioned medium were also tested. The "tube formation" assays then proceeded as described above.

Further, to allow comparison with previously published results (Kasper et al., 2007), conditioned media from MSCs in loaded and unloaded fibrin constructs were also tested in the same way. The MSCs were obtained from a 52-year-old female and used at passage 3. One million cells were suspended in each 700 µl fibrin construct (fibrinogen and thrombin-S solutions (TISSUCOL-Kit 2,0 Immuno, Baxter, Unterschleißheim, Germany) both diluted 1/4 with culture medium) and the resulting gels sandwiched between bone spongiosa chips of 15 mm diameter. These were placed in a purpose-built bioreactor (Matziolis et al., 2006) with 25 ml culture medium (RPMI-1640-based) and subjected to a 1 Hz cyclic load, corresponding to a 20% compression of the gel, for 3 days. Unloaded controls were cultured in otherwise identical conditions. The conditioned media were aliquoted and stored at −80 °C before use in the "tube formation" assay as described above.

Validation of strain levels on coated plates

Digital image correlation was used to quantify local strains across the BioFlex membranes (Bieler et al., 2009; Boerboom et al., 2008). After Matrigel coating, a fine paint speckle pattern was applied to both gels and uncoated membranes using an airbrush. Each whole well was then digitally photographed over a range of nominal strains from 0% to 20%. The paint spots were digitally correlated between relaxed and stretched images using Vic-2D software (version 4.4.1, 2006, Correlated Solutions, Inc., Columbia, SC, USA), to calculate strain levels across the flat region of the loading post. The tensions were characterised as first principal strains, averaged over the surface.

Statistics

Statistical evaluations used SPSS (version 12.0, SPSS Inc., USA). In each experiment, differences between network lengths over ten fields were tested using the Mann–Whitney *U*-test, with significance judged as $p \leq 0.05$. To compare results between different strain levels, total network lengths for strained samples were normalised to unstretched controls and analysed by Mann–Whitney *U*-tests and the Kruskal–Wallis test. Measured strain magnitudes (first principal strains) with and without gel coatings were tested for linear correlation against the corresponding nominal strain levels. Zymography results were tested for significant differences in each of the four bands (MMP-2 and -9, active and pro- forms) using paired