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The Role of Interleukin-6 in the Formation of the Coronary Vasculature

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Abstract

The formation and the patterning of the coronary vasculature are critical to the development and pathology of the heart. Alterations in cytokine signaling and biomechanical load can alter the vascular distribution of the vessels within the heart. Changes in the physical patterning of the vasculature can have significant impacts on the relationships of the pressure-flow network and distribution of critical growth and survival factors to the tissue. Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates several biological processes, including vasculogenesis. Using both immunohistological and cardioangiographic analyses, we tested the hypothesis that IL-6-loss will result in decreased vessel density, along with changes in vascular distribution. Moreover, given the impact of vascular patterning on pressure-flow and distribution mechanics, we utilized non-Euclidean geometrical fractal analysis to quantify the changes in patterning resulting from IL-6-loss. Our analyses revealed that IL-6-loss results in a decreased capillary density and increase in intercapillary distances, but does not alter vessel size or diameter. We also observed that the IL-6^{-/-} coronary vasculature had a marked increase in fractal dimension (*D* value), indicating that IL-6-loss alters vascular patterning. Characterization of IL-6-loss on coronary vasculature may lend insight into the role of IL-6 in the formation and patterning of the vascular bed.

Keywords

fractal geometry; coronary vasculature; image analyses; Interleukin-6

Introduction

Cardiac vessel distribution and patterning are important for the proper maintenance and function of the myocardium. During cardiac development and exercise-induced physiological hypertrophy, an increase in organ size coincides with a subsequent increase in capillary bed density (Korecky et al., 1982; Rakusan & Korecky, 1982; Hudlicka et al., 1992; Hudlicka & Brown, 1996; Izumiya et al., 2006; Walsh & Shiojima, 2007). In disease states, a characteristic feature of the progression toward pathological hypertrophy is a significant reduction in the capillary density and increase in the intercapillary distance (Korecky et al., 1982; Rakusan & Korecky, 1982; Hudlicka et al., 1992; Hudlicka & Brown, 1996; Karch et al., 2005; Izumiya et al., 2006). Manipulation of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), have also been shown to protect the myocardium against ischemic injury, prevent myocardial apoptosis, preserve contractile function, and delay the onset of pathological

hypertrophy (Friebs et al., 2004, 2006a, 2006b; Izumiya et al., 2006). Taken together, these observations suggest that capillary bed density and patterning are critical for the normal functioning of the heart. Thus, alterations of pro-or anti-angiogenic factors leading to patterning or distribution changes in the vascular bed can have a critical impact on the function of the heart and its ability to withstand pathological insult.

The pleiotropic cytokine, Interleukin-6 (IL-6), regulates several biological processes in a cell-specific manner by binding to the IL-6R α /gp130 signal transduction complex and subsequent activation of signal transducer and activator of transcription-3 (STAT3) pathway (Kamimura et al., 2003; Kurdi & Booz, 2007). In pathological hypertrophy, IL-6 is significantly upregulated both in clinical and mouse models of hypertension (Baumgarten et al., 2002; Vivanco et al., 2005). Moreover, constitutive stimulation of IL-6/sIL-6R α has been shown to attenuate infarct size in murine models (Matsushita et al., 2005). IL-6-gp130-STAT3 signaling has also been implicated in formation of the vasculature. In cancer models and adipose tissue, IL-6 has been shown to stimulate VEGF expression and regulate formation of the vasculature (Adachi et al., 2006; Feurino et al., 2007; Rega et al., 2007). Furthermore, cardiac-specific STAT3-null mice have been shown to decrease capillary density (Hilfiker-Kleiner et al., 2004). These data have led several groups to hypothesize that IL-6 may act in a cardioprotective manner and play a critical role in the formation and patterning of the coronary vasculature.

Alterations in the coronary vasculature in relation to the local parenchyma can be analyzed as semiquantitative changes in the density of capillaries per mm². Although this is useful, it lacks a quantitative understanding of the dynamic patterning of the vascular bed that is critical in studying the effects of the vasculature on the distribution of growth factors and oxygen (Kalliokoski et al., 2003; Anderson et al., 2005; Grizzi et al., 2005). Analyses of the complexity of the vascular bed require the use of noninteger numbers. These noninteger numbers are defined as non-Euclidean, having values falling between two integer topological dimensions and define the fractal dimension (D) of an object (Fuseler et al., 2007). Several studies have utilized computational D values to quantify patterning of bronchial capillaries, capillary branching during angiogenesis, and vascular patterning during exercise training (Kalliokoski et al., 2003; Anderson et al., 2005; Grizzi et al., 2005). Thus, utilizing fractal analyses we can quantify the complexity and the changes in the vascular bed of the heart in response to pathological stimuli, developmental conditions, and other fluctuations within the tissue. In this study, we investigate the hypothesis that IL-6-loss will (1) decrease the density of the coronary vasculature, (2) increase the inter capillary space, and (3) alter the D values of the vessel architecture.

Materials and Methods

Animal Procedures

Animals were humanely sacrificed via cervical dislocation. This investigation conforms to the *Guide for the Care Use of Laboratory Animals* (NIH, 1996). For this study we used IL-6 ($^{-/-}$) mice (Jackson Laboratories, Bar Harbor, ME, USA) and age-matched wild-type (WT) littermates (12–14 weeks) on a C57BL/6 background.

Immunohistochemistry

Freshly isolated hearts were snap frozen in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA) using liquid nitrogen. 7–10 μ m frozen sections were obtained and immunohistochemical staining was performed using the DakoCytomation Animal Research Kit (ARK) (#K3954), as per manufacturer's instructions. Briefly, sections were fixed in ice-cold acetone and blocked at room temperature in 3% H₂O₂. Samples were then stained with biotinylated (Dako ARK Biotinylation Reagent) rat anti-mouse CD31/PECAM (BD

Biosciences, #550274) at room temperature for 45 min. Slides were washed and then incubated with Streptavidin-HRP solution (Dako ARK) for 20 min. Samples were then washed and incubated with DAB+ HRP (Dako ARK) for 5 min. Samples were visualized and analyzed using the Dako Chromavision Systems ACIS 3 microscopy system and associated software. Images were taken of concurrent areas in the left ventricular free wall as well as the intraventricular septa of each animal, three animals per condition. Total CD31 staining was normalized to total nuclei to obtain percentage of CD31 staining.

Cardioangiography

WT and IL-6^{-/-} 12-week-old mice were anesthetized using a ketamine and xylazine cocktail in saline. Coronary vasculature was first perfused with saline supplemented with heparin and then with FluroSpheres Red (580/605) (Molecular Probes, F8801). 42 μ m sections were cut and imaged by confocal microscopy using the Zeiss LSM 510 META microscope (Zeiss, NY). Images were taken of 10 to 15 concurrent areas in the left ventricular free wall as well as the intraventricular septa of each animal, three animals per condition. LSM 510 software was used to take 1 μ m z-slices and reconstruct the three-dimensional image. Reconstructed images were used for subsequent fractal and density analyses.

Image Analyses: Intercapillary Space, Fiber Breadth and Length, Integrated Optical Density, and Fractal Box Analyses

Confocal color images of cardioangiography were first converted to 8-bit monochrome images for both image and fractal analysis. The fluorescence vasculature in an image is defined as a region of interest (ROI) by being thresholded using the “set threshold” subroutine of MetaMorph Image analysis software (v 6.1). The morphological descriptors of fiber breadth are a representative measure of blood vessel diameter. Fiber length is measured using the integrated morphometry algorithm of MetaMorph. Intercapillary space was measured using MetaMorph measure distance tool.

Images were analyzed for fractal analyses and integrated optical density (IOD) as previously described (Grizzi et al., 2005; Fuseler et al., 2006, 2007; Rogers & Fuseler, 2007). Briefly, the IOD of the region of fluorescence delineated by the thresholded boundaries is considered to be the “mass” of the region and is an accurate measurement of the total amount of labeled material in the region (Grizzi et al., 2005; Fuseler et al., 2006, 2007; Rogers & Fuseler, 2007). The IOD was defined as the weighted sum of the image histogram in which each term in the histogram was multiplied by the gray value it represents. When applied to thresholded boundaries, the IOD was defined by the following expression:

$$\text{IOD}(T1, T2) = \sum_{GV=T1}^{T2} H(GV) \times GV,$$

where the upper and lower thresholds defining the ROI in the histogram are given by T1, T2, GV is the gray value of each pixel, and $H(GV)$ is the gray level histogram. Area and IOD measurements were further refined by setting boundary conditions for acceptance of the fluorescent signal from the labeled vascular elements and eliminate any nonspecific and/or background autofluorescence using the software's optical calipers.

Fractal dimension (D) was determined as previously described (Grizzi et al., 2005; Fuseler et al., 2007). Briefly the vascular bed in WT and IL-6^{-/-} cardiac tissues have the appearance of a complex object composed of parts at different levels of resolution (blood vessels of different bore sizes) that are functionally and physiologically similar (self-similar) to the whole object. Under the conditions of these properties, the vasculature bed in WT and IL-6^{-/-} hearts can be considered fractal objects, and their topological dimension, the fractal dimension (D), can be

expressed by a noninteger number lying between two Euclidian integer topological dimensions (Grizzi et al., 2005). The values of D characterizing the vasculature bed in normal and pathological cardiac tissue are therefore fractional.

In applying fractal analysis in WT and IL-6^{-/-} hearts, the box-counting method of determining D was utilized (Glenny et al., 1991; Fernández & Jelinek, 2001; Fuseler et al., 2007). The box-counting method has been utilized to apply fractal analysis to macro- and micromolecular biological structures (Fuseler et al., 2007). The box-counting method consists of a grid of boxes of size e superimposed over the image of the structure, and the number of boxes containing any part of the structure recorded as $N(e)$. A fractal object expresses a straight line when $\log[N(e)]$ is plotted against $\log(1/e)$. The box fractal dimension D_b can be determined from the slope of the regression line; that is, $D_b = \log[N(e)]/\log(1/e)$. The vascular bed of cardiac tissue of WT and IL-6^{-/-} mice satisfied the conditions of being fractal objects, and D_b values were determined using HarFA software (Zmeskal et al., 2001). The HarFA software for the 40X images assigned mesh sizes of boxes with e values ranging from 2 to 179 pixels, and 30 steps within this range were calculated to generate the $\log[N(e)]$ versus $\log(1/e)$ lines to determine D_b . In these 40X oil-immersion images, one pixel is equal to $\sim 0.2287 \mu\text{m}$.

Statistical Analyses

Data obtained from image analyses were measured for significance using student's t -test or ANOVA test with a Mann-Whitney test. Analyses were performed on Sigma-stat software (SYSTAT Software Inc.).

Results

IL-6^{-/-} Hearts Displayed Decreased Coronary Vascular Density

Given that IL-6-loss decreases VEGF production and cardiac specific STAT3-deficient animals display decreased capillary density compared to WT animals, we evaluated the total capillary density in IL-6^{-/-} hearts (Hilfiker-Kleiner et al., 2004; Adachi et al., 2006; Feurino et al., 2007; Rega et al., 2007). Using immunohistological analyses of CD31 area normalized to total nuclei per field, we determined that IL-6^{-/-} animals had significantly decreased CD31 (Fig. 1, $P < 0.01$) when compared to age-matched WT animals (approximately 30% decrease normalized to control). These data suggest that IL-6-loss causes a semiquantitative deficiency coronary vasculature.

IL-6^{-/-} Hearts Displayed Increased Intercapillary Space, but No Changes in Fiber Breath or Fiber Length

Data from this study demonstrated that IL-6-loss resulted in a decrease in vessel density, suggesting that vessel spacing is altered in these animals. Therefore, we performed cardioangiography to visualize the coronary vasculature (Fig. 2). Intercapillary space was increased significantly in the IL-6^{-/-} heart ($11.23 \mu\text{m} \pm 1.79$) when compared to the WT control ($7.82 \mu\text{m} \pm 1.23$) (Fig. 3A, $P < 0.001$). However, fiber breadth and length were not significantly changed between IL-6^{-/-} and WT animals.

IL-6^{-/-} Hearts Were Observed to Have Decreased IOD and Increased D Values

IOD analyses were performed to determine the amount of fluorescent mass in the coronary vasculature of IL-6^{-/-} animals. Similar to CD31 analyses, IL-6^{-/-} hearts ($246,187 \pm 57,908$) had significantly decreased IOD when compared to WT hearts ($313,561 \pm 46,704$) (Fig. 4A, $P < 0.001$). These data underscore that IL-6-loss causes a general decrease in the relative density of the coronary vasculature. Given the indicated decreases in capillary density, IOD, and intercapillary space, we examined the D values in the IL-6-knockout mouse. D values in the

IL-6^{-/-} coronary vasculature (1.69 *D*-value $\pm$ 0.034) were significantly increased when compared to those in WT animals (1.60 *D*-value $\pm$ 0.066) (Fig. 4B, *P* < 0.001).

Discussion

We examined the effects of IL-6-loss on the distribution and patterning of the murine coronary vasculature using both quantitative distributive analyses and fractal box-counting analyses. Alterations in the vascular patterning and distribution are critical to the regulation of myocardial development and pathology. As the heart grows or is challenged by pathological stimuli, the alteration in physical stress causes changes in the both pro- and anti-angiogenic factors that change the pattern, distribution, size, and shape of the vascular bed and the blood vessels themselves (Kassab, 2000; Izumiya et al., 2006; Walsh & Shiojima, 2007). These fluctuations ultimately play a role in the ability of the vessels to distribute growth factors and oxygen to the parenchyma. Understanding the effects of those changes in factors that affect the formation of the vascular bed is critical to the study of cardiovascular pathology and development.

IL-6-Loss Alters Capillary Density and Diminishes Intercapillary Space but Not Fiber Breadth or Length

Previous studies have demonstrated that IL-6 can regulate VEGF expression in a myriad of cancer cell lines and in adipose tissue *in vivo* (Adachi et al., 2006; Feurino et al., 2007; Rega et al., 2007). Furthermore, cardiac-specific STAT3-null animals display decreased coronary capillary density (Hilfiker-Kleiner et al., 2004). Together, these data suggest that IL-6 acts as a pro-angiogenic regulator of coronary vasculature formation and that IL-6-loss will be detrimental to the formation of the vascular bed. Indeed, our data indicate that IL-6-loss attenuates the percentage of CD31 positive cells in the IL-6^{-/-} mouse hearts (Fig. 1). In addition to altering the vessel density, the distance between the capillaries was increased in the IL-6^{-/-} when compared to the WT animal (Fig. 3). Other groups have indicated that both of these are indications of a pathological vascular bed (Rakusan & Korecky, 1982; Hudlicka et al., 1992; Kassab, 2000; Hilfiker-Kleiner et al., 2004; Karch et al., 2005). IOD analyses, which measure the fluorescent “mass” of the angiographic image, indicate that IL-6-loss decreases the amount of the labeled vasculature (Figs. 2, 4A). Further experimentation still is needed to understand exactly what gene patterns IL-6-loss alters in the angiogenic cascade. However, the current data are significant in that they suggest that IL-6 can act in a pro-angiogenic manner in the formation of the coronary vasculature.

Vessel diameter and length can also alter the mechanical properties of the blood vessels and is important in the stress distribution and pressure-flow relationships of transport (Kassab, 2000). Analyses of blood vessel diameter and fiber length of the vessels indicate that the general size of each vessel is not changed in the vascular bed in response to IL-6-loss (Fig. 3B,C). Furthermore, these data suggest that the alterations in capillary density and intercapillary space seen in the IL-6^{-/-} heart are due to decreases in numbers of vessels and not decreases in vessel diameter or length.

IL-6-Loss Increases the D Values of the Vascular Bed

The vascular geometry is critical for understanding both the biomechanical and distributive properties of the cardiac blood vessels. Alterations in the vascular bed can alter local and global pressure flow of the vascular bed (Kassab, 2000). Also, patterning changes can alter the ability to distribute factors to the surrounding cells, as seen in the formation of tumor vasculature (Di Ieva et al., 2008). However, given the irregularity of the vascular bed and its branch patterning, quantifiers such as vessel density and intercapillary space are only semiquantitative in their nature, and fractal analyses must be used to describe the vascular pattern. Our data indicate

that IL-6^{-/-} hearts have an increased *D* value when compared to the WT coronary vascular bed (Fig. 4B). These data suggest that the IL-6-loss alters not only the vessel distribution, but the patterning of the vessels leading to an increase in the chaos in the vessel bed. The changes in the branch patterning also act to alter the ability of the vasculature to distribute growth factors and oxygen to the local tissue environment, possibly compensating for the loss of the vessel density. Furthermore, the morphological irregularity within the IL-6^{-/-} coronary vascular bed indicates a change in local pressures within the vasculature, again indicating possibly causing a change in cell patterning and overall cardiac function.

Summary/Conclusions

The formation and the patterning of the coronary vasculature are essential for the normal function of the heart. Alterations in angiogenic factors can critically alter the balance of this system and have detrimental or beneficial effects. In this study, we characterize several changes in the coronary vasculature of the IL-6^{-/-} mouse. We observed a decrease in capillary density and increase in intercapillary space, suggesting that IL-6 is playing a critical role in the formation of the vascular bed. Furthermore, the *D* value in the IL-6^{-/-} animal is increased suggesting an increase in distributive and biomechanical properties. Given that these animals have a global IL-6 deficiency, it is possible that the observed changes are due to developmental defects. As such further studies of neonatal IL-6^{-/-} animals need to be performed to understand the effects of IL-6-loss on the formation of the coronary vasculature. However, these data taken together underscore the importance of IL-6 in coronary vasculature formation and cardiac function. Further experimentation is under way in our lab to explore the alterations in downstream targets of IL-6 to better characterize the mechanisms behind the changes in the vasculature.

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Figure 1. IL-6-loss decreases CD31 distribution: representative immunohistochemical images of CD31 left ventricular free wall of (A) WT and (B) *IL-6*^{-/-}, respectively. (C) Quantifications of CD31 area versus total nuclear stained area. (# = $P < 0.01$, $N = 3$ animals.)

Figure 2. Representative cardioangiography images: representative reconstructed z-series images of coronary vasculature, perfused with microspheres (red) and stained with DAPI (blue) to visualize nuclei. (A) WT and (B) IL-6^{-/-} (representative of $N = 3$ animals).

Figure 3.

IL-6-loss alters intercapillary space but not fiber breadth or length: **A:** Intercapillary space analyses of *IL-6*^{-/-} and WT cardioangiographic images. **B:** Fiber length analyses of *IL-6*^{-/-} and WT cardioangiographic images. **C:** Fiber breadth analyses of *IL-6*^{-/-} and WT cardioangiographic images. (* = $P < 0.001$, $N = 3$ animals.)

Figure 4.

IL-6-loss decreases IOD and increases *D* values: **A:** IOD analyses of *IL-6*^{-/-} and WT cardioangiographic images. **B:** *D* values of both *IL-6*^{-/-} and WT coronary vascular beds. (* = $P < 0.001$, $N = 3$ animals.)