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ARTICLE

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Fractal dimension of K1735 mouse melanoma clones and spheroid invasion in vitro

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Abstract An in vitro tumour-host confrontation method to investigate the invasion behaviour of cancer has been applied to K1735 mouse melanomas. Fluorescently labelled spheroids of cancer cells and host cells were confronted and the temporal course of cancer invasion into the host was investigated using confocal laser scanning microscopy. To improve the quantitative data of this method, the boundary images of the fluorescently labelled confrontation pairs were treated as fractals. The physical and mathematical framework for determination of the fractal capacity dimension is widely used in biology and medicine and has proved to be a very useful tool for describing the cancer invasion process. The fractal capacity dimension determination was carried out by dilation of the binary boundaries of the objects, which were treated as an estimate of the Minkowski-Bouligand dimension. The fractal dimension correlated well with the degree of invasion of the K1735-M2 clone. Control experiments, with host-host confrontations and various K1735 clones with reduced invasiveness, support these results.

Keywords Fractal geometry · Confocal laser scanning microscope · Cancer invasion · Metastasis · Image processing

Introduction

Invasion of cancer into the surrounding tissues is one of the major mechanisms by which cancer is able to form metastases at various locations in the human body. As the rehabilitation and survival chances of a cancer patient are correlated in the case of metastatic cancers to the state and the number of secondary tumours, further insights into the behaviour of cancer during invasion has a significant impact on the fight against cancer. Recent observations (Ahammer et al. 2001) using the spheroid confrontation technique for investigation of cancer invasion has lead to the conclusion that application of this physical method for determining the fractal capacity dimension can support this fight, if more thoroughly investigated.

Fractal related work on cancer has been mostly successful in determining the mammographic density of breast carcinoma (Byng et al. 1997), MCF-7 breast cancer cells (Losa et al. 1998), leukemic cells (Losa et al. 1992; Losa and Nonnenmacher 1996), pigmented skin lesions (Cross et al. 1995), malignant melanoma (Claridge et al. 1992), benign and malignant breast epithelial cell nuclei (Einstein et al. 1998), mastopathic and mammary cancer tissue (Mattfeldt 1997), dysplastic lesions of cervix uteri (Sedivy et al. 1999), gallbladder adenocarcinomas (Waliszewski 1999), retinoid-induced differentiation of cancer cells (Waliszewski et al. 1999, 2000) and quantification of nuclear pleomorphism using an asymptotic fractal model (Landini and Rippin 1996); it has also been used for the study of the role of fractals, chaos and cancer (Sedivy and Mader 1997).

In comparison to single cells or monolayers, spheroids are well suited for the investigation of invasion in vitro (Bjerkvig 1992). Here dual fluorescent staining is used, as previously described (DeVaney et al. 1997; Ahammer et al. 1998), in order to observe the invasion optically and make possible the digital image analysis of the resulting spheroid confrontations. Analysis of the degree of invasion is carried out making use of fractal analytic and image analysis techniques.

Optical sections of the confronted spheroid pairs are digitally recorded and processed in order to obtain binary images. Dilating the binary images of the boundaries enables the implementation of a double logarithmic plot of the object areas as a function of the dilation disk radii. With the value of the slope of these

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plots, the fractal capacity dimension is calculated and serves as an estimate of the Minkowski-Bouligand dimension. The results of this dimension estimate are compared to the quantitative parameter INVASLOG, which has been proven in prior work to be a quantitative parameter well suited to describe the invasiveness of the considered confrontation cultures in vitro (Smolle et al. 1992).

Materials and methods

Spheroids, staining and confrontation

K1735-M2, K1735-Cl16 and K1735-Cl23 mouse melanoma cells (kindly provided by Dr. I.J. Fidler, Institute of Cell Biology, MD Anderson Hospital, Houston, Texas) and spheroids from freshly trypsinized embryonal chick heart cells were reaggregated to spheroids as described previously (Mareel and Meyvisch 1981; DeVaney et al. 1997). Before reaggregation the mouse melanoma cell monolayer was stained with the fluorescent dye CMFDA (chloromethylfluorescin diacetate) or diI [DiIC_{18}(3)] and the chick heart cell monolayer was stained with the fluorescent dye CMTMR (tetramethylrhodamine derivative) or diO [DiOC_{18}(3)]. With a stain concentration of 10 μM and a staining duration of 2 h, these dyes are appropriate for staining live cells and are stable for up to 5 days. All dyes were purchased from Molecular Probes Europe (Leiden, The Netherlands).

After reaggregation to a size of about 50 μm, the mouse melanoma spheroids were mechanically confronted (touching one another) with the chick spheroids, forming cancer-host spheroid pairs. These pairs were incubated in a microcultivation chamber at 37 °C, 95% RH and 5% CO_2.

Confocal laser scanning microscope

The confronted cancer-host spheroid pair images were recorded using a confocal laser scanning microscope (Leica, TCS NT, Heidelberg, Germany). A 10×0.3 NA objective and a zoom factor of 2 was used and the 512×512 pixel images had an image size of 500 μm×500 μm. A single pixel has, at this magnification, 0.977 μm side length; a single cell has, typically for K1735, a size of 10–15 μm. This "pixel resolution" was in practice sufficient to calculate the estimate of the fractal capacity dimension, as can be seen in the double logarithmic plot in Fig. 1, and was near the theoretically obtainable optical maximum for the apparatus used. Several optical sections (up to 16) were recorded as tif files from the Leica system. These files were not converted into other formats to circumvent any degradation by converting algorithms (especially data compressing algorithms). Both fluorophores were excited simultaneously with an ArKr laser ($\lambda = 488$ nm and 568 nm). The fluorescence light was detected with a standard fluorescine/rhodamine (FITC/TRITC) filter arrangement using a 530/30 nm bandpass filter and a 590 nm longpass filter.

Image processing

Several image processing steps were carried out before calculating the fractal capacity dimension from a single optical section of each of the spheroid confrontation pairs. The software KS300 (Zeiss, München, Germany) was used to eliminate cross-talk signals, which resulted from an overlap of the fluorescence spectra. This step has been previously published in detail (Ahammer et al. 1999). Binary images (representing the cross-talk eliminated regions) were generated for calculating the quantitative invasion parameter INVASLOG which was calculated by:

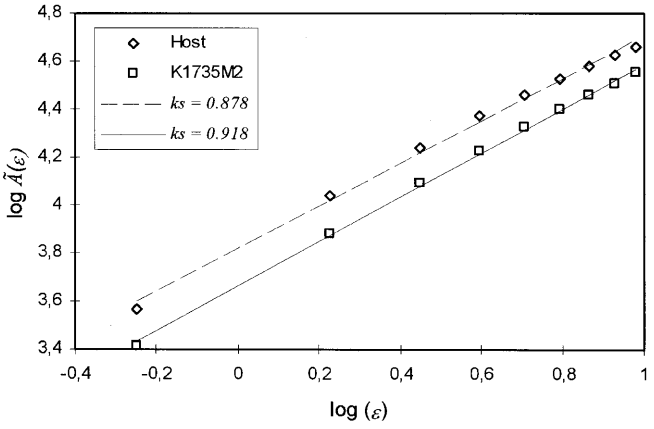

Fig. 1 Exemplary log-log plot for determining the slope k_s for calculating the capacity dimension D_s. $\tilde{A}(\varepsilon)$ is the approximated area in pixel^2 of the dilated area of the object and ϵ is the approximated radius calculated by $\varepsilon = \sqrt{\frac{\tilde{B}_\varepsilon}{\pi}}$, where $\tilde{B}_\varepsilon$ denotes the approximate dilation element area in pixel^2

$$\text{INVASLOG} = -\log(\text{Formfactor}) \tag{1}$$

with:

$$\text{Formfactor} = \frac{4\pi \times \text{area}}{\text{perimeter}^2} \tag{2}$$

The parameter INVASLOG was calculated for the host as well as for the cancer region. Previous investigations (Smolle et al. 1990, 1992) included subjective inspections by trained pathologists and showed especially that the parameter INVASLOG for the host region is able to quantify the invasiveness of the cancer spheroid.

Boundary images for determination of the fractal dimension were generated from the binary images using the Roberts operator. This operator is an edge detection function with a 2×2 matrix. From this matrix the differences of the diagonal elements are taken and the root of the sum of the squared differences gives the new value for the matrix. The results with the Roberts operator were compared with other edge detection operators, such as the Laplacian or the Sobel operator, which both use a 3×3 matrix. In comparison to the Roberts operator, the Laplacian operator led to very thin edges which were often broken and artificially segmented; the Sobel operator, on the other hand, led to very thick edges which tended to smooth out fine details.

Fractal dimension

To calculate the fractal capacity dimension (Mandelbrot 1982; Barnsley 1993; Liebovitch 1998), the boundaries of the images of both cancer and host regions were used. Several methods have been investigated in a prior publication (Ahammer et al. 2001), including box counting, the sausage method and a local method (Stoyan and Styoan 1994), as well as the influence of subsequent image processing steps, such as median filtering, a combination of binary closing and opening and a special hole filling and scrapping step and also the influence of noise. For the investigations here, solely the sausage method without any additional image processing steps was implemented.

The capacity dimension yielded by the sausage method, D_s, is based on the Minkowski cover, which was further examined by Bouligand (1929) using dilation. Outgoing from a set A in R^2 the set of all points $A(\epsilon)$ with:

$$A(\varepsilon) = \{\vec{y} : \vec{y} \in B_\varepsilon(\vec{x}), \vec{x} \in A\} \tag{3}$$

is the Minkowski cover of the set A, where $B_\varepsilon(\vec{x})$ is the structuring or dilation element and is a disk of radius ϵ located around the point defined by $\vec{x}$. Increasing/decreasing the radius ϵ will increase/decrease the set $A(\epsilon)$. Simply, the set $A(\epsilon)$ is the area of the dilated set A. Taking the infinitesimal limit $\epsilon \to 0$, the Minkowski-Bouligand dimension or sausage dimension D_s from a set A can be calculated by:

$$D_s = D_s(A) = \lim_{\varepsilon \to 0}\left(2 - \frac{\ln A(\varepsilon)}{\ln \varepsilon}\right) \tag{4}$$

All the capacity dimensions should theoretically give the same value; D_s is one of these. Despite the mathematical differences in their determination, the practical implementation of different algorithms such as dilation, box counting or local methods prevent the dimensions from reaching the same value. When determinig D_s from pixelated images the limit $\epsilon \to 0$ cannot be reached because the minimal ϵ is equal to the pixel size of the image. This non-zero pixel size is furthermore the reason why the dilation element $B_\varepsilon(\vec{x})$ cannot be determined exactly. Implementing a practical approach, boxes with pixel sizes of 1×1, 3×3, 5×5, ... 17×17 were used to approximate the disks. The corresponding approximated radius ϵ in pixels was calculated by:

$$\varepsilon = \sqrt{\frac{\tilde{B}_\varepsilon}{\pi}} \tag{5}$$

where $\tilde{B}_\varepsilon$ denotes the approximated dilation element area in pixel^2.

By varying ϵ the corresponding applied dilation element (box) results in a varying area $\tilde{A}(\varepsilon)$ of the object. $\tilde{A}(\varepsilon)$ denotes the approximated area of the dilated object, again in pixel^2. The slope k_s of a double logarithmic plot of $\tilde{A}(\varepsilon)$ versus ϵ gives the estimated fractal capacity dimension, D_s:

$$D_s = 2 - k_s = 2 - \frac{\log \tilde{A}(\varepsilon)}{\log \varepsilon} \tag{6}$$

This procedure was implemented with the software IDL 5.3 (Research Systems, Gilching, Germany). The sausage method gave very reliable results when compared to the box counting method, which was also implemented with IDL 5.3 (Ahammer et al. 2001). For testing the reliability of the procedures further, both methods were implemented with the image processing software KS300 and gave exactly the same numerical values for some arbitrary sample images. As KS300 is interpreter-based, the calculation times were far beyond the compiler-based IDL, leading to our preference for IDL.

Results

In order to evaluate quantitatively the invasiveness of the K1735 mouse melanomas, making use of the fractal capacity dimension D_s, binary fluorescence signal images of the spheroid confrontation pair were investigated. Figure 1 shows a sample double logarithmic plot for the determination of D_s. The scaling window for the determination of the slope k_s was set to $(2n+1) \times (2n+1)$ pixels with $n = 0, 1, 2, 3 \ldots 8$. To verify that the invasiveness and no other factors would be quantitized by D_s, additional control experiments with host-host confrontations and various K1735 clones were investigated.

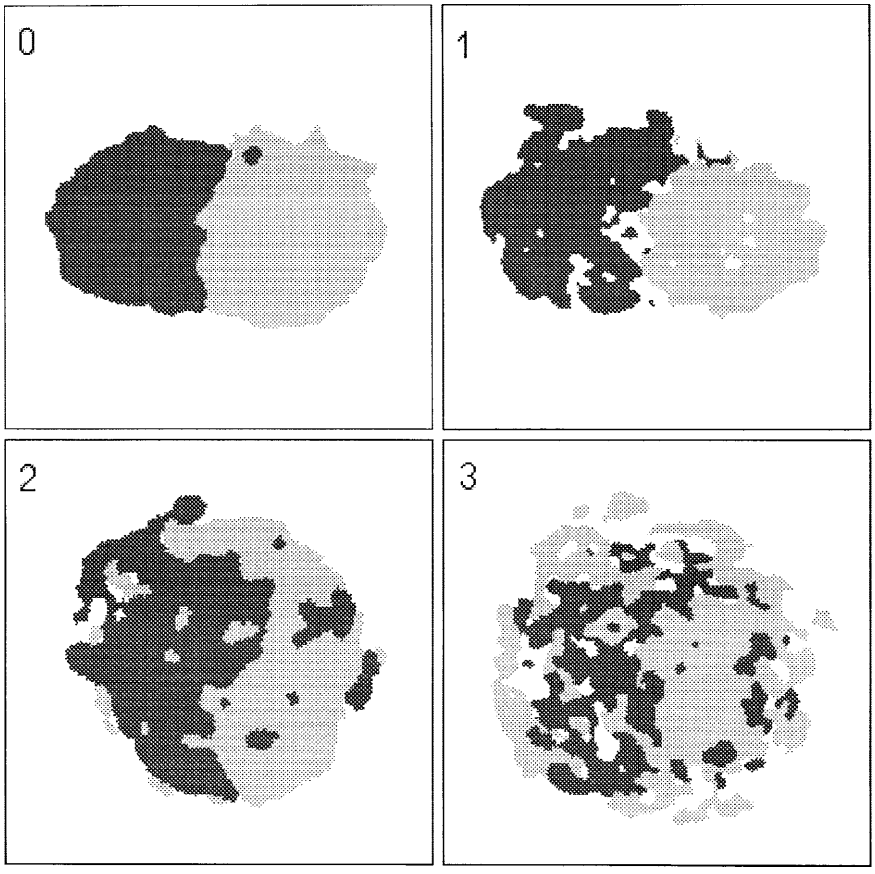

Fig. 2 Combined binary images of confronted cancer-host spheroid pairs. Each image is a single section recorded in tif format with a confocal laser scanning microscope. *Dark grey areas* represent the cancer and *light grey areas* represent the host. *Numbers* are the days of invasion

The values of D_s not only increased during the subjectively visible process of invasion but were beyond that compared to the parameter INVASLOG of the host spheroid, which served for this study as a standard value giving the standard quantitative values. The qualitative process of the invasion is depicted exemplarily in Fig. 2. As a single pixel is far smaller in size than a single cell, the two-dimensional section of the three-dimensional spheroid shows areas which are not directly correlated with multiples of the single cell size, as fractions of single cells may be present. Beginning with day 0 (Fig. 2), shortly (about 4 h) after the initial confrontation, both spheroids are structurally well separated but touching. Only one cancer cell (little dark grey area in the light grey region) has invaded the host spheroid. On the following days (Fig. 2, days 1, 2, 3) an increasing state of invasion with increasing clefting of the spheroids and increasing irregular forms could be observed. The quantitative results of this behaviour are shown in Fig. 3 for K1735-M2 mouse melanomas. First, the value of the fractal dimension D_s shows a dependence on the state of invasion for both the cancer and the host regions (Fig. 3a). Second, this increase of D_s is in accordance with the increase of the standard parameter INVASLOG for the host region. The INVASLOG values for the cancer were in this case equally accordant.

Quantitatively all the parameters correlated very well, with a failure probability $P < 0.001$. The actual values of the linear correlation coefficient can be seen in Table 1.

Confronting a host spheroid with another host spheroid instead of creating a cancer-host pair shows and verifies further whether this increase in the D_s or INVASLOG values is really dependent on the invasiveness of the cancer. Qualitative inspections of such confrontations showed subjectively and clearly that there was no distinct invasion (images not shown). Although both spheroids were attached, they remained distinct. Nevertheless, the shape of the spheroids had increasing irregularities at the boundaries. Figure 4 shows that for both methods (Fig. 4a and Fig. 4b) no recognizable increase in the parameters during the course of the invasion is detectable.

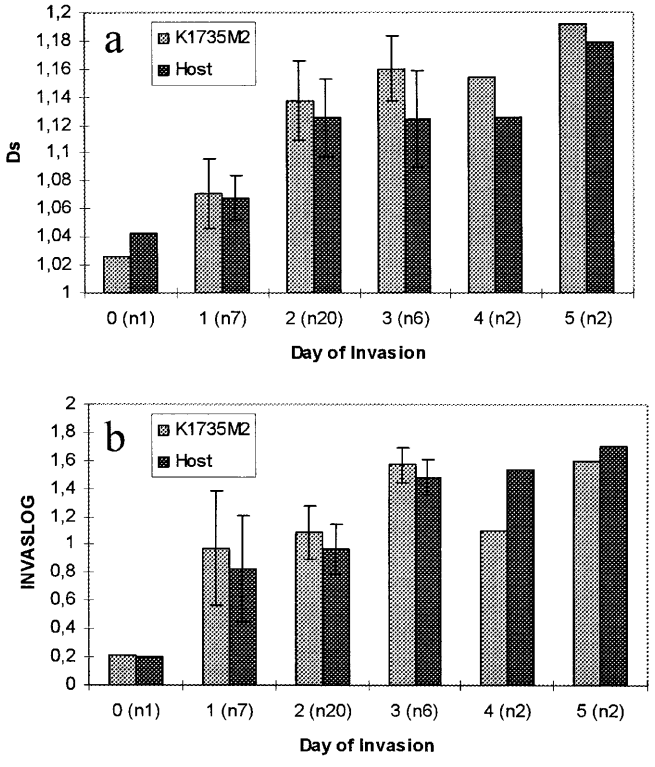

Fig. 3 Fractal capacity dimension D_s (**a**) and the quantitative parameter INVASLOG (**b**) of K1735-M2 mouse melanoma-host confrontations up to 5 days. The *n* numbers in parentheses denote the number of experiments

Table 1 Linear correlation coefficient, measuring the correlation between the capacity dimension D_s or the quantitative parameter INVASLOG and the day of invasion for both spheroids (K1735M2 and host). All values indicate that all parameters correlate very well within $P < 0.001$

	D_s	INVASLOG
K1735M2	0.732	0.598
Host	0.632	0.786

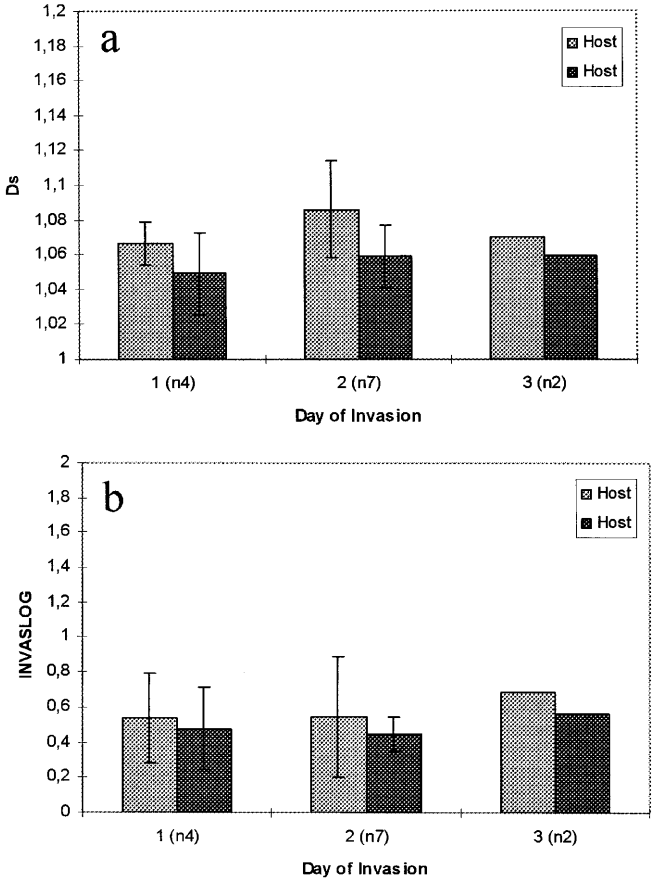

Fig. 4 Fractal capacity dimension D_s (**a**) and the quantitative parameter INVASLOG (**b**) of control host-host confrontations in dependence on the confrontation duration from 1 to 3 days. The *n* numbers in parentheses denote the number of experiments

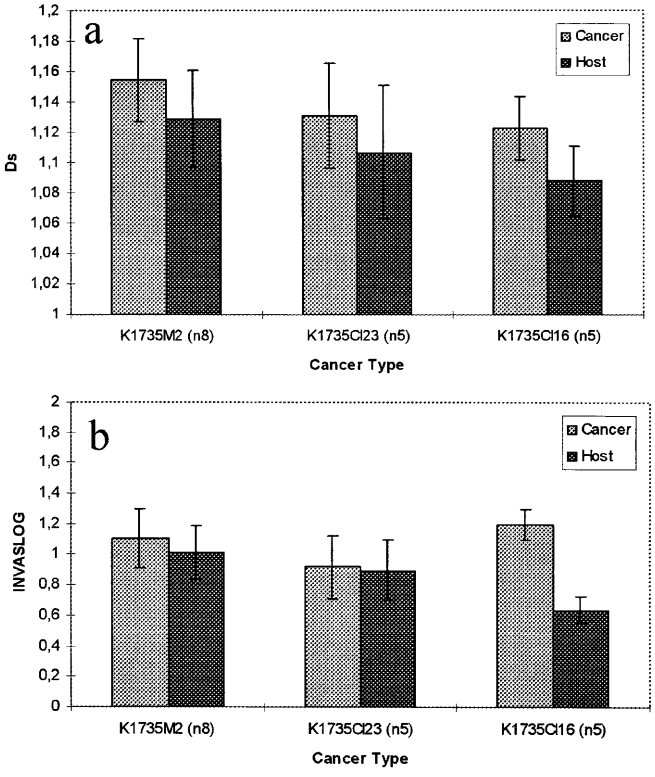

Fig. 5 Fractal capacity dimension D_s (**a**) and the quantitative parameter INVASLOG (**b**) of K1735 mouse melanoma-host confrontations for M2, Cl23 and Cl16 clones at the second day of confrontation. The *n* numbers in parentheses denote the number of experiments

A further experiment investigated the various clones of the K1735 mouse melanoma, which are distinct by their differing degrees of invasiveness (Albini et al. 1989; Staroselsky et al. 1991). The data were examined exclusively on the second day of invasion in comparison to the M2 clone. The principal accordance of both parameters D_s and INVASLOG is intrinsically visible in Fig. 5. Only the INVASLOG value of the Cl16 cancer spheroids showed a raised value.

Discussion

The use of reaggregated cell spheroids and tissue confrontation (Bjerkvig 1992) has been in use for many years. The quantification of invasion in vitro for the experimental analysis of the invasive process has been made possible through the selection of appropriate and clinically relevant parameters (Smolle et al. 1990). Recent developments in cancer research have demonstrated the effective use of fractals for the early detection of cancer. Here we demonstrate the use of a fractal dimension for the determination of invasion. Various clones of the same melanoma have been compared. For all experiments the value of the fractal dimension D_s showed a clear and strong dependence on the invasion process. Following the invasion from day 0 up to day 5 in Fig. 3, it is clear that the increase of D_s accords well with the state of invasion. The values of the linear correlation coefficients in the table show that both parameters D_s and INVASLOG highly (within $P < 0.001$) correlate with the course of invasion. The values for D_s are, for both spheroids (K1735-M2 and host), closer together than the values for INVASLOG. The high value of the parameter INVASLOG with the host spheroid confirms the prior work of the reliability of this parameter as a quantitative indicator of the invasiveness (Smolle et al. 1990, 1992). As the correlation coefficients for D_s are evenly very high, the indication that the fractal capacity dimension could serve as a quantitative invasiveness parameter is strongly supported.

Evaluation of host-host confrontations showed only low values for both parameters, confirming further that the fractal dimension D_s could serve as a quantitative parameter for the invasion process. D_s probably quantifies the overall dynamic state of the object observed and this state is, despite the absence of invasion, definitely not zero because only living specimens were investigated. The overall dynamic state of the living objects can be assumed to vary with time as seen in the cancer host confrontations (Ahammer et al. 2001), but only small changes would be expected in the control experiments. This results in the observed fluctuations of the quantitative parameters seen in Fig. 4.

Finally, the results gained by investigations of various clones corroborate and harden the importance of D_s, because again the accordance with the parameter INVASLOG is obvious. As D_s presents similar values for the host as well as for the cancer (except for a small absolute difference) and these values show exactly the same dependence on the experimental conditions, it can be assumed that both values are equally suitably for the quantitative description of cancer invasion.

In order to significantly increase the relevance of the presented methods and data with regard to in vivo applications, it would be necessary to calculate D_s under various pharmaceutical conditions or under heat or ionizing radiation treatment. This could demonstrate further the properties of the fractal capacity dimension in relation to the invasiveness of cancer. This would enable the replacement of some animal experiments that are used for the determination of the quantitative data of invasion under various forms of chemo- or radiotherapy that is not only painful but also distressing for the animals involved.

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