Quantitative Evaluation of Melanoma Cell Invasion in Three-Dimensional Confrontation Cultures In Vitro Using Automated Image Analysis

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Tumor invasion is a crucial feature of tumor growth in vivo. Confrontation cultures of multicellular melanoma spheroids and embryonic chick heart fragments provide a model for invasive growth in vitro. We have developed an image analysis method, which facilitates the objective measurement of tumor cell invasion in this model. Cryostat sections of confrontation cultures were immunohistochemically stained with an antiserum directed against the stromal component for automated recognition of the stroma tissue. The slides were automatically processed by a grey level based computerized image analysis system. On Spearman's rank correlation test, 25 out of 39 parameters correlated with the reference value of invasion, which was derived from the subjective evaluation of five independent observers. Two parameters combining the stroma margin and the total amount of stroma tissue completely reproduced the judgement of the morphologists in our test set. The quantitative evaluation of tumor invasion in vitro by automated image analysis may be helpful in pharmacologic and pathogenetic studies of tumor growth. *J Invest Dermatol 94:114–119, 1990*

Malignant tumor growth and metastasis formation are characterized by both tumor cell proliferation and tumor cell invasion into the surrounding host tissue [1–4]. Whereas tumor cell proliferation has been extensively studied in the past, scientific interest has focused on tumor cell invasion particularly in recent years [5–9]. Invasion involves dissociation of tumor cells from the tumor bulk, active movement, interaction with stromal components, possible destruction of stromal components, and the ability to proliferate.

Invasion is difficult to assess in histologic sections. The situation is complex, the observer has to deal with a single view of a dynamic process, and the diagnosis of "invasive growth" is based largely on qualitative and subjective criteria [10–12]. A more detailed study of the invasive process has been achieved with complex in vitro models, including the confrontation of tumor cells with aorta, vein, lung, diaphragm [13] and embryonic chick heart fragments [10,14,15]. The model employing embryonic chick heart fragments, as described by Mareel and co-workers [14], has shown promising correlation with the infiltrative potential of tumor cell lines in animal experiments [10]. Most of the studies with this model have been evaluated qualitatively, and only occasionally has a quantitative evaluation method been reported [16]. However, objective quantitative methods would be helpful in the subtle analysis of this model, particularly in pharmacologic investigations.

In this study, we present a new set of methods for the quantitative evaluation of invasiveness of melanoma cell lines in confrontation with embryonic chick heart fragments. The procedure is based on automated computerized image analysis and facilitates an objective measurement of the complex process of tumor cell invasion under experimental conditions.

MATERIALS AND METHODS

Cell Lines The K 1735 melanoma cell line developed in a female C3H mouse after short exposure to UV radiation followed by skin painting with croton oil [17]. The sublines K 1735-cl16 and K 1735-M2 were kindly provided by Dr. I.J. Fidler (Institute for Cell Biology, M.D. Anderson Hospital, Houston, TX). The B 16 mouse melanoma parent cell line and the sublines B 16-F1 and B 16-F10 were provided by Dr. Schlick and Dr. Keilhauer (Knoll AG, Ludwigshafen, FRG).

Cell Culture Cells were grown as a monolayer on plastic in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Meckenheim, FRG) containing 4.5 g/l glucose and supplemented with 10% fetal calf serum (PAA-Laborgesellschaft, Linz, Austria), penicillin 100 IU/ml, and streptomycin 100 μg/ml (Flow Laboratories, Meckenheim, FRG). The cell cultures were maintained at 37°C in an atmosphere of 5% CO2 and 95% relative humidity. Subconfluent monolayers were harvested by mild trypsinization with 0.05% trypsin/0.02% EDTA in Ca++/Mg++-free phosphate-buffered saline (PBS).

Tumor-Multicell-Spheroids Multicellular spheroids were obtained by performing spinner culture for approximately 1 week. Cell suspensions with $2-4\times10^5$ cells/ml were stirred with a magnetic stirrer system at a speed of 120/min. Individual spheroids with a diameter of 200 μm were selected under a stereomicroscope.

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Abbreviations:
DMEM: Dulbecco's modified Eagle's medium
EDTA: ethylenediamine-tetraacetate
M: molar
PBS: phosphate-buffered saline

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Table I. Quantitative Evaluation of Tumor Cell Invasion In Vitro. Definition of Primary Morphologic Measuring Parameters

Parameter	Definition
TOTAREA	Total area in μm^2
STRAREA	Stromal area in μm^2
TUMAREA	Tumor area in μm^2
ENGAREA	Engulfed tumor area in μm^2
INVAREA	Invaginated tumor area in μm^2
STRCONT	Marginal stromal area in μm^2
CLCONT2	Marginal stromal area after 2 closures in μm^2
CLCONT4	Marginal stromal area after 4 closures in μm^2
ENGCONT	Marginal area of engulfed tumor area in μm^2

Precultured Heart Fragments Heart tissues of 8-d old chick embryos were aseptically dissected with microscissors into approximately 400 μm fragments and maintained in spinner culture with a magnetic stirrer system at a speed of 120/min for 4 d. The resulting rounded fragments were used as a stromal model in the invasion assay.

Invasion Assay For analysis of invasiveness in vitro we used the embryonic chick heart assay established by Mareel et al [14]. Preselected tumor-multicell spheroids, 200 μm in diameter, were placed into close contact with rounded chick heart fragments, 400 μm in diameter, on top of a semisolid agar medium [14] in 24-multiwell plates and allowed to attach to each other in a humidified incubator at 37°C. After an incubation period lasting between 2 h up to 7 d, confrontation cultures were withdrawn and prepared for light microscopy by fixing in Bouin Holland's solution, immersing in 1 M sucrose solution, and snap-freezing in liquid nitrogen.

Immunohistochemistry Serial sections of each confrontation culture were stained with a polyclonal rabbit anti-chicken heart antiserum [15] (provided by Dr. Mareel and Dr. De Bruyne, Ghent, Belgium). Five-micrometer cryostat sections were incubated with the primary antiserum for 30 min. After careful rinsing in PBS, a peroxidase-conjugated goat-anti-rabbit antiserum (Dako, Copenhagen, Denmark) was applied as a second reagent. Staining was achieved with aminoethyl-carbazole and hydrogen peroxide. Counterstaining was omitted. For further details see the technique of Huber et al [18].

Subjective Evaluation of Invasion From the collection of immunohistochemical sections of confrontation cultures, 10 slides obtained at different time intervals were selected for subjective evaluation. Five independent dermatopathologists provided a ranking of the 10 slides based on the following definition of invasion: "Functionally, tumor invasion is defined by dissociation of tumor cells, active tumor cell movement, and disintegration and destruction of preexisting tissue components. Morphologically, tumor invasion is characterized by an irregularity of the tumor border, the extension of tumor strands into the neighboring stroma, and by the presence of tumor nests and/or single tumor cells within the neighboring stroma. Tumor and stroma components are intermingled to a variable degree, and the stroma may show disintegration and destruction." The median ranking of the five investigators served as a standard for the subsequent testing of quantitative parameters.

Image Analysis Procedure The immunohistologic slides of the confrontation cultures were examined with an Axiomat bright field microscope (Zeiss, Oberkochen, FRG) at a magnification of 100× with use of a green filter. The TV image from a Vidicon black and white camera (Bosch, Hamburg, FRG) was fed into the image analyzer VIDAS (Kontron, Munich, FRG). A blank part of the slide was used as a reference image for shading correction. A 3 × 3 median filter, histogram normalization, and interactive editing were subsequently performed [19]. The area of the total culture and the area of the stromal component were discriminated by interactive grey level segmentation. For the measurement of grey value parameters, a lowpass filtered image was generated [19]. The primary morphologic parameters were measured after various binary image operations (Table I; Figs 1–5). Erosion is a morphologic operation that removes a certain amount of pixels at the margin of each object using a particular structuring element. "Marginal area" was determined by erosion applying an octagon operator as the structuring element (1 pixel = 2.43 μm^2) [19]. Closure is the application of dilation (which adds a certain amount of pixels at the margin) and subsequent erosion, which finally results in a 'smooth' contour of the object. The definitions of the measuring parameters are given in Tables I–III.

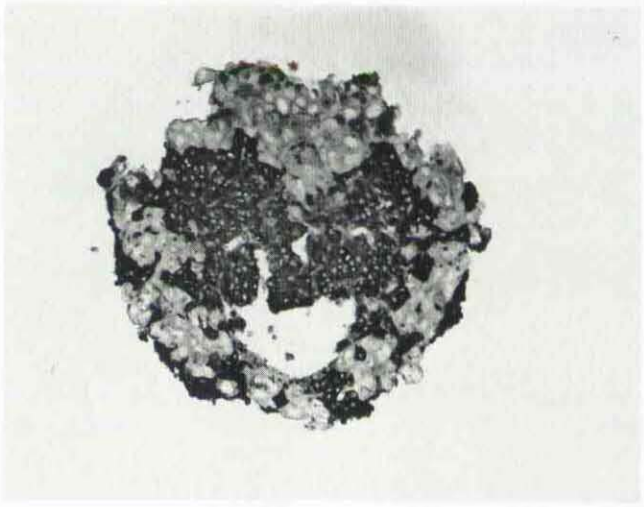

Figure 1. Melanoma cell invasion in vitro. Confrontation of a B 16-F 1 spheroid with an embryonic chick heart spheroid after 3 d. Rabbit anti-chick heart antiserum, two-step immunoperoxidase method (×100).

Statistics The ranking obtained by the median judgement of the five dermatopathologists was compared with the ranking obtained with each individual measuring parameter by Spearman's rank correlation test [20]. A p value of less than 0.05 was considered to indicate a statistically significant correlation.

RESULTS

General Observations In all specimens, the embryonic chick heart component could be clearly distinguished from the tumor component (Fig 6). During the course of confrontation, the tumor components tended to surround the stroma tissue. Subsequently, finger-like protrusions of tumor nests were found at the margin of

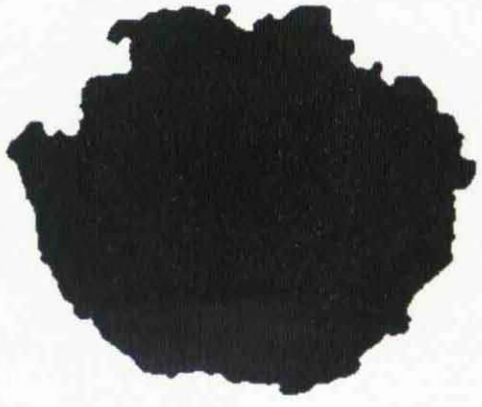

Figure 2. Melanoma cell invasion in vitro. Example of segmentation of the whole confrontation culture by automated image analysis. The artificial cleft within the section has been removed by interactive editing.

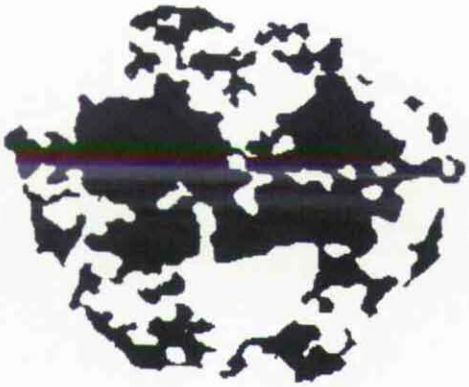

Figure 3. Melanoma cell invasion in vitro. Example of segmentation of the stroma component by automated image analysis.

Figure 4. Melanoma cell invasion in vitro. Example of segmentation of the invaginated tumor component by automated image analysis.

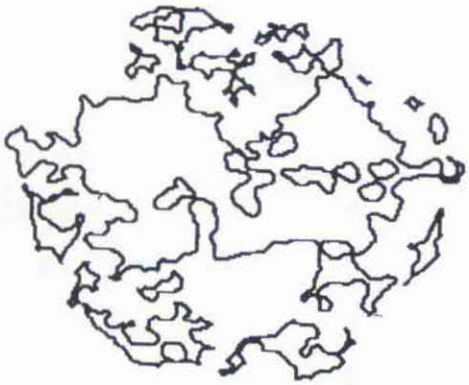

Figure 5. Melanoma cell invasion in vitro. Example of segmentation of the marginal stromal area by erosion using an octagon operator.

the stroma tissue (Fig 7). Finally, isolated sections of tumor nests appeared within the stroma, and the stroma showed signs of disintegration. The amount of stroma gradually decreased as the amount of tumor tissue increased (Fig 8). At the final stage, only small fragments of stroma tissue remained dispersed within the tumor tissue.

Primary Morphologic Parameters Six out of nine primary morphologic parameters showed a significant correlation with the ranking of the dermatopathologists (Table IV). The best parameters of the primary morphologic parameter group were INVAREA and STRCONT (Fig 9). INVAREA indicates the area occupied by tumor nests, which either extend into the stromal compartment in narrow invaginations or are engulfed in small stromal clefts.

Table II. Quantitative Evaluation of Tumor Cell Invasion In Vitro. Definition of Secondary Morphologic Measuring Parameters Calculated From Primary Parameters

Parameter	Definition
STREL	Relative stromal area (STRAREA/TOTAREA)
TUREL	Relative tumor area (TUMAREA/TOTAREA)
AREAREL	Relation of tumor and stromal area (TUMAREA/STRAREA)
ENGREL	Relation of engulfed tumor area and total area (ENGAREA/TOTAREA)
ENGSTR	Relation of engulfed tumor area and stromal area (ENGAREA/STRAREA)
ENGTU	Relation of engulfed tumor area and total tumor area (ENGAREA/TUMAREA)
INVREL	Relation of invaginated tumor area and total area (INVAREA/TOTAREA)
INVSTR	Relation of invaginated tumor area and stromal area (INVAREA/STRAREA)
INVTU	Relation of invaginated tumor area and total tumor area (INVAREA/TUMAREA)
ENGINV	Relation of engulfed tumor area and invaginated tumor area (ENGAREA/INVAREA)
STRCREL	Relation of marginal stromal area and total area (STRCONT/TOTAREA)
STRCSTR	Relation of marginal stromal area and total stromal area (STRCONT/STRAREA)
STRCTUM	Relation of marginal stromal area and tumor area (STRCONT/TUMAREA)
STRCINV	Relation of marginal stromal area and invaginated tumor area (STRCONT/INVAREA)
CLC2REL	Relation of marginal stromal area after 2 closures and total area (CLCONT2/TOTAREA)
CLC2STR	Relation of marginal stromal area after 2 closures and stromal area (CLCONT2/STRAREA)
CLC2TUM	Relation of marginal stromal area after 2 closures and tumor area (CLCONT2/TUMAREA)
CLC2CONT	Relation of marginal stromal area after 2 closures and marginal stromal area before closure (CLCONT2/STRCONT)
CLC4REL	Relation of marginal stromal area after 4 closures and total area (CLCONT4/TOTAREA)
CLC4STR	Relation of marginal stromal area after 4 closures and stromal area (CLCONT4/STRAREA)
CLC4TUM	Relation of marginal stromal area after 4 closures and tumor area (CLCONT4/TUMAREA)
CLC4C2	Relation of marginal stromal area after 4 closures and marginal stromal area after 2 closures (CLCONT4/CLCONT2)
CLC4CONT	Relation of marginal stromal area after 4 closures and of marginal stromal area before closure (CLCONT4/STRCONT)
ENGCREL	Relation of marginal area of engulfed tumor area and total area (ENGCONT/TOTAREA)
ENGCSTR	Relation of marginal area of engulfed tumor area and stromal area (ENGCONT/STRAREA)
ENGCTUM	Relation of marginal area of engulfed tumor area and total tumor area (ENGCONT/TUMAREA)
ENGCENG	Relation of marginal area of engulfed tumor area and engulfed tumor area (ENGCONT/ENGAREA)

Table III. Quantitative Evaluation of Tumor Cell Invasiveness In Vitro. Definition of Grey Value Parameters Based on a Lowpass Filtered Image of the Confrontation Cultures

Parameter	Definition
GVMEAN	Mean grey value
GVSDEV	Standard deviation of grey value
GSDEVREL	Relation of standard deviation of grey value and mean grey value (GVSDEV/GVMEAN)

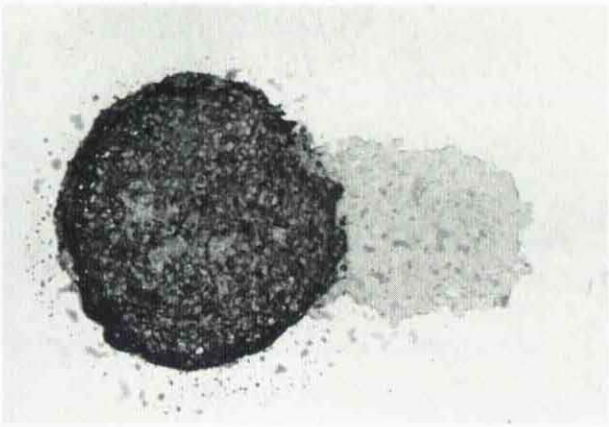

Figure 6. Melanoma cell invasion in vitro. Example of an early stage of invasion. K 1735-M2 confronted with embryonic chick heart fragment for 1 d. Rabbit anti-chick heart antiserum, two-step immunoperoxidase method (×100).

Figure 7. Melanoma cell invasion in vitro. Example of an intermediate stage of invasion. K 1735-M2 confronted with embryonic chick heart fragment for 3 d. Rabbit anti-chick heart anti-serum, two-step immunoperoxidase method (×100).

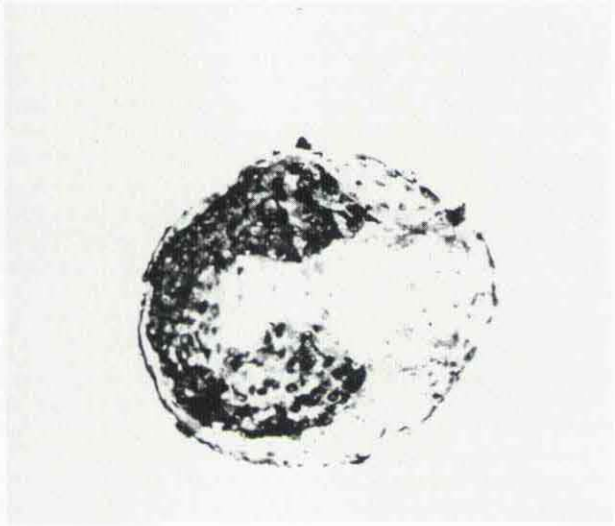

Figure 8. Melanoma cell invasion in vitro. Example of a late stage of invasion. K 1735-M2 confronted with embryonic chick heart fragment for 6 d. Rabbit anti-chick heart antiserum, two-step immunoperoxidase method (×100).

Table IV. Quantitative Evaluation of Tumor Cell Invasion In Vitro. Rank Correlation (Spearman's test) of Measuring Parameters Compared with the Median Ranking Based on Five Independent Observers[a]

Parameter	r	t	p =<
	Primary morphologic parameters		
TOTAREA	0.309	0.919	—
STRAREA	−0.684	2.658	0.05
TUMAREA	0.789	2.844	0.05
ENGAREA	−0.169	0.487	—
INVAREA	0.890	5.548	0.0005
STRCONT	0.903	5.945	0.0005
CLCONT2	0.793	3.693	0.005
CLCONT4	0.684	2.658	0.05
ENGCONT	−0.169	0.487	—
	Secondary morphologic parameters		
STREL	−0.757	3.282	0.01
TUREL	0.757	3.282	0.01
AREAREL	0.757	3.282	0.01
ENGREL	−0.169	0.487	—
ENGSTR	−0.169	0.487	—
ENGTU	−0.169	0.487	—
INVREL	0.878	5.208	0.0005
INVSTR	0.963	10.199	0.0005
INVTU	0.806	3.852	0.005
ENGINV	−0.169	0.487	—
STRCREL	0.836	4.315	0.005
STRCSTR	1.000	>18.000	0.0001
STRCTUM	0.551	1.870	0.05
STRCINV	0.878	5.208	0.0005
CLC2REL	0.830	4.213	0.005
CLC2STR	1.000	>18.000	0.0001
CLC2TUM	0.466	1.492	—
CLC2CONT	−0.527	1.755	—
CLC4REL	0.843	4.422	0.005
CLC4STR	0.987	18.000	0.0005
CLC4TUM	0.236	0.668	—
CLC4C2	−0.575	1.991	0.05
CLC4CONT	−0.612	2.189	0.05
ENGCREL	−0.169	0.487	—
ENGCSTR	−0.169	0.487	—
ENGCTUM	−0.169	0.487	—
ENGCENG	−0.181	0.522	—
	Grey value parameters		
GVMEAN	0.845	4.653	0.005
GVSDEV	−0.739	3.106	0.01
GSDEVREL	−0.903	5.945	0.0005

[a] A p value of less than 0.05 is considered to indicate a statistically significant correlation (r = coefficient of correlation, t value based on Spearman's test).

STRCONT refers to that part of the stroma which is in the immediate neighborhood of tumor tissue. Both parameters increase in value when the stromal compartment is irregularly outlined.

The significant correlation of CLCONT2 and CLCONT4, which refers to the marginal stroma area after "smoothing" of the stromal contours, shows that not only small clefts, which disappear during the smoothing process (closing operation), but also larger clefts, contribute to the irregularity of the stromal contour. Additionally, there was a positive correlation for the size of the tumor area and a negative correlation for the size of the stromal area. Total area and the amount of tumor tissue engulfed within the stromal compartment did not correlate significantly with tumor invasion.

Secondary Morphologic Parameters Sixteen out of 26 features correlated with the reference ranking (Table IV). There were two parameters that completely reproduced the ranking obtained by the morphologists. These parameters were STRCSTR (Fig 10) and CLC2STR, which refer to the relationship of the marginal stromal area to the total stromal area before and after two closing steps, respectively. These parameters are superior to the simple measure-

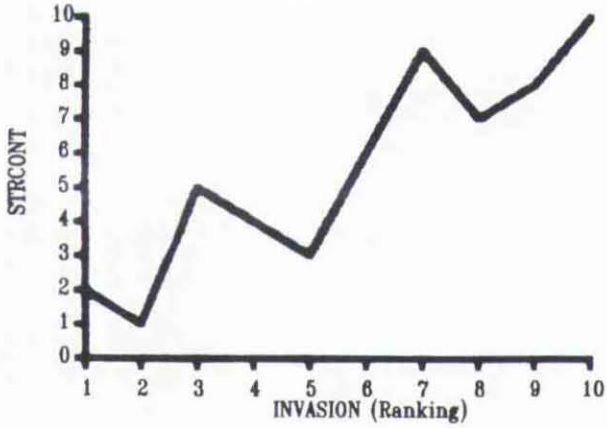

Figure 9. Melanoma cell invasion in vitro. Correlation of the measuring parameter STRCONT. The *x-axis* shows the ranking obtained by five independent observers, the *y-axis* the ranking provided by the parameter STRCONT. Spearman's rank correlation test: $r = 0.903$, $t = 5.945$, $p = < 0.0005$.

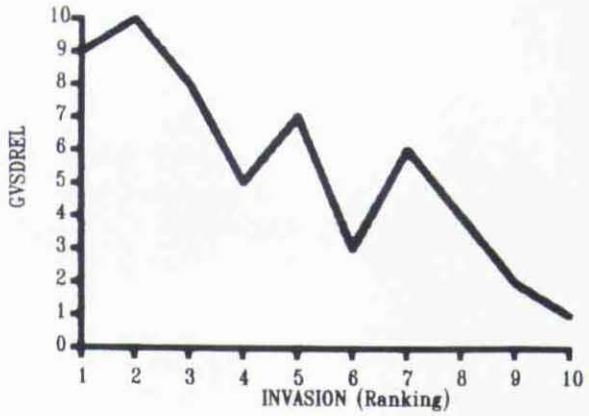

Figure 11. Melanoma cell invasion in vitro. Correlation of the measuring parameter GVSDREL. The *x-axis* shows the ranking obtained by five independent observers, the *y-axis* the ranking provided by the parameter GVSDREL. Spearman's rank correlation test: $r = -0.903$, $t = 5.945$, $p < 0.005$.

ment of the marginal stromal area, particularly in late stages of invasion, when the total amount of stromal tissue is already greatly reduced.

Another useful secondary morphologic parameter was INVSTR, indicating that the amount of invaginated tumor area is particularly consistent when related to the total stromal area.

CLC4CONT relates the marginal stromal area after four closing operations with the original marginal stromal area and indicates the presence of small clefts. Although there was a significant correlation with invasion, the parameter was far less sensitive than those using the original marginal stromal area or the invaginated tumor area.

Grey Value Parameters All three grey value parameters showed a significant correlation with invasion (Table IV). The relation of grey value standard deviation and mean grey value (relative standard deviation; Fig 11) appeared to be of particular accuracy. As the grey value parameters are all assessed after a large lowpass filter, a small standard deviation indicates a small stromal area or stromal tissue scattered more or less evenly over the whole section. A high standard deviation, on the other hand, indicates a compact stromal compartment opposed to a compact tumor compartment. As a particular advantage, the assessment of the grey value parameters does not require a previous segmentation of stroma and tumor components.

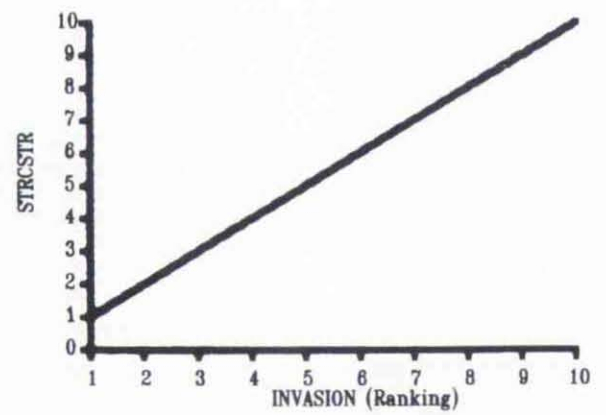

Figure 10. Melanoma cell invasion in vitro. Correlation of the measuring parameter STRCSTR. The *x-axis* shows the ranking obtained by five independent observers, the *y-axis* the ranking provided by the parameter STRCSTR. Spearman's rank correlation test: $r = 1$, $t > 18.000$, $p < 0.0001$.

DISCUSSION

Our study supports the previous finding that invasion can be simulated in an in vitro model of multicellular spheroids [8–10,13–15,21]. The method developed by Mareel et al [14] using embryonic chick heart fragments has the advantage of providing a living stromal substrate, which more closely resembles the in vivo conditions than non-cellular substrates [10,14,15].

The model used in this study clearly yields morphologic pictures that resemble the invasive growth observed in tumors in situ. Thus dermatopathologists had no difficulty in relating observations made in this model to the process of invasion in complex tumor tissues.

Our study shows that the method developed by Mareel et al [14] is suitable for quantification. Because subjective bias should be eliminated from any measuring procedure, the model should be evaluated objectively by automated image analysis [19]. Analysis is facilitated by the selective staining of the stromal component by an immunohistochemical technique using an anti-chick heart antiserum [15]. The specific staining enables a clear distinction between the tumor tissue and the heart fragments. User interaction is largely restricted to the editing of artificial folds and clefts in the immunohistologic section.

Mathematical morphology [19] provides a variety of tools to generate measuring parameters beyond basic values. The image analysis procedure of this study utilizes these tools to create a large number of parameters which in some way resemble tumor invasion. The parameters in our study are superior to those reported previously [16]. The method of De Neve et al [16] is based on the assumption that a "direction of invasion" can be calculated in each confrontation culture. However, our melanoma cell lines usually surround the chick heart fragment prior to invasion. Thus an unequivocal "direction of invasion" is no longer evident. All our parameters are independent of tissue orientation and do not rely on the direction of invasion. Care should be taken, however, that suitable "index" sections, cut through the largest diameter of the confrontation culture, are submitted to the image analysis procedure. "Cups," which only contain one or another tissue component, would definitely introduce a bias in the measuring procedure.

Evaluation of the various criteria generated by the image analysis procedure requires a suitable reference value of invasion. As the morphologic correlate of invasion is a complex and subjective feature [10–12], we used the median ranking obtained by independent dermatopathologists. The five investigators have previously been involved in the formulation of a working definition of invasion. The good interobserver correlation (Table V) indicates that the

Table V. Qualitative Evaluation of Confrontation Cultures by Five Independent Observers. Rank Correlation (Spearman's test) Between Each of the Individual Observers and Between Individual Observers and the Calculated Median Rank[a]

Observer no.	1	2	3	4	5
Observer no.					
1		r = 0.951	r = 0.975	r = 0.939	r = 1.000
2			r = 0.903	r = 0.927	r = 0.951
3				r = 0.866	r = 0.975
4					r = 0.939
Median of five observers	r = 1.000	r = 0.951	r = 0.975	r = 0.939	r = 1.000

[a] r = coefficient of correlation.

median ranking based on subjective estimates of invasion can serve as a suitable reference value.

Twenty-five out of 39 quantitative morphologic parameters generated by our procedure correlated with invasion. Some of them clearly resemble a single aspect of the working definition of invasion: TUMAREA and TUREL indicate an absolute and relative increase of the tumor component, STRAREA and STREL a concomitant decrease of the stromal component. ENGAREA shows the amount of isolated tumor nests within the stroma, and INVAREA resembles the tumor component projecting into clefts of the stroma. The secondary parameters, STRCSTR and CLC2STR, exactly reproduce the reference judgement in our test set. The parameters are particularly useful, because they take into account the total stromal area (which decreases during invasion) and the amount of stroma being in contact with tumor tissue (which usually increases during invasion). Obviously, the relationship of both values provides the best mathematical reference to the applied working definition of invasion. However, because of the multiple statistical comparisons performed, future application on large series will have to further elucidate the reproducibility of the criteria selected on the basis of this study.

The grey value parameters provided less reliable results. However, they might be useful in future studies, because they do not require a clear cut segmentation of the tumor and the stroma component. With respect to future simplification of the system, grey value parameters could be applied without the sophisticated histotechnical procedure used in this study.

We conclude that a consistent measurement of melanoma cell invasion is possible in this complex in vitro model using automated image analysis. The measuring model can be useful in the quantitative evaluation of biologic and pharmacologic effects on the in vitro system. Furthermore, the application on various tumor systems might help to elucidate the correlations between in vitro and in vivo observations.

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