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Dynamic Study of the Extraembryonic Vascular Network of the Chick Embryo by Fractal Analysis

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Fractal analysis is widely used in many scientific fields, including the study of vascularization. It is a convenient method that defines the complexity of natural structures. The chorioallantoic membrane of the chick embryo is a standard experimental model for the study of vasculogenesis and angiogenesis. The aim of this investigation was to demonstrate that fractal geometry is more appropriate than any other method to describe and analyse the evolution of a vascular network, i.e. the extraembryonar vascular network of the chick embryo. We used an original methodology to evaluate the complexity of this network in the first stages of embryo development (day 3 until 6). We demonstrated an increase of fractal dimension, indicating an increasing complexity of the vascular tree, until an asymptotical value of about 1.70 at day 4. The fractal approach is more accurate than other usual semi-quantitative or quantitative methods evaluating the complexity of a growing vascular tree.

Introduction

Fractal geometry has been widely used to characterize irregular structures (Mandelbrot, 1982), e.g. vascular networks (Vico & Cartilier, 1993; Vico *et al.*, 1994). The degree of complexity is estimated by fractal dimension (D) which may have a non-integer value, and which can be determined, for instance, by the box-counting method (Feder, 1988). Fractal analysis allows the complexity of a bidimensional vascular network to be easily quantified objectively and reproducibly. Current methods used to evaluate this complexity and its eventual variations in physiological or pathological circumstances lack precision or remain semi-quantitative. The aim of the present study was to demonstrate that fractal geometry is more appropriate than any other method to describe and analyse the evolution of a vascular network. Employing our own methodology, we investigated the evolution of a vasculature in the process of formation, i.e. the extraembryonar vascular network of the chick embryo. Several parameters (D, fractal

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dimension; total length of vessels, S_l; total area of the *area vasculosa*, S_t; and vascular density, D_v) were evaluated. The superiority of the fractal approach was demonstrated, and the physiological information it generated is discussed.

Materials and Methods

The chorioallantoic membrane (CAM) of chick embryo has been chosen for methodological and practical reasons. This model is easy to manipulate, inexpensive, well established and widely accepted. It is the largest extraembryonar organ, easily accessible because of its superficiality. It is a bidimensional, vascularized structure, growing in a topologically two-dimensional tissue sheet. Therefore, its D will lie between 1.0 and 2.0 and can be treated easily with standard image analysis. Moreover, the CAM is a standard experimental model for the study of angiogenesis (Folkman, 1974), and tumor graft culture (Ausprunk *et al.*, 1974).

ANIMAL EXPERIMENTS

Chick embryos were grown by the windowed method (Auerbach *et al.*, 1974). Twenty fertilized eggs (*Gallus domesticus*) were incubated in a standard egg incubator at 38°C for 6 days under atmospheric conditions (21% oxygen level). The atmosphere was constantly humidified (100% relative humidity). The eggs were windowed on the second day of incubation, after the shell had been wiped with chlorhexidine and allowed to dry, and after removal of 6 ml albumen. An oval window (approximately 5.0 × 3.5 cm) was created with sterile scissors, and sealed with parafilm to avoid desiccation of the embryos. Under our experimental conditions, the first blood vessels in the CAM were visualized by the 60th hour. The vascular networks were controlled every 8 hours for the

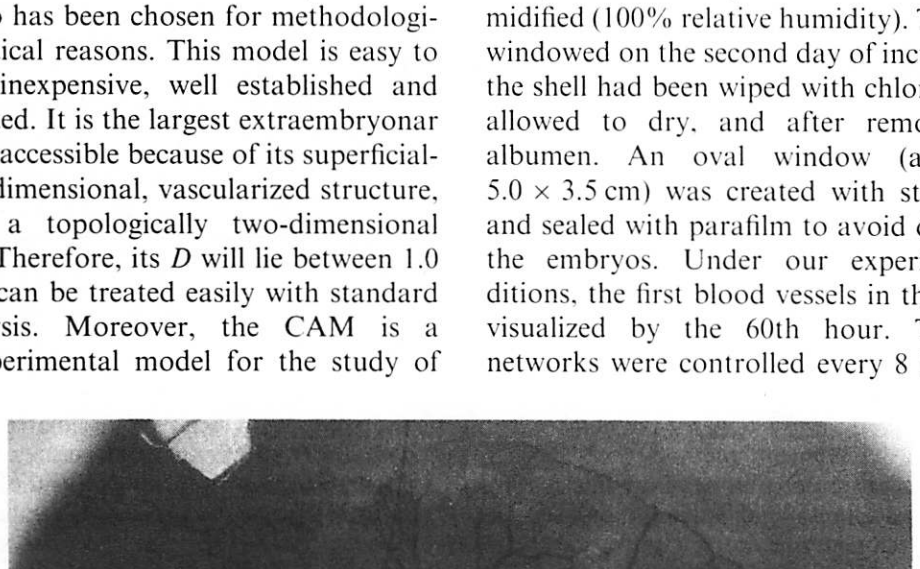

FIG. 1. Chick embryo at 96 hours.

3 following days, and every 12 hours on the last day. Pictures were taken with a Macrolens 60 mm Nikon F801 (Kodak T-Max 100) (Fig. 1). Only nine embryos reached the 142th hour; the others died before this time period and/or were not considered because of incomplete visualization of the vascular network. Vascular networks were hand traced on overhead transparencies for image analysis. No distinction was made between arterial and venous trees.

IMAGE ANALYSIS

Images were grabbed using Neotech Image Grabber software 2.1 (NeotechTM, Eastleigh, U.K.). Gray level images were obtained on a graphic window of 640 × 480 pixels and processed with Ultimage/X 1.41 software (Graftek, Meudon-LaForet, France) on a Quadra 900 Apple Macintosh

II. To extract blood vessels, the images were thresholded for the production of binary images. Thresholding consisted of segmenting images into two regions where the structure and back ground were characterized by two different intensities: pixels belonging to a given gray level interval were highlighted while others became black. An approximately same gray level interval was considered for all images. Then, the images were subjected to an L-skeleton command (Russ, 1990). Skeletons allow representation of the structural shape of an object by reducing its thickness to 1 pixel. L-skeleton uses masks known as structuring elements with an L-shape. As this study was concerned with the spatial distribution of vessels, vessel width was ignored.

FRACTAL ANALYSIS

Classically, the D of a vascular tree is determined by the box-counting method. It relies on the fact that boxes of different sizes used to cover a vessel structure yield different estimates of its length. The equation is $N(\epsilon) = k.\ \epsilon^{-D}$ where ϵ is the box size, $N(\epsilon)$ is the number of boxes of size ϵ needed to cover the structure studied, and D is the fractal dimension. A detailed explanation of the experimental procedure has been published elsewhere (Vico & Cartilier, 1993).

A more efficient box-counting method has been developed recently in our laboratory (Kyriacos *et al.*, 1994). It is based on a modification of the classical computerized box-counting method (Feder, 1988) in which the counting method, the type of step sequence and range of length scales have been defined precisely in order to provide an accurate estimation of D. Briefly, the method consists of dividing the embedding space into a sequence of boxes of decreasing size (ϵ), and counting the number of boxes $N(\epsilon)$ of size ϵ that contain at least 1 pixel of the structure. Thus, using boxes of different sizes to cover an object yields different estimates of $N(\epsilon)$ because the smaller the boxes, the more details of the structure are taken into account. For $\epsilon \to 0$, the relationship, if any, is $N(\epsilon) = k.\ \epsilon^{-D}$ where D is the fractal dimension. D is calculated from the slope of the points (log $(1/\epsilon)$, log $N(\epsilon)$) which normally lie on a straight line, because of the power-law relationship. Counting is performed in a range defined by ϵ_{min} to ϵ_{max} Hence, ϵ_{min} corresponds to a box size larger than the thickness of the structure (in our case, $\epsilon_{min} = 2$) and ϵ_{max} is what gives a box number equal to 2. One important and specific feature of this method is that the step sequence is determined by an arithmetic subset, where repetitions of the same values of $N(\epsilon)$ as well as plateaus are ignored (Kyriacos *et al.*, 1994). It was applied to estimate the D of the human retinovasculature to obtain one of the components used to support existing growth models that have been proposed so far (Kyriacos *et al.*, 1997). Digitization, image processing and fractal analysis required a few minutes for a single vascular network.

Other usual parameters were also evaluated: S_i and D_r which is defined as the ratio between S_i and the total area (S_t) of the *area vasculosa* considered (pixel/pixel).

Results

The first vessels appeared by about the 60th hour. D increased from about 1.30 by the 64th hour to 1.68 by the 112th hour. By the 112th hour, a plateau was reached for $D = 1.70$ asymptotically. The measurement of D is shown in Figs 2 and 3, D being the slope of the log–log plot of $N(\epsilon)$ vs. $1/\epsilon$, for the ascending and plateau phases, respectively. These figures show the excellent linearity of the plots with $R^2 > 0.99$.

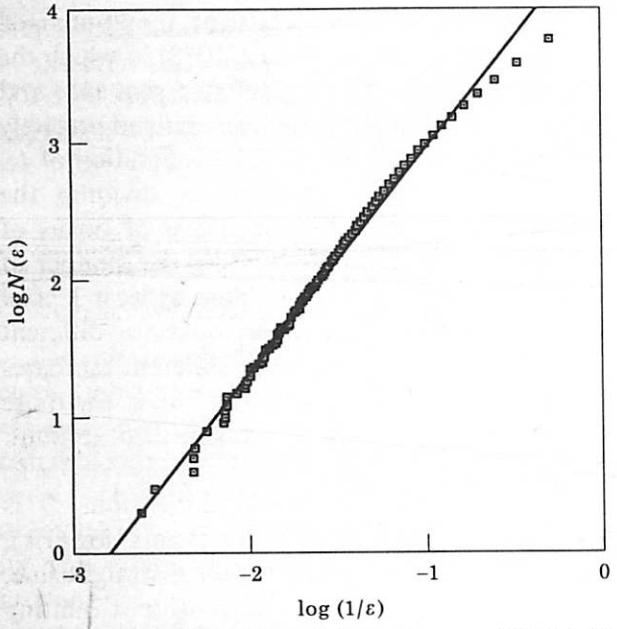

FIG. 2. Estimation of fractal dimension on a CAM at 88 hours. $D = 1.64$; $R^2 = 0.992$.

The measures were widely distributed in the early stages of vasculogenesis, but the distribution became narrower as vasculogenesis developed (Fig. 4). S_i as well as D_r increased linearly with time (Fig. 5). The distribution of S_i and D_r measures remained wide, whatever the stage of development of the vascular tree. When D was expressed as a function of S_i, asymptotical behavior was demonstrated (Fig. 6). Also, D showed a similar asymptotic behavior when related to D_r (Fig. 7). Finally, the coefficient of variation (CV) of each parameter revealed an obvious superiority for D, with a very low value of 2 to 3%, specially when considering this is biological material, in contrast to 15 to 35% for S_i and D_r (Fig. 8).

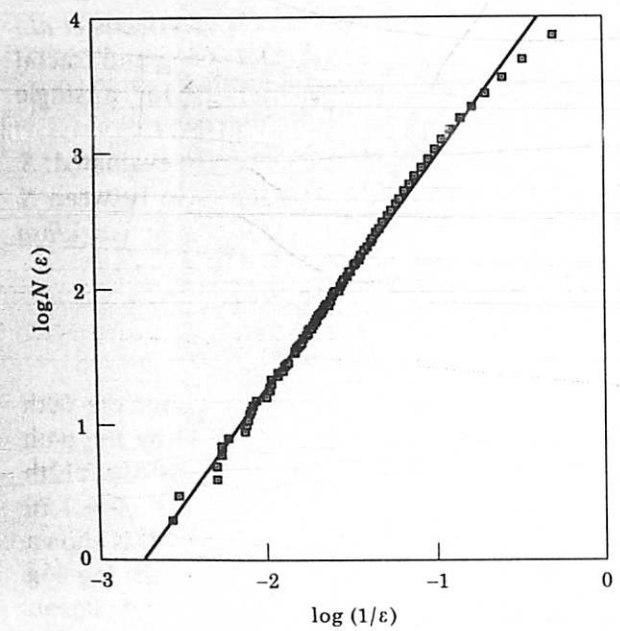

FIG. 3. Estimation of fractal dimension on a CAM at 112 hours. $D_f = 1.70$; $R^2 = 0.995$.

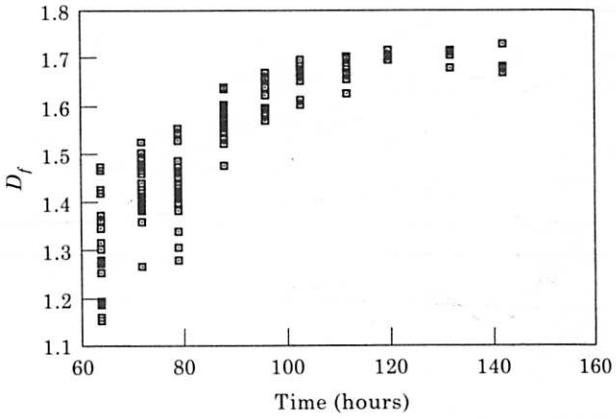

FIG. 4. D vs. time. D increases continuously from about 1.30 by the 60th hour, until about 1.68 by the 112th hour. Then, a plateau is reached and D remains stable with time at approximately 1.70. Note that, although the measures are widely distibuted in the early stages of vasculogenisis, the distribution becomes narrower for later and ultimate stages, when studies concerning angiogenesis are usually performed.

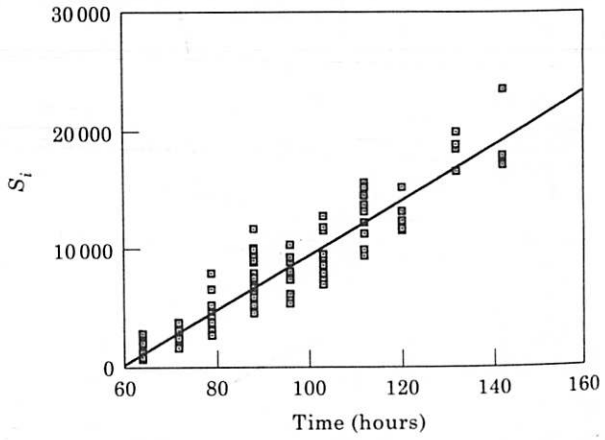

FIG. 5. S_i vs. time. The total vessel length, S_i, increases linearly with time, with wide distribution of results whatever the stage of development.

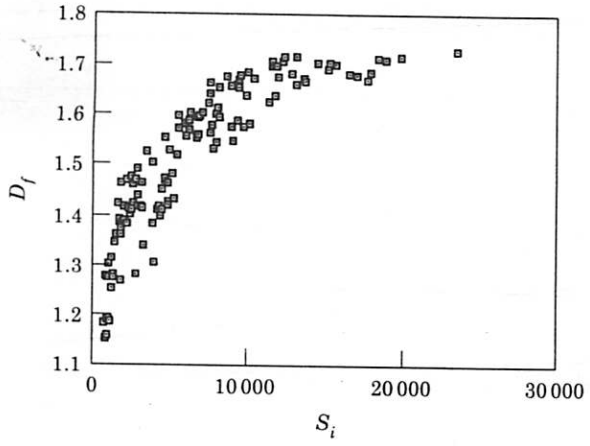

FIG. 6. D vs. S_i. D is asymptotically linked with S_i.

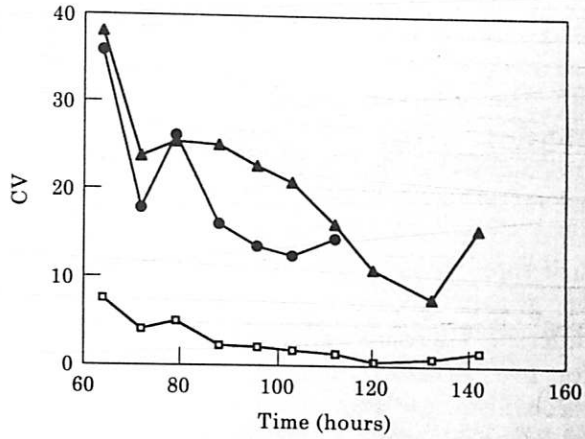

FIG. 8. The CV of D, D_v and S_i vs. time. (▲) S_i, (●) D_v, (□) D_f.

Discussion

Several studies have been performed to understand the logic of vascular network growth. Experimental models have been developed: the newborn rat lung (Caduff *et al.*, 1986), the CAM of quail embryos (Pardanaud *et al.*, 1987; Poole & Coffin, 1988), the CAM of chick embryos (Folkman, 1974), and the cornea of rabbits and rats (Gimbrone *et al.*, 1974). The CAM has been used as a standard and experimental model for angiogenesis (Folkman, 1974), and for tumor graft culture (Ausprunk *et al.*, 1974). Fractal organization of the vascular tree was suggested by Mandelbrot a long time ago (Mandelbrot. 1982). It was later demonstrated not only in the retina by several authors (Masters *et al.*, 1992), but also in the skin (Vico & Cartilier, 1993; Vico *et al.*, 1994), kidney (Cross *et al.*, 1993), and lungs (Gan *et al.*, 1993; Jiang *et al.*, 1994). Until recently, quantitative and objective measures of vascular growth were lacking. Grading or scoring methods comprising visual assessment of the vascular network are too approximative, subjective and unreproducible. The use of ^{3}H amino acids has been suggested to evaluate angiogenesis in the CAM (Splawinski *et al.*, 1988; Thompson *et al.*, 1985). Some geometrical approaches have been proposed, but are difficult to apply in practice (Barnhill & Ryan, 1983). Among all these methods, D_v and related measurements are more objective in quantifying vascular structures (Hayek *et al.*, 1991).

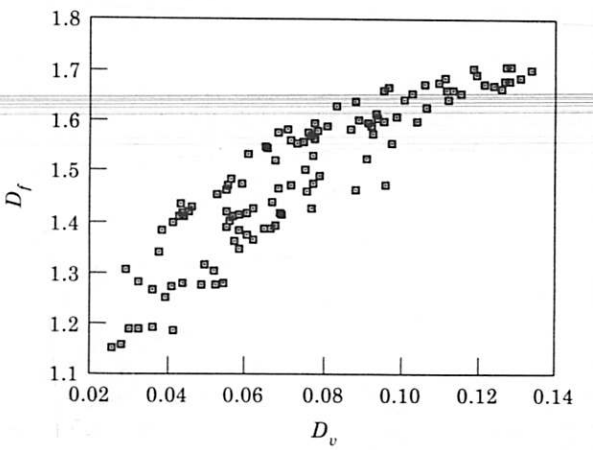

FIG. 7. D vs. D_v. The vascular density, D_v, has a similar evolution as the total vessel length, S_i, when related to D.

Some studies based on fractal analysis were performed on the CAM of the chick embryo to evaluate vasculogenesis and angiogenesis. Tsonis & Tsonis (1987) described a D close to 5/3 for the 4-day-old embryo. They suggested diffusion-limited aggregation (DLA), largely used in physics (Witten & Sander, 1981, 1983), as a possible model for vascular growth. However, the method used for D determination was not described. Moreover, DLA was proposed by these authors and others only on the basis of a roughly similar macroscopic pattern aspect, and similar D of about 1.70. Based on mathematical and physical arguments, it was shown recently that DLA cannot be accepted as a valid model for vessel growth (Kyriacos *et al.*, 1997). Briefly, it was demonstrated that there exists a preferential angle of bifurcation of 36° ($\pi/5$) for structures generated by the DLA process

(Arneodo *et al.*, 1992); in the case of the retina, the angles observed for vessels are higher, above 60° for arteries and about 90° for veins. Moreover, DLA is an irreversible growth process, while vasculogenesis and angiogenesis are reversible with regression and remodeling. It must be remembered that there is an infinity of structures with a given D for a geometric pattern or growth process that can be completely different. Therefore, at steady state, a different D for two structures implies a different growth mechanism, while an identical D does not imply an identical growth process. Hence Laplacian process governing growth mechanisms such as DLA are not responsible—at least alone—for the formation of the retinal vasculature, as static and dynamic analyses do not display characteristics similar to the existing retinal vasculature. More complex phenomena seem to account for the formation of retinal vessels that involve mechanical and physico-chemical factors (Kyriacos *et al.*, 1997). Thus, one should be cautious before considering DLA as the only growth mechanism involved on the sole basis of a D close to 1.70.

Kurz *et al.* (1994) investigated the development of blood vessels in the CAM of chick embryos between days 6 and 19 under other experimental conditions They examined fragments of the vascular tree (the CAM surface studied was 1 cm^2), while our experiment involved its entirety. They showed a triple scatter plot establishing relations between D, total vessel length density, and the density of proliferating cells, suggesting a higher proliferation rate in the capillary layer in the first part of vasculogenesis (Kurz *et al.*, 1994). They reported a stable D of about 1.1 for capillaries (smaller boxes), a D ranging from 1.3 to 1.5 with no further increase for intermediate vessels, and a D approaching 2.0 for greater vessels. This could be related to the multifractal concept, corresponding to the possibility of a given natural structure exhibiting different fractal behaviours in function of the scale of observation (Stanley & Meakin, 1988). Our method permits us to study vessels as a single entity: our D presents a different behaviour with time than that described by these authors, as we observed an asymptotic increase of D until values of about 1.70.

More recently, fractal analysis was used to quantify angiogenesis in the CAM (Kirchner *et al.*, 1996). This study was also performed on a limited area of the vascular tree, at a higher magnification. The D determined by the classic box-counting method showed a nearly linear increase from day 6 until days 11–12. The rate of increase peaked at approximately 1.80 around day 13, and by day 16, D began to decrease slightly. These results are in better accordance with our own, as the methodology for image analysis is closer to ours: the vascular network becomes progressively more complex up to a maximum value. However, these authors showed a decrease of D after a peak of similar value as the plateau we observed. This may be due to the fact that they tried to establish a polynomial relationship in base 2. It seems inappropriate to use polynomial regression ($y = ax^2 + bx + c$) in this case, since that kind of function will always lead to a maximum followed by a decrease which could continue till value $y = 0$; this means that vessels would spontaneously regress completely, which is a false assumption.

In our study, an asymptotic value of $D = 1.70$ reveals a stable vascular architecture in terms of fractal organization: even if S_i and D_r increase, the structure remains the same in fractal language. S_i and D_r appeared to be linearly related to the time of incubation; therefore D was also asymptotically related to S_i and D_r. One reason why S_i and D_r continued to increase linearly with time while D remained stable is that the entire vascular network grows proportionally within the growing embedding tissue (the total area of the *area vasculosa* increases with time). Another reason is that new iterations appear following the rule of division of vessels. More likely, we should have a combination of both reasons. Our results suggest progressive vascular network formation until a stable vascular architecture is reached after about 100 hours of incubation under our experimental conditions. It is hypothesized that a network of such a complexity should correspond to a more efficient vascular supply in terms of metabolism regarding spatial occupation; in this hypothesis, efficient should be understood as being the capacity for a vascular network to maximally cover (perfuse) the surface while minimally

occupying the tissue itself. Variations of D are considerable at the beginning of vasculogenesis, but become narrower along the time course (Fig. 4). This could signify that some embryos show faster and/or larger growth than others, but all will develop until that final stage characterized by $D = 1.70$. This point is also suggested by the asymptotic behavior of D vs. S_i and D_r: a stable vascular architecture is achieved whatever the S_i and D_r. However, one must remain cautious before assigning the value of $D = 1.70$ to any physical growth model, particularly the DLA model often mentioned in the literature, as discussed previously. On the other hand, D appears as a sensitive parameter to evaluate a vascular network and its evolution or modification. The disparity of the results decreases with time and becomes narrower at periods of incubation where the CAM is used to study angiogenic factors.

Furthermore, S_i and D_r appear to be less than ideal parameters, showing a linear relationship with time and a higher CV. With our methodology (windowed eggs), it is not possible to follow these parameters for periods of time longer than 100 hours, because the CAM is not completely visualized at these stages. Because of the asymptotic evolution of D in function of S_i and D_r, these cannot be accurate to evaluate the quality of a vascular network in terms of tissue perfusion. Indeed, it is clear that greater S_i (above 13000) and greater D_r (above 0.11) do not signify better tissue perfusion, as D remains constant. This shows that S_i and D_r are parameters which are not accurate enough to estimate the quality of a vascular tree, while D accurately estimates its complexity and perfusing capacity.

Finally, the CV for the parameters studied also favors the superiority of D. The CV of D is small, and is practically constant after 90 hours of incubation (2–3%). One could take advantage of these minimal variations of D in further studies evaluating the effects of angiogenic factors on the vascular network.

Conclusion

Based on the general concept of fractal analysis and on a modified box-counting method, we are able to evidence a stable vascular architecture when studying the evolution of the branching patterns of extraembryonar vascular structures of the chick embryos CAM. During growth of the embryo, D increases up to an asymptotic value of about 1.70. D, as compared with S_i or D_r, appears to be the most sensitive parameter to evaluate the evolution of an embryonic vascular network, and could be used to study the influence of angiogenic factors on the geometry and branching patterns of vascular structures. The advantages of this parameter over other more usual methods are: it is an easy, precise and reproducible automated method for evaluation of the complexity of the vascular tree; the CV for the biological pattern is very small and there is an asymptotical relationship with time with a stable plateau.

REFERENCES

ARNEODO, A., ARGOUL, F., BACRY, E., MUZY, J. F. & TABARD, M. (1992). Golden mean arithmetic in the fractal branching of diffusion-limited aggregates. *Phys. Rev. Lett.* **68,** 3456–3459.

AUERBACH, R., KUBAI, R., KNIGHTON, R. & FOLKMAN, J. (1974). A simple procedure for the long-term cultivation of chicken embryos. *Dev. Biol.* **41,** 391–394.

AUSPRUNK, D. H., KNIGTHON, D. R. & FOLKMAN, J. (1974). Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. *Dev. Biol.* **38,** 237–248.

BARNHILL, R. L. & RYAN, T. J. (1983). Biochemical modulation of angiogenesis in the chorioallantoic membrane of the chick embryo. *J. Invest. Dermatol.* **81,** 485–488.

CADDUFF, J. H., FISCHER, L. C. & BURRI, P. H. (1986). Scanning electron microscope study of the developing microvasculature in the postnatal rat lung. *Anat. Rec.* **216,** 154–164.

CROSS S. S., START, R. D., SILCOCKS, P. B., BULL, A. D., COTTON, D. W. K. & UNDERWOOD, J. C. E. (1993). Quantitation of the renal arterial tree by fractal analysis. *J. Pathol.* **170,** 479–484.

FEDER, J. (1988). *Fractals*. New York: Plenum Press.

FOLKMAN, J. (1974). Tumor angiogenesis factor. *Cancer Res.* **34,** 2109–2113.

GAN, R. Z., TIAN, Y., YEN, R. T. & KASSAB, G. S. (1993). Morphometry of the dog pulmonary venous tree. *J. Appl. Physiol.* **75,** 432–440.

GIMBRONE, M., COTRAN, R. & FOLKMAN, J. (1974). Tumor growth neovascularization: an experimental model using rabbit cornea. *J.N.C.I.* **52,** 413–427.

HAYEK, A., BEATTIE, G. M., LOPEZ, A. D. & CHEN, P. (1991). The use of digital image processing to quantitate angiogenesis induceby basic fibroblast growth factor and transplanted pancreatic islets. *Microvasc. Res.* **41,** 203–209.

JIANG, Z. L., KASSAB, G. S. & FUNG, Y. C. (1994). Diameter-defined Stralher system and connectivity matrix of the pulmonary arterial tree. *J. Appl. Physiol.* **76,** 882–892.

KIRCHNER, L. M., SCHMIDT, S. P. & GRUBER, B. S. (1996). Quantitation of angiogenesis in the chick chorioallantoic membrane model using fractal analysis. *Microvasc. Res.* **51,** 2–14.

KURZ, H., WILTIN, J. & CHRIST, B. (1994). Multivariate characterization of blood vessel morphogenesis in the avian chorioallantoic membrane (CAM): cell proliferation, length, density and fractal dimension. In: *Fractals in Biology and Medicine* (Nonnenmacher, T. F., Losa, G. A. & Weibel, E. R., eds), pp. 132–140. Basel: Birkhaüser Verlag.

KYRIACOS, S., BUCZKOWSKI, S., NEKKA, F. & CARTILIER. L. (1994). A modified box-counting method. *Fractals* **2,** 321–324.

KYRIACOS, S., NEKKA, F., VICO, P. & CARTILIER, L. (1997). The retinal vasculature: towards an understanding of the formation process. In: *Fractals in Engineering* (Levy-Vehel, J., Lutton, E. & Tricot, C., eds), pp. 383–397, London: Springer-Verlag.

MANDELBROT, B. (1982). *The Fractal Geometry of Nature*. San Fransisco: Freeman.

MASTERS, B. R., SERNETZ, M. & WLCZEK, P. (1992). Image analysis of human retinal blood vessels and their characterisation as fractals. *Acta Stereol.* **11 (Suppl. 1),** 355–360.

PARDANAUD, L., ALTMANN, C., KITOS, P., DIETEREN-LIEVRE, F. & BUCK, C. A. (1987). Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* **100,** 339–349.

POOLE T. J. & COFFIN, J. D. (1988). Developmental angiogenesis: quail embryonic vasculature. *Scanning Microsc.* **2,** 443–448.

RUSS, J. C. (1990). *Computer-Assisted Microscopy—the Measurement and Analysis of Images*. New York: Plenum Press.

SPLAWINSKI, J., MICHNA, M., PALCZAK, R., KONTUREK, S. & SPLAWINSKI, B. (1988). Angiogenesis: quantitative assessment by the chick chorioallantoic membrane assay. *Methods Find. Exp. Clin. Pharmacol.* **10,** 221–226.

STANLEY, H. E. & MEAKIN, P. (1988). Multifractal phenomena in physics and chemistry. *Nature* **335,** 405–409.

THOMPSON, W. D., CAMPBELL, R. & EVANS, T. (1985). Fibrin degradation and angiogenesis: quantitative analysis of the angiogenic response in the chick chorioallantoic membrane. *J. Pathol.* **145,** 27–37.

TSONIS, A. A. & TSONIS, P. A. (1987). Fractals: a new look at biological shape and patterning. *Perspect. Biol. Med.* **30,** 355–361.

VICO, P. G. & CARTILIER, L. H. (1993). A new approach in the study of skin vascularization. *Plast. Reconstr. Surg.* **92,** 463–468.

VICO, P. G., BOYER, H. & CARTILIER, L. H. (1994). New concepts in the study of tissue vascularization: a mathematical model of skin vascularization. *Plast. Reconstr. Surg.* **94,** 174–179.

WITTEN, T. A. & SANDER, L. M. (1981). Diffusion limited aggregation, a kinetic critical phenomenon. *Phys. Rev. Lett.* **47,** 1400–1403.

WITTEN, T. A. & SANDER, L. M. (1983). Diffusion limited aggregation. *Phys. Rev. B* **27,** 5686–5687.