Mature Adipocytes and Leptin Influence Angiogenic Processes

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Abstract

The process of wound healing requires the transportation of substrates needed for the rebuilding of tissue through vessel formation from pre-existing vessels, i.e., angiogenesis. Adipose tissue extract is often used to facilitate the wound healing process, which is attributed to its potent angiogenic properties. Most angiogenic processes are regulated through growth factors such as vascular endothelial growth factor, angiopoietin, fibroblast growth factor, and leptin. Leptin is predominantly synthesized and secreted by adipocytes; therefore, it was hypothesized that mature adipocytes augment angiogenic processes in dispersed adipose tissue, which is attributed to leptin. Subcutaneous adipose tissue was collected from 4 prepubertal female pigs (gilts) at 148 + 12 days of age weighing 80 + 6 kg and enzymatically dispersed (collagenase type II). Cells were separated into 2 groups 1) mature lipid-filled adipocytes (MA) and 2) all other cell types (AOC; vascular endothelial cells (VEC), preadipocytes, fibroblasts). The AOC were incubated for 24 hr in cell culture media with 10% fetal bovine serum (FBS) to promote attachment, which conditions the environment to promote the morphological changes required for vessel tube formation. After the AOC attachment period, media was removed and replaced with MA in culture media with or without leptin (10-11 M) or rabbit polyclonal leptin antibody (1:100 dilution) and incubated for an additional 24 hrs. The MIXED procedure of SAS was utilized to determine the effect of treatment on angiogenic cellular processes in-vitro. The addition of MA stimulates (P<0.05) the progression in each stage of angiogenesis; however, leptin antibody mitigated the effect of MA on vessel tube formation. Although exogenous leptin did not augment morphological progression of vessel tube formation, leptin antibody suppressed VEC elongation and vessel tube formation (P<0.05). Mature adipocytes stimulate VEC angiogenic progression, which is attributed, in part, to endogenous adipocyte leptin production.

Introduction

Wound healing is an intricate process that is initiated after a trauma transpires and is executes in 3 stages: inflammation, proliferation, and remodeling (1,2). The inflammatory stage includes coagulation of blood and an influx of leukocytes to the trauma site to protect against infection. The proliferative stage is responsible for wound closure, which includes fibroplasia, re-epithelialization, and angiogenesis. In the remodeling stage, the trauma site is restructured to resemble the original tissue organization. Critical to all 3 stages is appropriate re-vascularization (angiogenesis), which transports nutrients, cytokines, leukocytes, and waste to and from the wound site. Adipose tissue extracts applied to wounds has been reported to accelerate the wound healing process (3). Adipose tissue wound healing properties are likely attributed to one or more factors such as multiple growth factors, stem cells, and dense vascularization. Leptin is a predominant hormone and is synthesized and secreted by MA's. Leptins numerous properties include being a main adipokine, possessing inflammatory factors, and inducing the synthesis and secretion of proteases and adhesion molecules needed for the progression of angiogenesis (4).

Materials & Methods

- **Animals**
  - Four crossbred (Yorkshire x Hereford x Hampshire) pre-pubertal female piglets, ~150d of age.
- **Tissue Collection**
  - Piglets were anesthetized and subcutaneous adipose tissue (3 grams) was harvested from the cervical vertebral region.
  - Tissue Transport: Harvested adipose tissue were placed in pre-warmed (37°C) Hanks solution (HyClone; Logan, UT) for transportation. Adipose tissue was enzymatically digested for cell culture.

Materials & Methods

- **Cell Preparation**
  - **Cell Isolation**: Tissue was digested in phenol-free Dulbecco's Modified Eagle's Medium Nutrient Mixture F12 Ham (DMEM; Sigma Aldrich; St. Louis, MO) with Type II Collagenase (1.5 mg/ml; Sigma) in a vigorously shaking water bath at 37°C for 60 min.
  - **Cell separation**: vascular endothelial cells (VEC), fibroblasts and preadipocytes (all other cells; AOC) were separated from mature adipocytes (MA, floating cells) for pre-treatment culture.
- **Cell Culture:**
  - **Pre-treatment Cultures:**
    - AOC Attachment cultures:
      - AOC (~8.0 x 10^5 cells/well) were cultured in triplicate for 24 hr in attachment media [phenol-free DMEM, 10% charcoal stripped fetal bovine serum (CFBS, Hyclone), 0.1 mg/ml streptomycin, 100 U/ml penicillin, 2.5 mM L-glutamine (Hyclone)] at 37 ºC in an atmosphere of 5% CO_2 in air with 95% humidity to promote cellular aggregation and attachment.
      - Following 24 hrs, cultures were analyzed for evidence of angiogenic progression.
    - MA Acclimation culture:
      - MA (~3.0 x 10^5 cells/well) were cultured in triplicate in standard non-attachment media [phenol-free DMEM, 0.1 mg/ml streptomycin, 100 U/ml penicillin, 2% CFBS, 1.5% bovine serum albumin, 2.5 mM L-glutamine] at 37 ºC in an atmosphere of 5% CO_2 in air with 95% humidity for 24 hr to acclimate cells to culture conditions prior to treatment.
  - **Treatment Cultures:**
    - Attachment media was removed from AOC cultures and replaced with standard media. MA cells were added to AOC cultures w/without treatment. Treatments were 1) 0M (control, media only), 2) porcine Leptin (10^{-11}, Cell Sciences), or 3) rabbit polyclonal Leptin Antibody (Leptin Ab, Thermo Fisher). Antibody treatment was utilized to inhibit endogenous Leptin to confirm that MA influenced angiogenesis through Leptin. Cells were incubated for 24 hr under the same incubation conditions. At termination, angiogenic progression was documented.
- **Angiogenic Progression Analysis**
  - **Evidence of VEC angiogenic progression:**
    - VEC were categorized relative to the state of progression: *Elongation* (elongated and tapered on 2 ends), *Sprouting* (cytoplasmic pseudopod projections, 3-or more), *Nascent Tube* (sprouts from one VEC connecting to a sprout from another VEC), *Tube formation* (cylinder structure resembling the beginning formation of a lumen).

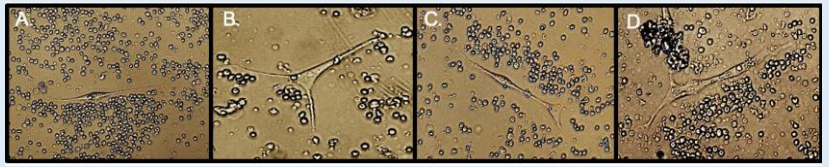

Figure 1. Representative pictures of cells during angiogenic progression. A: Elongation. B: Sprouting. C: Nascent Tube. D: Tube formation

- **Statistical Analysis**:
  - The MIXED procedure of SAS was utilized to determine the effect of MA addition on angiogenic progression and the effect of Leptin and Leptin Ab on angiogenic progression. The PDIFF option was conducted to identify significant differences among treatment means.
  - All means are reported as LS MEANS±SEM.

Results

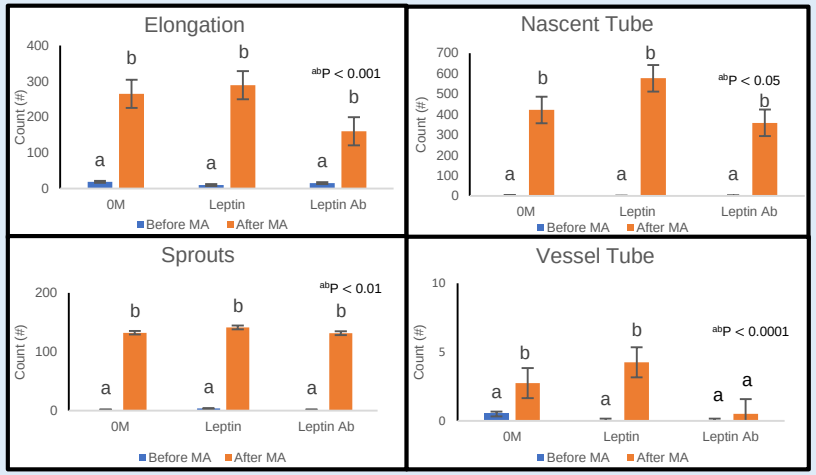

Figure 2. Angiogenic progression of cells with addition of MA and with or without Leptin or Leptin Ab treatment (n=4). Blue bars represent AOC culture only. Orange bars represent MA added to AOC after 24 hr with or without Leptin or Leptin Ab treatments added. After MA were added elongation, (P<0.01) sprout formation (P<0.05), nascent tubes, and (P<0.0001) vessel tube formation increased.

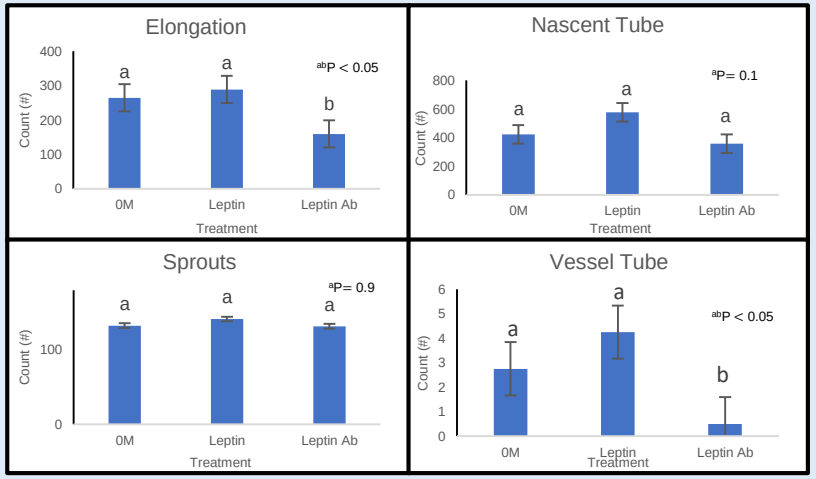

Figure 3. Angiogenic progression of cells after addition of MA only with or without Leptin or Leptin Ab treatment (n=4). Antibody treatment reduced (P<0.05) the number of elongated VEC and total number of vessel tubes formed.

Conclusion

- Addition of MA alone augmented the morphological process of angiogenesis, which may be attributed to endogenous leptin.
  - Leptin Ab appeared to suppress progression of different stages of the process.

Acknowledgements

McNair Staff at Texas A&M University – Kingsville

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