Quantitative and morphometric evaluation of the angiogenic effects of leptin

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Abstract. Angiogenesis is a dynamic process that requires an interaction of pro-and antiangiogenic factors. It is known that the cytokine leptin stimulates endothelial cell growth and angiogenesis, but further quantitative analysis is necessary to understand leptin angiogenic effects. The quail chorioallantoic membrane (CAM) assay has been used to study angiogenesis *in vivo* by focusing on morphometric parameters that quantify vascular complexity and density. We quantify the angiogenic activity of leptin using the CAM assay by digital morphometry and a computer-assisted image analysis to evaluate more precisely vessel length, diameter, branching, and tortuousity. CAM images are obtained from *ex ovo* cultures of E8-E9 quail embryos. MATLAB® and custom software are used for our analysis. The effects of leptin, vascular endothelial growth factor-165 ($VEGF_{165}$), and their corresponding neutralizing antibodies are compared. Our results show that CAM treated with leptin and $VEGF_{165}$ has a significant increase in vascular complexity and density. A corresponding decrease is observed using neutralizing antibodies. Notably, leptin induced more significant changes than VEGF in vessel length and tortuousity. Conversely, VEGF induced a greater increase in vessel branching than leptin. These results underscore the importance of using multiparametric quantitative methods to assess several aspects of angiogenesis and enable us to understand the proangiogenic effects of leptin.
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1 Introduction

The formation of new blood vessels, angiogenesis, is a complex biological process that requires a successful interaction between many cellular and molecular components to generate mature, stable, and functional blood vessels.[1] This vascularization process is of paramount importance in both disease and normal states.[2,3] To accomplish this process, soluble factors stimulate endothelial cells to proliferate, produce molecules that degrade extracellular matrix, migrate, avoid apoptosis, and finally differentiate and organize to form mature and functional new vascular tubes that are stabilized by the recruitment of smooth muscle cells known as pericytes.[4]

Angiogenesis can occur by one of the two known mechanisms: sprouting or intussusception, both resulting in increased vascular branching and vessel density.[5] Different approaches have been used to evaluate angiogenesis.[6] For example, the avian chorioalantoic membrane (CAM) model is one of the *in vivo* assays most widely used to study these biological phenomena.[6–14] The CAM model offers many advantages over other models, including easy access to the blood vessel network, function in low or absence of immunocompetence, shorter time frame for study completion, and no concern because of animal sacrifices. Additionally, this assay is relatively simple and inexpensive, and is suitable for large-scale screening.

It is well known that vascular endothelial growth factor (VEGF) causes an increase in vascular density and branching in the CAM assay, and it is often viewed as the "gold standard" angiogenic factor.[9,13] A quantitative method has been developed[10–14] to evaluate changes in vascular complexity and vascular density in response to angiogenic modulators in the CAM. This method uses computer-assisted image analysis of CAM obtained from *ex ovo* quail embryo cultures. The approach involves two morphometric techniques: (1) assessment of vascular complexity by measuring the space-filling vascular branching pattern with fractal dimension (D_f) analysis and (2) evaluation of vascular density by the grid intersection (ρ) method. The effects of proangiogenic molecules such as basic fibroblast growth factor (bFGF) and antiangiogenic molecules such as angiostatin have been quantified by these morphometric methods.[10,12]

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Discovered more than a decade ago, leptin, a 16-kDa product of the obese (ob) gene[15] is a cytokine with multiple biological effects.[16–22] Leptin is expressed predominantly by adipocytes with an important role in energy balance.[23] It is also known that leptin has mitogenic effects[24,25] and wound healing capabilities by acting as a potential proangiogenic factor on endothelial cells.[26] It was recently demonstrated[27] that leptin upregulates the expression of VEGF as well as its receptor VEGF-R2.

In this paper we explore in detail the proangiogenic effects of leptin on a CAM model using two quantitative image-analysis methods. One method is based on ImageJ 1.30v software, and a second is a multiparametric computer-assisted method that we developed using the image processing toolbox of MATLAB® to measure more precisely other vascular parameters such as branching, length, diameter, and tortuousity.

To quantify vascular density and complexity, we used fractal dimension and horizontal and vertical grid intersection analyses applied to CAM images. VEGF and anti-VEGF antibodies were used in these studies as positive and negative controls, respectively.

The changes induced by leptin were qualitatively and quantitatively different from those induced by VEGF. Our results indicate that leptin increased the vascular density and vascular complexity in the CAM compared to controls. It also had an enhancing effect on tortuousity and overall vascular length in comparison to VEGF. The results reported herein also highlight the importance of measuring multiple parameters in the morphometric assessment of angiogenic modulators, which enabled us to quantify more precisely the proangiogenic effects of leptin and compare these effects with other proangiogenic molecules.

2 Methods

2.1 *Embryo Culture*

Fertilized Japanese quail (*Coturnix coturnix japonica*) eggs (Boyd's Bird Co., Pullman, Washington) were disinfected and incubated at 37 °C under ambient atmosphere until E2.5. They were then opened under sterile conditions in a laminar flow hood, and each embryo was place in one well of a six-well cell culture cluster (Costar Inc., Corning, New York) and cultured further under the same conditions.

2.2 *Embryo Treatment*

Some of the embryos were kept without perturbation to evaluate the normal angiogenesis and these served as baseline controls. Other embryos were treated with phosphate-buffered saline (PBS), mouse leptin 4.0 and 0.4 μg/ml [AFP352C, National Hormone & Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIDDK, NIH)], rabbit polyclonal antibodies to mouse leptin at different dilutions (1:10, 1:100, 1:1000) (lot AFP3011199, National Hormone & Peptide Program, NIDDK, NIH), chicken leptin at different concentrations (0.1, 0.05, 0.025, 0.012, 0.006, and 0.003 μg/ml) (lot AFP8942E, National Hormone & Peptide Program, NIDDK, NIH), and rabbit polyclonal antibodies to chicken leptin at different dilutions (1:500, 1:1000, 1:10000) (lot AFP1082300, National Hormone & Peptide Program, NIDDK, NIH).[†] Human recombinant VEGF-165 ($hrVEGF_{165}$) 500 ng/ml and 5 μg/ml (Genentech, Inc., San Francisco, California) and monoclonal antibodies to $hrVEGF_{165}$ 5 μg/ml (lot 74839, G180CL, Genentech, Inc., San Francisco, California) were used as a positive and negative controls, respectively, in all the experiments. All of the substances used were prepared in a sterile laminar flow hood to the desired concentration and prewarmed at 37 °C. Each treatment was prepared in a total volume of 500 μl and was added gently onto each CAM at E7.

2.3 *Fixation and Processing of the CAM*

After 24 48 h of treatment, the embryos were fixed *in situ* with Karnovsky fixative (16% paraformaldehyde, 25% glutaraldehyde, and 0.2 M sodium phosphate) (Electron Microscopy Sciences, Hatfield, Pennsylvania), and allowed to fix for at least 24 h. The whole embryos were then taken out of their wells and placed in PBS for further dissection to separate the CAM from the rest of the embryo. Each CAM was carefully rinsed with PBS to flush out the blood within the veins. Arterial blood remained inside the vessels after the fixation. Then the edges of the CAM were dissected without affecting the midterminal zone of the arteries. Finally, each CAM was mounted onto a glass slide and covered with a glass coverslip or with Crystal Mount (Biomeda, Foster City, California).

2.4 *Image Analysis*

Images from the midterminal region of the CAM were acquired at 1600×1200 pixels, a magnification of 10×, and a resolution of 1041 pixels/cm with a stereoscopic microscope (MZ 12.5, Leica, Bannockburn, Illinois) attached to a CCD camera (IN1120, Diagnostic Instruments, Inc., Sterling Heights, Michigan). We used two methods to analyze the images. In the first method, we evaluated the vascular complexity and density using the tools of ImageJ 1.30v software (Wayne Rasband National Institutes of Health; United States). Each image was converted to 8-bit binary (black and white) and then the image was skeletonized. In the images taken with our system, each pixel corresponds to 9.6 μm. The skeletonized images were analyzed by fractal dimension (D_f) with the box-counting method.[6] This method overlays the image with a series of square boxes of decreasing size. The number of boxes that contain at least one black pixel is counted. The value of the slope of a squared regression yields D_f value. The D_f value was used as an indicator of vascular complexity that combines branching and tortuousity but does not discriminate among these two parameters. The method of grid intersection was used to evaluate vessel density with the same software[28] (ImageJ, 1.30v). This method takes into account the number of intersections between a skeletonized vessel and a superimposed rectangular vertical and horizontal grid of 64-pixel spacing.

We use a second method to quantify more precisely other vascular parameters such as vessel length, diameter, tortuousity, and branching. This image-analysis method consisted of a program that we developed based on the image process-

[†]All of these hormones and antibodies were kindly provided by Dr. A. F. Parlow, Scientific Director, National Hormone and Peptide Program, Harbor-UCLA Medical Center. Torrance, California 90502 (parlow@humc.edu).

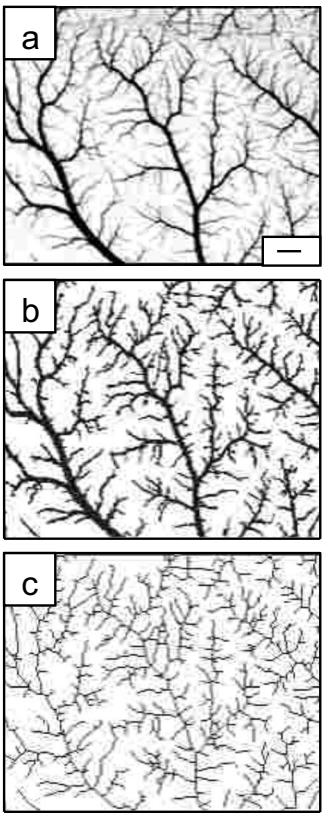

Fig. 1 Image processing of the CAM. The images were taken in a stereoscopic microscope at a 10× magnification. Then, they were transformed to (a) 8-bit gray scale, (b) binarized, and (c) skeletonized images before applying morphometry quantification. Bar=1 mm.

ing toolbox of MATLAB® (The MathWorks, Natick, Massachusetts). An automatic-histogram-derived threshold selection, as proposed by Otsu,[29] was used to obtain the CAM vascular binarization images. A sequential thinning method was then used to skeletonize images from the binarized vascular images (Fig. 1). The distance D_{sb} between each skeleton pixel in the skeletonized image and its nearest background pixel in the corresponding binarized image can be calculated based on Euclidean distances. In this method, the vascular radius of each skeleton/center pixel is equal to the corresponding D_{sb}, thus generating a diameter map. With this map, several measurements were evaluated, includins length, area, and average diameter of the vessels. For example, the length of a branch was defined as the total number of branch pixels after the preceding processing.

We obtained a node-structural map from every skeletonized vascular image. This node-structural map provided a functional tool for reconstructing the total vessel network and we could completely characterize the vascular organization by specifying the connectivity properties of each pixel. We also could evaluate the total number of branches (end nodes) and the degree of branching (furcation/junction nodes) in these regions as well as vessel tortuousity. The vessel tortuousity has been quantified in the vessels of the retina and is defined as the arc to chord ratio[30,31] (see Fig. 4 in Sec. 3.2). The arc length L_{arc} is defined as the length of the vessel section, and the chord length is the Euclidean distance between start point

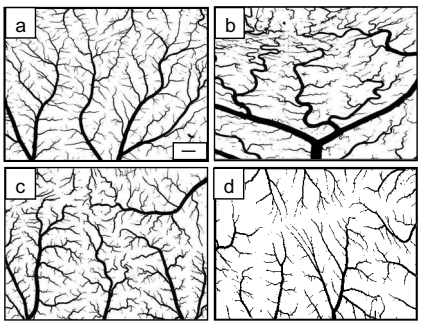

Fig. 2 Effect of mouse leptin on CAM from E8-E9 embryos. Examples of some binarized CAM images captured at 10× of magnification; (a) CAM without any treatment, (b) CAM treated with mouse leptin (0.4 μg/ml), (c) CAM treated with hrVEGF$_{165}$ (500 ng/ml), and (d) CAM treated with antimouse leptin (5 μg/ml). Bar=1 mm.

(S) and endpoint (E) of this vessel section. According the definition, the greater the ratio value, the greater the tortuousity.[30,31]

2.5 *Statistics*

Data are expressed as mean ± standard error from 50 samples for each treatment group of five independent experiments. The statistical significance was assessed by Student's t test.

3 Results

3.1 *Effect of Mouse Leptin, Chicken Leptin, and VEGF on CAM*

Similarities between the chicken and mammalian leptin receptor genes have been reported.[32] We treated the CAM with mouse leptin and chicken leptin (as proangiogenic factors) and the corresponding antimouse leptin and antichicken leptin antibodies (as antiangiogenic factors) to evaluate the angiogenic responses. The hrVEGF$_{165}$ and anti-hVEGF effects were used as controls. The CAM treated with mouse leptin for 24 h showed a clear increase in vascular density, complexity, and tortuousity [Fig. 2(b)] compared to the CAM controls [Fig. 2(a)]. An increase in vessel tortuousity and density was also observed in CAM treated with hrVEGF$_{165}$ [Fig. 2(c)]. Antimouse leptin antibodies clearly diminished the vascular density and complexity [Fig. 2(d)] Quantification of these vascular parameters using morphometric measurements such as grid intersection and fractal dimension are shown in Fig. 3. A significant increase in vessel density and complexity is evident with mouse leptin and hrVEGF$_{165}$ in E8-E9 embryos compared to controls treated with PBS. These effects were totally repressed by antimouse leptin and anti-hVEGF antibodies. Surprisingly, when these antibodies were used directly, and alone, on the CAM, a decrease in these parameters was observed, particularly at higher concentrations.

Table 1 summarizes these results and data from those CAM treated with chicken leptin and antibodies antichicken leptin. A significant increase was observed in vascular complexity and density at lower concentrations (0.025 μg/ml) of chicken leptin compared to mouse leptin (0.4 μg/ml). Those CAM treated with antichicken leptin antibodies alone also showed a significant decrease in vascular density and complexity compared to controls.

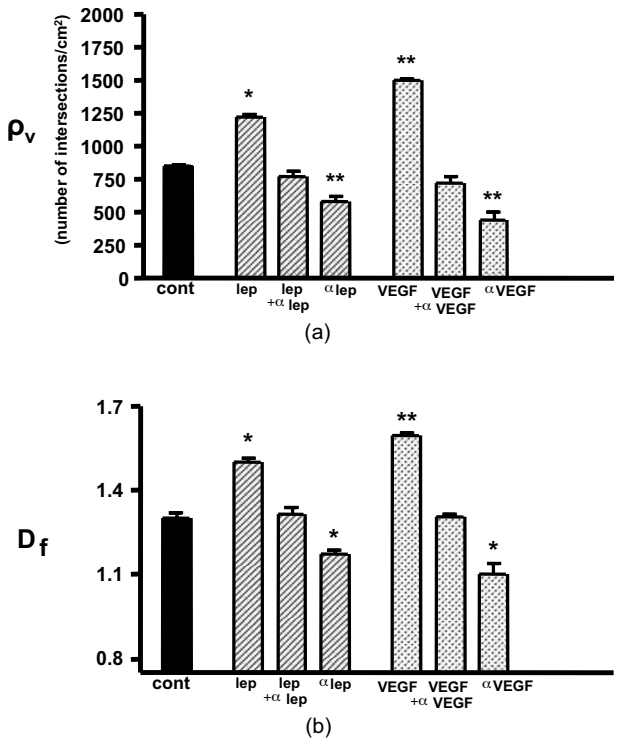

Fig. 3 Quantification of the effects of mouse leptin on CAM using the image tool box of ImageJ, 1.30v. Conventional vascular parameters were applied to CAM images. (a) Vascular density was measured applying grid intersection analysis (ρ_v) and (b) vascular complexity was evaluated using fractal dimension analysis (D_f). Note: Cont, CAM controls without treatment; Lep, CAM treated with mouse leptin, αLep, CAM treated with antibodies antimouse leptin; VEGF, CAM treated with VEGF; αVEGF, CAM treated with antibodies antiVEGF; *, $P<0.05$; and **, $P<0.0001$.

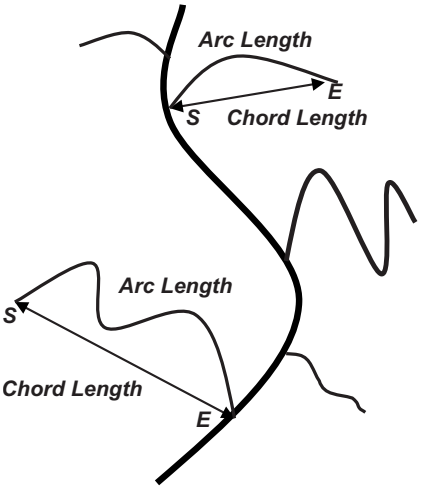

Fig. 4 Evaluation of vessel tortuousity based on the node-structure map. The vessel tortuousity is defined as the arc-to-chord ratio. The arc length (L_{arc}) is defined as the length of blood vessel section. The chord length is the ED between start point (S) and endpoint (E) of the blood vessel. According to the definition, the greater the ratio value, the greater the tortuousity.

3.2 *Other Specific Vascular Parameters Measured on the CAM*

As mentioned, mouse leptin and $hrVEGF_{165}$ increased the vascular complexity and density in CAM. These vascular parameters have been measured previously using morphometric techniques.[6,10–14] To measure other vascular parameters for specific angiogenic changes such as vessel length, vessel diameter, branching, and tortuousity, we designed a new computer-assisted method, similar to the one used by Parsons-Wingerter et al.[13] We used transformed images used in the binarized and skelenotized mode for these analyses. Based on the node-structure map that we developed with the software, we could easily evaluate the vessel length, diameter, branching, and tortuousity.

As mentioned in Sec. 2, the vessel tortuousity is defined as the arc to chord ratio.[30,31] Figure 4 shows an example of two branches with different tortuousity. The arc length (L_{arc}) is defined as the branch length of the vessel and the chord length is the Euclidean distance (ED) between start point (S) and endpoint (E) of that branch. By this definition, the radio (L_{arc}/ED) will be greater in those vessels that exhibit more tortuousity.

Figure 5 shows the results of the image quantification of the vascular parameters already mentioned in those CAM treated with mouse leptin, antimouse leptin, $hrVEGF_{165}$ and anti-hVEGF. Table 2 summarizes the results obtained using chicken leptin, antichicken leptin, $hrVEGF_{165}$, and anti-

Table 1 Quantification of the effects of chicken leptin, antichicken leptin, $hrVEGF_{165}$, and anti-hVEGF (controls) on the quail CAM using conventional vascular parameters.

Vascular Parameter	PBS	Chicken Leptin	Antichicken Leptin	VEGF	Anti-VEGF
Vascular complexity[a] (fractal dimension)	1.36±0.014	1.53±0.05*	1.1±0.02**	1.63±0.06*	1.29±0.02*
Vascular density[b] (grid intersection)	795±22	1231.3±38**	534±11**	1501.6±12**	578.9±45**

[a]The vascular complexity corresponds to the value of the least-squares regression slope of the plot of log $N(p)$ versus log p [where $N(p)$ is the number of boxes that contain at least one pixel in the box-counting method].
[b]The vascular density is expressed in the number of intersections/cm^2.

Each value represents mean±SEM (standard error of the mean); $n=50$ samples for each treatment group; *, $P<0.05$; **, $P<0.001$.

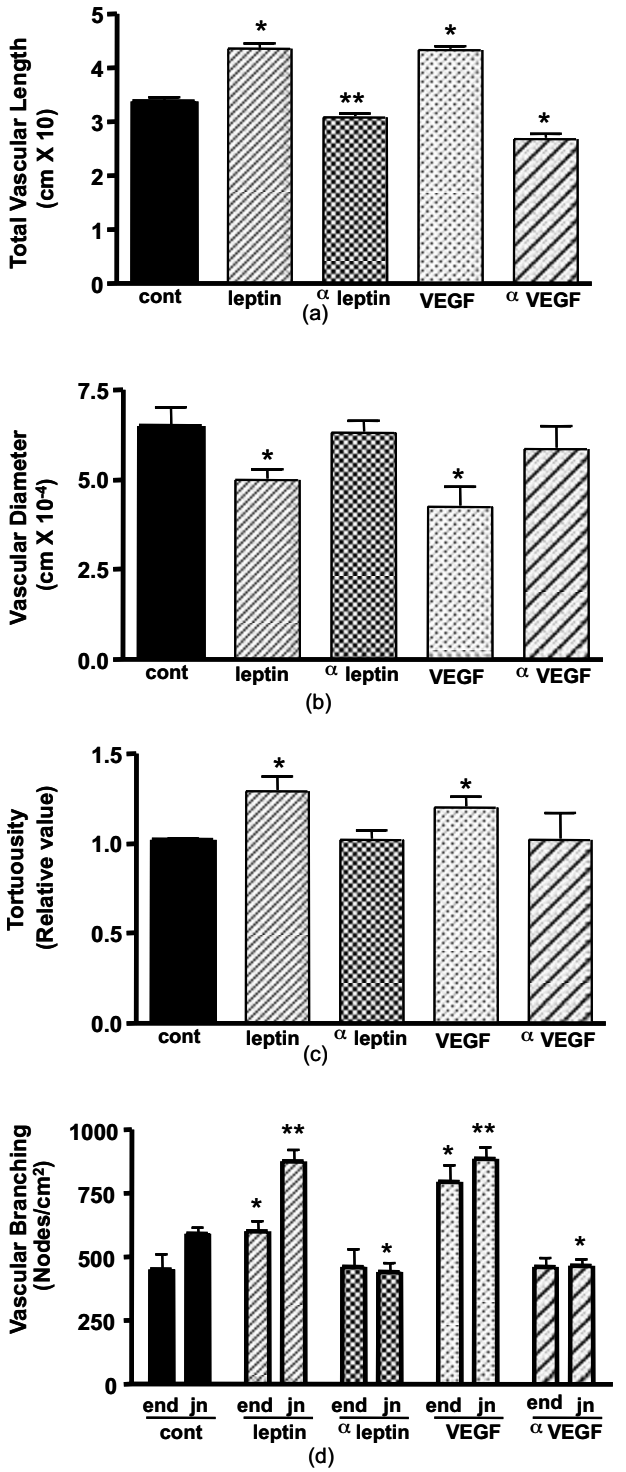

Fig. 5 Quantification of specific vascular parameters on CAM treated with different angiogenic and antiangiogenic factors using the image processing toolbox of MATLAB®: (a) total vessel length, (b) vascular diameter, (c) tortuousity, and (d) vascular branching (end and furcation branch number) were measured with this method (*, $P<0.05$; **, $P<0.001$).

hVEGF on CAM. Mouse leptin increased the total vascular length [Fig. 5(a)] along with a decrease in vessel diameter [Fig. 5(b)]. Similar results were observed in those CAM treated with $hrVEGF_{165}$. Antimouse leptin and anti-hVEGF antibodies significantly decreased the vascular length without any modification in the vascular average diameter [Figs. 5(a) and 5(b)]. Chicken leptin also increases the total vascular length and decreases the average diameter of CAM blood vessels (Table 2). Antichicken leptin affects only the total vascular length.

A tortuousity pattern was evident in most of the first-, second-, and third-generation blood vessels treated with mouse leptin and $hrVEGF_{165}$ [see Figs. 2(b) and 2(c)]. A increase in tortuousity was observed in CAM treated with mouse leptin, chicken leptin, and $hrVEGF_{165}$ [Fig. 5(c) and Table 2]. The vessel tortuousity was more dramatic in those CAM treated with mouse and chicken leptin [Fig. 2(b), Table 2]. Antimouse leptin, anti-hVEGF, and antichicken leptin antibodies had no effect on vessel tortuousity [Fig. 5(c), Table 2].

A drastic effect in the formation of end nodes, which represent terminal branches, and furcation nodes, which represent emerging branches, was observed after treatment with mouse leptin, chicken leptin, and $hrVEGF_{165}$ [Fig. 5(d), Table 2]. Antimouse leptin, antichicken leptin, and anti-hVEGF antibodies produced a significant decrease, however, in the number of furcation nodes. No changes were observed in the number of end nodes.

4 Discussion

The formation of new and functional blood vessels is a dynamic process that requires the participation of factors and cells that interact harmoniously.[4] Because angiogenesis is critical in both normal and pathological conditions, it is essential to use new methodologies for quantification of the effects of pro- and antiangiogenic factors for a deeper understanding of their possible role *in vivo*. Many *in vitro* and *in vivo* models have been proposed to study the angiogenic responses to several angiogenic factors and cytokines.[6,7,10–14,33]

Angiogenesis can occur by sprouting and/or intussusception.[5] These mechanisms increase the complexity and the number of branches of the developing vessel network.[5] It has been demonstrated that leptin is able to stimulate human endothelial cell proliferation and stimulate angiogenesis *in vivo* and *in vitro*.[26,34] Leptin is a circulating hormone that regulates adipose tissue mass through hypothalamic effects on satiety and energy expenditure.[35] Whereas adipose tissue is considered to be the key site for leptin production, this cytokine is also produced in actively angiogenic tissues such as placenta and fetal tissues such as heart, bone, and hair follicles, suggesting an important role in neovascularization in these tissues.[36] However, the mechanisms through which leptin increase the vascular density and complexity during angiogenesis are not fully characterized.

In this work, we designed a program based on the image-processing toolbox of MATLAB® to measure additional vascular parameters such as vessel length, diameter, branching, and tortuousity to study in more detail the effects of mouse leptin, chicken leptin, $hrVEGF_{165}$, and neutralizing antibodies against these cytokines on a CAM model.

In this paper, we have demonstrated that leptin induces an increase in vascular complexity and density. The proangiogenic effects of leptin quantified in our CAM model may be the result of a direct effect of leptin in vascular receptors,

Table 2 Quantification of the effects of chicken leptin, antichicken leptin, hrVEGF$_{165}$ and anti-hVEGF (controls) on quail CAM using other specific vascular parameters.

Vascular Parameter	PBS	Chicken Leptin	Antichicken Leptin	VEGF	Anti-VEGF
Total vascular length[a]	32±0.77	45±0.86**	26±1.06**	42±0.75**	25±0.77**
Average vascular diameter[a]	62±5	44±3*	60±4	40±5*	57±6
Tortuousity[b]	1.4±0.10	1.9±0.14*	1.4±0.17	1.7±0.26	1.2±0.15
End nodes[c]	454±60	680±30*	378±62	831±58**	453±30
Furcation nodes[c]	604±22	907±23**	377±20**	907±15**	453±28*

[a]Values expressed in centimeters. Only for the average vascular diameter the values are expressed in centimeters $\times 10^{-4}$.
[b]Values expressed in relative units (see Sec. 2).
[c]Values expressed in number of end or furcation nodes per square centimeter. The total area analyzed in each image was 76 cm^2.

Each value represents mean±SEM; $n=50$ samples for each treatment group, *, $P<0.05$; **, $P<0.001$.

and/or an indirect effect through the activation of VEGF or a synergistic activity with VEGF or bFGF (Refs. 37–39). The increase in these parameters may be made possible by two known mechanisms: sprouting and intussusception.[5] The increase in vascular length along with the decrease in vascular diameter support the idea of an intussusception mechanism exerted by leptin in a quail CAM model. This nonsprouting angiogenesis has been demonstrated[40] in chicken CAM. This idea is also supported by the fact that antileptin and anti-VEGF antibodies decreased the junction nodes (sites where intussusception may take place for division of larger branches) without affecting the end nodes (distal terminal branches growing by a possible sprouting mechanism). Leptin and hrVEGF$_{165}$ increased the end node number, suggesting an angiogenic effect by the sprouting mechanism. However, the increase in the end node number was more dramatic with hrVEGF$_{165}$, suggesting that this cytokine stimulates the sprouting mechanism more than leptin. Therefore, it is possible that leptin stimulates angiogenesis through the intussusception mechanism, while VEGF may increase density and complexity by both intussusception and sprouting processes. The sprouting mechanism implies endothelial cell proliferation, which has been widely demonstrated[41] as an effect of VEGF.

Neutralizing antibodies, antimouse leptin, and antichicken leptin inhibited the vascular density, complexity, and length and had no effect on vascular diameter. This fact suggests that the antibodies may inhibit the intussusception mechanism without affecting the sprouting mechanism, which could be regulated for more than one proangiogenic factor. This also suggests[40] that intussusception may be the main angiogenic process during the development of a nondisturbed CAM.

The vascular density inhibited in normal CAM using antileptin suggests a possible role of endogenous leptin during normal maturation of the vascular network. This antileptin effect could also affect proangiogenic molecules that act synergistically with leptin such as VEGF (Refs. 13, 37, and 39).

Most of the CAM treated with leptin showed a change in the vessel morphology. The tortuousity induced by leptin was more evident than that observed after treatment with hrVEGF$_{165}$. These alterations induced by leptin will require further analysis to understand the biological significance; however, it is possible that this morphology is the result of the effects of molecules that modify the extracellular matrix.[42] This fact also suggests a direct effect of leptin not mediated by VEGF since treatment with this cytokine was less dramatic in vessel tortuousity.

In this work, a node-structural map was developed that can be easily derived from skeletonized images. Based on such node-structural maps and thickness maps, angiogenically significant multiparameter measurements such as morphometrics, structure, and branch generation can be implemented and evaluated. In addition, the quantitative method described here was used as an important tool to discriminate between effects of proangiogenic and antiangiogenic molecules. Through this analysis, we evaluated some of the proangiogenic effects of leptin *in vivo*. Further studies involving the interactions between this cytokine and other pro- and antiangiogenic molecules will be necessary for a better understanding of its biological effects on the differentiation and maturation of functional blood vessels.

Acknowledgments

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