Leptin enhances endothelial cell differentiation and angiogenesis in murine embryonic stem cells

Silvia Kurtovic [a], Tina T. Ng [a], Ankur Gupta [a], Vaithilingaraja Arumugaswami [a,b], Kira L. Chaiboonma [a,b], Mohammad Amin Aminzadeh [c], Raj Makkar [c], Donald C. Dafoe [a,b,*], Dodanim Talavera-Adame [a,b,**]

[a] Comprehensive Transplant Center, Department of Surgery, Cedars-Sinai Medical Center, 8700 Beverly Blvd., Los Angeles, CA 90048, USA
[b] The Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, 8700 Beverly Blvd., Los Angeles, CA 90048, USA
[c] The Heart Institute, Cedars-Sinai Medical Center, 8700 Beverly Blvd., Los Angeles, CA 90048, USA

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A B S T R A C T

The metabolic regulation of leptin and its angiogenic effects have been well characterized in adult mammals. However, the role of leptin in the differentiation of embryonic stem cells (ESCs) to endothelial cells (ECs) has not been characterized. We hypothesized that leptin enhances the generation of ECs derived from ESCs and, in this way, promotes angiogenesis in embryonic vessels. To address this hypothesis, we utilized an in vitro model consisting of murine ESCs-derived embryoid bodies (EBs). Vascular density, EC and angiogenesis markers as well as phosphorylation levels of signal transducer and activator of transcription 3 (pSTAT3) were investigated in leptin-treated EBs and in untreated EBs as controls. ESC-derived ECs were isolated by magnetic sorting based on the expression of platelet endothelial cell adhesion molecule (PECAM-1/CD31). Significant upregulation of EC and angiogenic markers as well as higher vessel density were found in leptin-treated EBs compared to controls. CD31 positive enriched cells derived from leptin-treated EBs had improved proliferation and survival rate and showed higher levels of pSTAT3. These results suggested that leptin promotes EC differentiation and angiogenesis in mouse EBs and that janus tyrosine kinase (JAK)/STAT pathway can play a role in this biological process. Leptin-mediated EC differentiation and angiogenesis in ESCs can be a useful application towards regenerative medicine and tissue engineering.

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Introduction

Leptin is a 16-kDa product of the obese (ob) gene with multiple biological effects (Coppari and Bjørbæk, 2012; Harvey, 2003; Margetic et al., 2002; Morioka et al., 2007; Yang and Barouch, 2007). Leptin plays an important role in the regulation of food intake and body weight in humans (Friedman and Halaas, 1998). Leptin has also been shown to have a potent angiogenic effect which is essential for tissue regeneration (Sierra-Honigmann et al., 1998). Through the use of computer-assisted image-analysis software, we characterized the angiogenic effects of leptin in quail chorioallantoic membranes (CAMs) and concluded that leptin promotes blood vessel growth in CAMs through a mechanism known as intussusception or splitting angiogenesis (Talavera-Adame et al., 2008). In addition, it has been reported that leptin-treated human endothelial progenitor cells form tube-like structures in vitro via the long form of leptin receptor (Wolk et al., 2005). Therefore, leptin promotes not only angiogenesis but also blood vessel remodeling (Talavera-Adame et al., 2008). Endothelial cells (ECs) and other cells respond to leptin via leptin membrane receptors (Wolk et al., 2005). The leptin receptor (Ob-R) was first characterized by Tartaglia et al. (1995). Leptin receptor is encoded by the diabetes gene (db) and six isoforms of this receptor have been identified (Chen et al., 1996; Harvey, 2003; Margetic et al., 2002; Tartaglia et al., 1995). These isoforms are generated by alternate splicing (OB-R_{a-f}) in mice (Harvey, 2003). Most of these isoforms are membrane-spanning proteins with extracellular and intracellular domains (Chen et al., 1996). The extracellular domains are identical for all leptin isoforms (Lee et al., 1996). In contrast, the intracellular domain can be short and define the short forms of leptin receptor (OB-$R_{a,c,d,f}$) while another isoform has longer intracellular domain and it is known as the long form of leptin receptor (OB-R_b) (Harvey, 2003; Tartaglia et al., 1995). Another form of leptin receptor named OB-Re has no transmembrane domain and circulates

* Correspondence to: D. C. Dafoe, the Board of Governors Regenerative Medicine Institute &, Comprehensive Transplant Center, Department of Surgery, 8635 W. Third Street, Suite 590W, Los Angeles, CA 90048, USA. Fax: +1 310 423 3864.
** Correspondence to: D. Talavera-Adame, the Board of Governors Regenerative Medicine Institute & Comprehensive Transplant Center, Department of Surgery, Cedars-Sinai Medical Center, 8700 Beverly Blvd., AHSP A8104-R, Los Angeles, CA 90048, USA. Fax: +1 310 248 8066.
E-mail addresses: donald.dafoe@cshs.org (D.C. Dafoe), Talaverad@cshs.org (D. Talavera-Adame).

as soluble receptor (Kratzsch et al., 2002). Apparently, OB-R$_b$ leptin receptor has a major role in food intake in mammals since its mutation may result in the obese phenotype in mice (Harvey, 2003; Margetic et al., 2002; Tartaglia et al., 1995). In general, leptin receptor has homologies with the class I cytokine receptor superfamily including interleukin-6 receptors (IL-6R), leukemia inhibitor factor (LIF), and granulocyte-colony stimulating factor (G-CSF) (Harvey, 2003; Tartaglia et al., 1995). Activation of leptin receptor causes phosphorylation of janus tyrosine kinase 2 (JAK2) which in turn activates signal transducers and activators of transcription (STAT) proteins. JAK2 phosphorylates leptin receptor and activates STAT3 that translocates to the nucleus and function as transcription factor that targets multiple genes (Banks, 2000; Harvey, 2003; Margetic et al., 2002). Most of the leptin effects in ECs are exerted through this JAK/STAT pathway and have been evaluated mainly in adult tissues and organs (Margetic et al., 2002). With the emergence of embryonic stem cells (ESCs) that can be maintained undifferentiated in culture, the role of hormones such as leptin in EC differentiation and blood vessel development can be explored (Thomson, 1998). When these ESCs are plated in suspension, they form structures composed with the all three germ layers: ectoderm, mesoderm, and endoderm and are called embryoid bodies (EBs) (Karbanová and Mokrý, 2002). The mesoderm gives rise to several structures including the cardiovascular system (Talavera-Adame et al., 2013). Then, ECs can be derived from ESCs (Levenberg et al., 2002). The function of these derived ECs has been also evaluated in vivo (Huang et al., 2010). In addition, the effects of angiogenic molecule such as erythropoietin in cardiovascular enhancement in embryoid bodies have been also described (Müller-Ehmsen et al., 2006). It has been reported that leptin is crucial for embryo pre-implantation as well as the epithelial to mesenchymal cellular transition necessary for cardiac valve development, and adipocyte differentiation (De Vos et al., 1996; Herrid et al., 2006; Kallen and Lazar, 1996; Nath et al., 2008). Furthermore, the role of leptin in early hematopoietic differentiation has also been described (Mikhail et al., 1997). Apparently, endothelium interactions with different tissues during development is crucial for adequate organogenesis (Lammert et al., 2001; Matsumoto et al., 2001; Nikolova and Lammert, 2003; Talavera-Adame et al., 2011, 2013). In this work we used ESCs as an in vitro model to characterize leptin inductive effects in EC differentiation and embryonic angiogenesis.

Materials and methods

Cells and reagents

Mouse ESC line R1 (from [strains 129/Sv × 129/Sv-CP] F1 3.5-day blastocyst) (Samuel Lunenfeld Research Institute, ON, Canada) passages 20–25 were plated on Mitomycin C (Sigma, St. Louis, MO)-inactivated mouse embryonic fibroblasts (MEFs) (ATCC, Manassas, VA) as feeder layers. The culture medium for cell maintenance consisted of high glucose Dulbecco Modified Eagle Medium (DMEM-H) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Omega Scientific Inc., Tarzana), 1 mM sodium pyruvate, 0.1 mM non-essential amino-acids, 200 μM L-glutamine (Invitrogen, Grand Island, NY), 1000 U/mL leukemia inhibitor factor (LIF) (Chemicon, Temecula, CA) and 100 μM β-mercaptoethanol (Sigma, St. Louis, MO). MEFs were grown at 37 °C under 5% CO$_2$ in DMEM-H (Invitrogen, Carlsbad, CA) supplemented with 15% FBS (Omega Scientific, Tarzana, CA). To induce formation of embryoid bodies (EBs), mouse ESCs (R1) were cultured in hanging drops after disaggregating with Accutase (Innovative Cell Technologies, San Diego, CA). Six hundred cells were plated in each drop of 20 μL hanging on the lid of a petri dish for two days in DMEM-H supplemented with 20% FBS (Omega Scientific Inc., Tarzana, CA). After this time, complete media were added to the cells to keep them in suspension for an additional three days for EB formation (EBs day 5).

Leptin treatment

Recombinant mouse leptin (ProSpec, East Brunswick, NJ) was added directly at 1 nM or 10 nM concentration to the media consisting in DMEM-H supplemented with 10% KO-serum replacement, 0.1 mM non-essential amino-acids, 200 μM L-glutamine (Invitrogen, Grand Island, NY), and 100 μM β-mercaptoethanol (Sigma, St. Louis, MO) used for EB maintenance. Leptin was added to EB groups at 5 days of development for additional 10, 20, and 30 days respectively. The media were replaced with fresh media with leptin every three days. After these time points the EBs were harvested for analysis or cell sorting.

Immunocytochemistry and FACS

For immunocytochemistry, the EBs plated on coverslips treated or untreated with 1 nM leptin for 10 days were fixed with paraformaldehyde 4% (Polysciences, Inc., Warrington, PA) at different time points and permeabilized with 0.3% Triton X-100 in PBS for 5 min. After rinsing with PBS, cells were blocked with 5% BSA in 1× PBS for 1 h and then exposed overnight using primary antibodies to PARP (ab3565; dil. 1:100), Ki-67 (Millipore, Billerica, MA; dil. 1:100), PECAM-1/CD31 (BD Biosciences Pharmingen, San Diego, CA; dil. 1:10), pSTAT3 (Cell Signaling Technologies, Danvers, MA; dil. 1:100), angiopoietin 2 (Ang2; dil. 1:500), angiopoietin 1 (Ang1; 10 μg/mL), fetal liver kinase 1 (FLK-1; dil. 1:100), fms-related tyrosine kinase 1 (FLT-1; dil. 1:50), tyrosine kinase with immunoglobulin-like and EGF-like domain 1 (TIE1; dil. 1:10), TEK tyrosine kinase (TIE2; dil. 1:25), leptin receptor (Abcam, Cambridge, MA; dil. 1:100), mouse IgG1, rat IgG2a, and rabbit IgG (isotype controls; Santa Cruz, Biotechnology, Inc., Santa Cruz, CA; dil. 1:100). The secondary antibodies used were as follows: Alexa Fluor 555 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rat IgG, Alexa Fluor 555 goat anti-rat IgG, and Alexa Fluor 555 goat anti-rabbit (Molecular Probes, Eugene, OR). All the secondary antibodies were diluted 1:1000 in blocking solution (5% BSA in 1× PBS). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) contained in a ProLong Antifade solution used to mount the coverslips (Life Technologies, Grand Island, NY). Images were acquired with a multi-purpose zoom microscope (Nikon AZ 100, USA; http://www.nikon.com/) attached to a DS-Qi1 high-sensitivity CCD camera (http://www.nikon.com/) and analyzed using an imaging software NIS-Elements AR 3.10 (Nikon Instruments, Melville, N.Y.) and the image tools of ImageJ 1.30v software (Wayne Rasband National Institutes of Health; USA). Another group of images was acquired with a TCS SP5 X confocal microscope (Leica Microsystems, Mannheim, Germany).

For FACS analysis, about 1×10^6 cells were obtained from EBs either untreated or treated with 1 nM leptin for 10 days. The cell suspension was incubated for 30 min in blocking solution (1% BSA in 1× PBS) with rat anti mouse-CD31 in ice. After this time, the cells were washed several times and treated with the secondary antibody Alexa Fluor 488 goat anti-rat for 30 min in ice. After several washes, the cells were resuspended in 500 μL and analyzed by flow cytometry using BD LSRFortessa (BD Biosciences, San Jose, CA).

Image analysis

For image binarization, the images of the EB blood vessels were captured using a SPOT camera RT-KE slider 7.4.2 attached to a Nikon Eclipse TE2000-S fluorescent microscope (Diagnostic Instruments, Inc., Sterling Heights, MI). The SPOT v4.6 software (Diagnostic Instruments, Inc., Sterling Heights, MI) was used to obtain the higher resolution images that were analyzed using the image tools of ImageJ software 1.37v (Wayne Rasband National Institutes of Health; USA). The photos were binarized using the image tools of ImageJ software 1.37v (Wayne Rasband National Institutes of Health; USA).