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Research paper

Dermatopontin augments angiogenesis and modulates the expression of transforming growth factor beta 1 and integrin alpha 3 beta 1 in endothelial cells

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ABSTRACT

Dermatopontin (DPT) is a matricellular protein with cardinal roles in cutaneous wound healing. The protein is also reported to be altered in various anomalies including cancer. The present study is aimed to unravel the role of DPT in angiogenesis which is imperative in many physiological and pathological processes. DPT's capabilities on promoting angiogenesis were assessed using various *in vitro* and *ex vivo* systems. The results indicated that DPT enhances cell motility and induces lamellipodia formation in endothelial cells. Additionally, we noticed that DPT stimulates tube formation in endothelial cells when plated on a matrigel substrate. However, it was observed that DPT had no effect on the proliferation of endothelial cells even at higher concentrations and prolonged treatment periods. Additional experiments on CAM and aortic arch assays apparently depicted that DPT promotes neovascularisation and tube sprouting, thus unraveling its prominent role in angiogenesis. Further, PCR analysis revealed that endothelial cells are devoid of DPT expression, but when exogenously supplied, modulates the expression of transforming growth factor β1 and integrin α3β1 which are reported to have crucial roles in endothelial cell behaviour during angiogenesis. In conclusion, DPT possess vital pro-angiogenic properties and thus retains promising therapeutic values in managing chronic wounds and cancer.

1. Introduction

The process of branching of new capillaries from the existing blood vessels *in vivo* is called angiogenesis (Adair TH, 2010). It occurs during various physiological and pathological conditions like fetal development, wound healing, tumor metastasis, etc. and is predominantly governed by the microenvironment of the cells (Folkman, 1995). The overall process of angiogenesis requires precise harmonization of proliferation, migration, apoptosis and differentiation of the cells involved. The endothelial cells upon stimuli loses its contact inhibition, secrete proteins and proteases which perform functions like basement membrane degradation and facilitate the invasion of cells in to the surrounding matrix where they proliferate and differentiate to form new blood vessels (Madri and Pratt, 1988; Senger and Davis, 2011). Therefore, molecules which regulate any of the aforementioned steps are of high importance as they impart significant therapeutic value.

Extracellular matrix (ECM) provides a critical framework for angiogenesis by conferring molecular signals, immobilizing angiogenic regulators and delivering structural support necessary for all the stages of blood vessel formation including sprouting, lumen formation and vessel stabilization (Risau, 1997). Several ECM molecules are reported to have functions either as pro or anti angiogenic and sometimes both, depending upon its spatio-temporal expression (Sottile, 2004). Several of the fibril forming proteins and its partner proteins that regulate the process of fibrillation has been hitherto shown to influence angiogenesis (Bornstein and Sage, 2002; Yang et al., 2000). For instance, decorin which has functional abilities in modulating the ECM architecture inhibits angiogenesis potentially (Jarvelainen et al., 2015). Other ECM molecules like collagen and fibronectin, both having a tendency to form fibrillar structure are also reported to have vital abilities in modulating the

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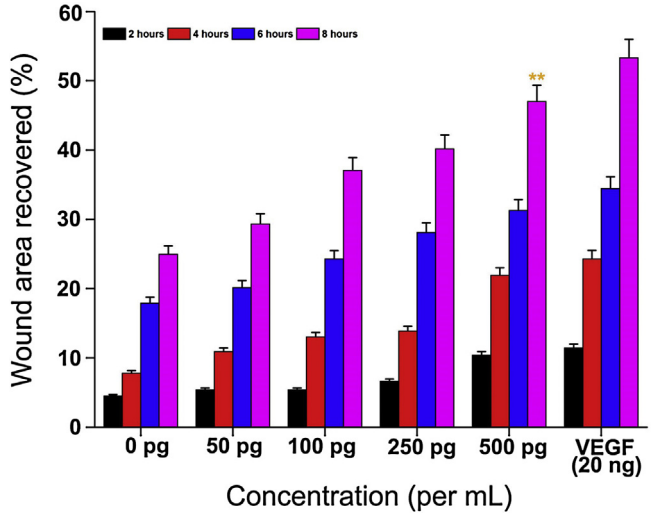

Fig 1. Graphical representation of the wound area recovered after treatment with DPT and VEGF. ** *P*-value (0.03) was calculated using Student's *t*-test for the indicated concentration by comparing with the untreated cells at the same time point (n = 3).

process of angiogenesis (Astrof and Hynes, 2009; Twardowski et al., 2007).

Dermatopontin (DPT), a skin abundant protein first identified in bovine dermis plays a significant role during wound repair (Forbes et al., 1994; Neame et al., 1989). This tyrosine rich matrix protein induces collagen fibrillation and its levels are reported to be altered during various dermatopathological conditions(Okamoto and Fujiwara, 2006). DPT, which regulates the biological activity of transforming growth factor beta 1 (TGF β1) and decorin, is also attributed in oral squamous cell and hepatocellular carcinomas (Fu et al., 2014; Li et al., 2009; Okamoto et al., 1999; Okamoto et al., 1996; Yamatoji et al., 2012). Importantly, DPT is involved in the metastasis of oral cancer and pathogenesis of prostate cancer, where an abnormal angiogenesis is witnessed (Takeuchi et al., 2006; Yamatoji et al., 2012). Furthermore, the protein has been recognized to interact with integrins and its expression is elevated in experimentally induced myocardial infarction (Takemoto et al., 2002). We have previously reported that DPT promotes keratinocyte migration revealing its important role in re-epithelialization (Krishnaswamy and Korrapati, 2014). The altered expression of DPT in various cancers and its pivotal role in adhesion and migration of cells had prompted us to decipher its angiogenic potential.

2. Materials and methods

2.1. Cell culture

Human endothelial cell line, EA.hy926 was purchased from American Type Culture Collection (ATCC, USA). The cells cultured using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with streptomycin (100 μg/mL), penicillin (100 units/mL), gentamicin (30 μg/mL), amphotericin B (2.5 μg/mL) and 10 % FBS (Invitrogen, USA) were maintained at 37 °C in 25 cm^2 tissue culture flasks in an incubator supplied with 5% CO_2 and 95% air. All the chemicals, until otherwise mentioned, were procured from Sigma-Aldrich (USA) and are cell culture tested.

2.2. Chicken embryos

Fertilized white leghorn chicken eggs (Sp. *Gallus gallus domesticus*) were purchased from Tamilnadu government poultry station, Potheri, Chennai. The eggs were cleaned with 70 % ethanol and incubated at 37 °C in a humidified incubator. All the experiments were performed in accordance with the guidelines framed by the CPCSEA and institutional ethical committee.

2.3. Scratch wound assay

Equal density of 1.8×10^5 cells (EA.hy926) per well was seeded in a 24 well tissue culture plate (TCP) and allowed to become confluent overnight in a CO_2 incubator. A scratch wound was made on the monolayer of cells using a 200 μL pipette tip. The cells were then washed twice with warm phosphate buffered saline (PBS) and fresh medium sans serum containing different concentrations of human recombinant DPT (rDPT) (4629-DP, R&D systems, USA) and vascular endothelial growth factor (VEGF) (Sigma-Aldrich, India) were added in triplicates. The migration of the cells was captured using a phase contrast microscope (Leica Microsystems, Germany) at a time interval of 2 h and the wound area was calculated using image J software (Abràmoff, 2004). The percentage of wound area recovered was calculated using the formula given below and was analyzed for significance using student's *t*-test. After the study period, cells were fixed and actin fibers were stained for assessing the lamellipodia formation.

$$\text{Percentage of wound area recovered} = \frac{(\text{Initial wound area - Final wound area})}{\text{Initial wound area}} \times 100$$

2.4. Phalloidin staining

The lamellipodia formation was identified by staining the actin fibers. After treating the EA.hy926 cells overnight with rDPT (500 pg/mL), cells were washed twice with PBS and fixed with 3.7 % formaldehyde. The fixed cells (along with cells fixed after scratch assay) were washed, permeabilized with 0.1 % triton X 100 (Sigma-Aldrich, USA) and blocked with 1 % bovine serum albumin (BSA). The cells were then washed twice with PBS and stained with Oregon Green® 488 Phalloidin (O7466, Invitrogen, USA) for 30 min in a humidified dark chamber. The excess stain was removed by washing and the nucleus was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were then captured using a fluorescence microscope (Leica Microsystems, Germany).

2.5. Cell proliferation assay

The proliferative potential of DPT on EA.hy926 cells was assessed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Mosmann, 1983). Equal density of 12×10^3 cells per well was seeded in a 24 well TCP and incubated overnight at 37 °C in a CO_2 incubator. Next day, fresh serum free DMEM medium containing different concentrations of rDPT were added in triplicates. After 24, 48 and 72 h the cells were treated with MTT (0.5 mg/mL in PBS) for 3 h at 37 °C. The formazan complex formed by the live cells was dissolved in dimethyl sulfoxide (DMSO) and measured colorimetrically at 570/630 nm using a micro plate reader (Bio-Rad, USA).

2.6. Tube formation assay

In vitro endothelial tube formation was assessed by standard procedures using matrigel matrix as a substrate (Arnaoutova and Kleinman, 2010; Francescone et al., 2011). All the procedures were performed on ice in sterile conditions and the pipettes, tips and the TCP used for the experiment were pre-chilled. A 24 well TCP was coated with approximately 300 μL of suitably diluted growth