ACTA STEREOL 1999; 18/1: 27-32
ORIGINAL SCIENTIFIC PAPER

ANGIOGENESIS EVALUATED WITH A NEW COMPLEXITY MEASURE

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ABSTRACT

Growth factor effects on blood vessel patterns were assessed with a fast and automated method that measures complexity (extended counting method: XCM; Sandau, 1996). XCM is a robust estimator of complexity not only in theory but also in practice: 1) it fulfils the quasi-maximum-property; 2) it shows reduced variance due to pattern translation and rotation; 3) it is less sensitive to image manipulations and thresholding. XCM is superior to traditional fractal analysis for detecting changes in vascular complexity following growth factor and control treatment.

Key words: angiogenesis, avian embryo, blood vessel pattern, complexity, fractal, VEGF.

INTRODUCTION

The study of blood vessel differentiation and growth (angiogenesis research) may provide knowledge for an improved understanding of embryonic development, of pathological conditions and for therapeutical approaches to a broad variety of diseases. Growth factors play a key role in angiogenesis and their effects need to be quantitatively analyzed for an improved understanding. Blood vessel patterns in the chick chorioallantoic membrane (CAM) during normal development and after growth factor application (Wilting et al., 1993) have previously been characterized with estimates of vessel length density, endothelial proliferation intensity and complexity ("fractal dimension", cf. Kurz et al. 1994, 1995; Wilting et al., 1996). We observed in these studies that complexity could only be measured with conventional methods (e.g., box-counting method, BCM) under strictly standardized assay conditions and with user aided image analysis. Changes in vascularity in the CAM were also assessed by Kirchner et al. (1996) using a highly interactive BCM. For routine use of angiogenic CAM assays, a minimum of user interaction is required. In addition, the necessity to describe complexity without the need for assumptions about a fractal structure (Sandau and Kurz, 1994), and several flaws inherent in BCM and related methods prompted Sandau (1996a,b) to introduce the XCM. This new measure of complexity is applied here for evaluating growth factor effects on blood vessel patterns.

CAM ASSAY AND IMAGE ANALYSIS

Detailed description of the angiogenic CAM assay and of growth factors can be found in Wilting et al. (1993, 1996, 1998), Kurz et al. (1994, 1995, 1998a), and Birkenhäger et al. (1996). Briefly,

non-inflammatory carrier disks were applied for two days on the differentiated CAM. Salt-free solutions of homo- and heterodimers of Vascular Endothelial Growth Factor (VEGF) isoforms with 165 and 121 amino acids, or of Placental Growth Factor (PlGF) isoforms had been adsorbed to the carriers in µg quantities. CAMs were fixed in the egg and pieces of about 1 cm^2 were dissected and imaged in a petri dish. A CCD camera with macro objective was connected to a Matrox framegrabber in a 90 MHz Pentium-based PC running the image analysis software package analySIS (SIS, Münster). In each image, naive and treated CAM (with or without carrier) were visible. Images were digitized with 512x512x8 resolution (1 Pixel = 11 µm), and after shading correction were stored as TIF files. XCM and BCM measurements were implemented with routines (written in TurboPASCAL 7.0 by K.S.) that evaluated complexity of images within seconds. CAM assays were classified in two groups according to the XCM value without a priori knowledge of treatment.

PROPERTIES OF BCM AND XCM

The pertaining mathematical background of fractal dimension in general can be found in Falconer (1990) and in Stoyan and Stoyan (1994). Details on the BCM and on the XCM are given in Sandau (1996a,b), and in Sandau and Kurz (1997). We here use the abbreviations dim for true fractal dimension, bcmdim for the result of the BCM measurement, and x-dim for that using XCM.

XCM, in contrast to BCM, calculates a measure of complexity (x-dim) without regression. XCM searches the image for the most complex largest box (upper bound) by counting the number of intersecting smallest boxes (lower bound) contained in that box. Hence, x-dim grows monotonically with complexity, and is determined by the most complex part of the image. This corresponds to an important feature of fractal dimension, the maximum property, which is approximately fulfilled by x-dim: quasi-maximum-property.

A practical problem for BCM is the design of the regression, i.e. which values to use for calculating the regression. In many cases the image is a digital image of a set in the plane. Then the maximum resolution is given by the pixel size of the digital image. We used at least twice the pixel size for the lower bound to avoid artefacts in the measurements, and chose the larger values by doubling, as recommended by Stoyan and Stoyan (1994). The upper bound is close to the image size, but may be smaller. This bound defines the largest box, which can also be used for XCM. A second problem is image noise. Sandau and Kurz (1997) have shown that adding noise to a (fractal) pattern diminishes bcmdim, but not x-dim.

A third problem is the digitalization of the set. Fractal dimension is motion invariant. But the digitalization of a fractal set depends on the position of the fractal set with respect to the digitalization grid (pixel structure). Variability due to translation and rotation was tested with a von Koch curve. Results reported earlier (Sandau, 1996b) showed that BCM had a fourfold higher variance than XCM. BCM tends to underestimate dim, whereas XCM overestimates dim. The smaller variance of x-dim as compared to bcmdim is present for all dim values. Comparable observations were also made using other self-similar sets (Sandau, 1996a; Sandau and Kurz, 1997).

Further problems, which are not encountered in computer-generated self-similar sets, but in nearly all practical applications, are related to the transformation of a grey scale image into a binary image, and to further image processing that will be described below (cf. Fig. 3; Sandau and Kurz, 1997).

An important feature of the new complexity measure is its ability to automatically detect and measure the most complex part of a pattern. Therefore, no user interaction was needed once the image had been digitized.

EVALUATION OF CAM ASSAY

Blood vessel growth in the chorioallantoic membrane (CAM, the respiratory organ of chicks in the egg) proceeds from small, but finite sized capillaries to the formation of larger bifurcating tubes. This process apparently is controlled by local factors to adapt CAM growth globally to egg size and embryonic haemodynamics (Kurz et al., 1995; Kurz and Sandau 1997, Kurz et al. 1998b). If an endothelial growth factor is applied after CAM expansion has ceased, we expect that the pattern of capillaries, and of precapillary and postcapillary vessels becomes more complex. This can be verified by time-consuming histological techniques, or much faster by image analysis of the altered vessel arrangement in a CAM whole mount.

The complexity of the vascular pattern in the region of control or growth factor-treated CAMs (Fig. 1) was assessed with BCM and XCM. From the results shown in Fig. 2, two properties of the XCM are confirmed for the analysis of natural patterns: the higher mean value of the estimate, and the lower variance, as compared with BCM. The analysis of complexity with XCM clearly separates two groups: lower complexity (between 1.51 and 1.60) was observed for all control experiments, whereas an increased x-dim (between 1.60 and 1.68) was found in all VEGF-treated CAMs. Differences between various controls, or between various VEGF isoforms were not significant. Interestingly, PlGF/VEGF heterodimers induced angiogenesis, but PlGF alone did not

In addition, Fig. 2 shows that the effect of threshold variations (-5%, + 5%) for binarization was less pronounced for x-dim than for bcmdim. This stability may also be due to the quasi-maximum-property of XCM, and is a very favorable feature in classification based on quantitative image analysis. Image analysis-based classification of growth factor effects was in concordance with histological findings (not shown) in all 45 CAM assays reported here.

Fig. 1. Control CAM with unchanged vascular pattern under the polygonal carrier (left) and VEGF-treated CAM with increased complexity of the capillary and precapillary vascular pattern (right). Note that blood vessels form anastomoses in the normal and experimental CAM, indicating that branching self-similarity is not present. These images were digitized at 512x512x8 resolution, with 1 Pixel corresponding to 17 μm.

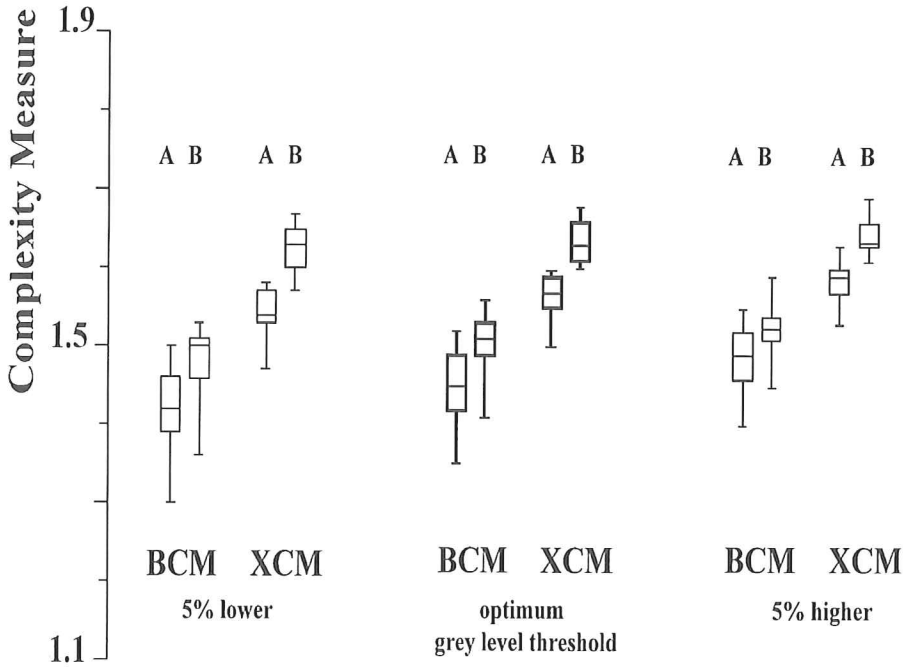

Fig. 2. Complexity of vascular patterns as estimated with BCM and XCM, and depending from binarization threshold (5% grey level deviations from global optimum). The lower bound for both methods was 2 pixels, the upper bpund was 128 pixels. XCM better separates control and PlGF-treated (A; n=23) from VEGF- and VEGF/PlGF-treated specimens (B; n=22), and is less sensitive to threshold variation. Automated evaluation required optimum thresholding and the use of XCM. Each box plot indicates median, quartiles and extreme values.

DISCUSSION

We have shown that 1) the XCM is superior to the BCM because of its reduced variance with respect to both motion and thresholding, and due to its noise insensitivity (monotonicity, quasi-maximum-property); 2) the CAM assay can be evaluated automatically and reproducibly with XCM; (3) the carrier material is well-suited for the CAM assay, because it does not induce changes in vascular complexity; 4) the endothelium-specific mitogenic effect of the 165 (Wilting et al., 1993; Kurz et al., 1995) and 121 (Wilting et al., 1996) amino acid isoforms of VEGF homodimers, and of heterodimers of VEGFs or of VEGF and PlGF results in a greater complexity of the vascular pattern at the site of application. The enhanced complexity corresponds to the histologically verified multiple layers of capillaries and precapillary vessels, and to the visual impression of a higher bifurcation density (Kurz et al. 1998a).

The maximum property is approximately fulfilled by XCM, but not by BCM. This is the most important argument against using BCM and related methods based on linear regression for measuring complexity via "fractal dimension". The regression works as a kind of average, and a noise affects bcm-dim severely. XCM additionally is less sensitive against variation of the binarization threshold (cf. Fig. 2; Sandau and Kurz, 1997).

Both stability and precision of estimate are highly desirable properties. In summary, the XCM has properties closer to the original concept of fractal dimension. However, it overestimates "true" fractal dimension (Sandau, 1996a), which is a minor drawback, because the absolute value of dim may be of little significance (Murray, 1995), whereas reliable comparisons between experimentally altered and control patterns are a prerequisite for unbiased evaluation. Our conclusions are supported by others (Dubuc et al., 1989; Soille and Rivest, 1996), who also warn of most commonly-used methods for "fractal analysis".

The CAM is a well-established assay for blood vessel formation (Wilting et al., 1993). Blood vessel patterns have been described with fractal geometry before (cf. literature in Mandelbrot, 1982; Weibel, 1991; Losa et al., 1994; Kurz et al., 1994; Sandau and Kurz, 1994; Kirchner et al., 1996; Kurz and Sandau, 1997; Kurz et al. 1998a,b), and one rather often comes across statements of the kind "blood vessels are fractals". However, the latter statement is a very strong idealization (Kurz and Sandau, 1998): 1) finite-sized capillaries mark the lower limit; 2) blood vessel arrangement changes within the system and self-similarity may be absent; 3) the blood vessel system is not organized as a tree, but as a network with loops, anastomoses, and blood sinus; 4) the complexity of real development (reviewed by Wilting et al., 1995) can not be achieved with a simple geometric generator. Therefore, the description of blood vessel patterns, particularly of patterns including capillaries, anastomoses or blood sinus, as fractals may be misleading (cf. Panico and Sterling, 1995). Similar considerations have been discussed by Tautu (1994) with respect to normal and pathological tissue growth. Nevertheless, complexity can be assessed without assuming a fractal structure.

When VEGF and PlGF were applied separately, but with two adjacent carriers on the same CAM, no interference could be detected (Kurz et al., 1998a). XCM classified CAM blood vessel patterns correctly into controls and PlGF-treated, and in VEGF-treated ones. This recommends the method as a complexity parameter in multivariate characterization of blood vessel patterning. For example, recent results show that VEGF-C mediates lymphangiogenesis (Oh et al., 1997; Wilting et al., 1998), but does not increase blood vascular complexity. The precision of XCM could be of particular value for screening VEGF-stimulated CAMs for blood vessel growth inhibition by putative anti-angiogenic substances.

In a more theoretical context, it was shown using XCM that the complexity of simulated (non-fractal) vascular growth patterns (Sandau and Kurz, 1994) was comparable to that of the CAM bifurcation pattern (Kurz and Sandau, 1997; Kurz et al., 1998b). However, our experience with vascular growth models supports the notion that complexity is but one aspect of (vascular) patterns that should be supplemented with other measures to more extensively characterize their structure. It remains to be shown whether methods like XCM can be used to measure complexity in medical imaging, or can even detect subvisual clues that contain important information on image texture.

ACKNOWLEDGMENT

We are obliged to Drs. R. Birkenhäger and H.A. Weich for kind donation of growth factors.

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