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Tumor Angiogenic Activity (TAA) Production *in Vitro* and Growth in the Nude Mouse by Human Malignant Melanoma

W. STENZINGER, J. BRÜGGEN, E. MACHER and C. SORG

Department of Experimental Dermatology, University of Münster, Von-Esmarch-Str. 56, D-4400 Münster, F.R.G.

Abstract—*The production of angiogenic activity by eleven human melanoma lines* in vitro *was compared with the extent of tumor growth in the nude mouse. Angiogenic activity was assayed by measuring the vascular response of the chick chorioallantoic membrane to serum-free supernatants. Growth in the nude mouse was determined after subcutaneous injection of cells* 60 days *later. Angiogenic activities ranged from negative to highly positive. In five lines angiogenic activity* in vitro *correlated with the extent of tumor growth in the nude mouse. In contrast, two lines did not show such a correlation, e.g. they produced large tumors without any detectable angiogenic activity. Histological examination of these two tumors revealed moderate degrees of vascularization and only low degrees of necrosis. It is concluded that the extent of tumor growth in the nude mouse is partly independent of the production of angiogenic activity by the tumor cells themselves.*

INTRODUCTION

IT HAS been shown that the growth of solid tumors beyond the size of a few millimeters in diameter is strictly dependent on the establishment of an adequate blood supply to the tumor [1]. It has also become clear that the blood vessels do not originate in the tumor but are elicited from the host [2] and that the tumorigenic potential and the ability of a tumor to induce angiogenesis are closely correlated [3–5]. Most of these experiments were performed by *in vivo* techniques, e.g. for melanoma by implanting tissue into the hamster cheek pouch, the rabbit eye or onto the chick chorioallantoic membrane ([6–8]; for review of methods see [9]). Since neovascularization is not a process confined to neoplasias but also occurs in a series of normal and pathological situations such as in wound healing, diabetes and inflammation [10, 11], it is not clear whether angiogenesis was induced directly by tumor cells or by inflammatory cells and their products. A soluble tumor angiogenic factor (TAF) has been extracted from various tumor tissues or from body fluids in several instances ([12, 13]; for review see [11]). Angiogenic activity obtained by serum-free wash of tissue-cultured tumor cells was reported in two instances only [14, 15]. Quantitative differences in angiogenic factor production could be shown between different cell types [14]. So far no comparison has been made between angiogenic activity production of human tumor cells *in vitro* and the extent of tumor growth in the nude mouse.

In previous studies we have shown that human melanoma lines are heterogeneous with respect to the expression of melanoma-associated surface antigens, the production of plasminogen activator and growth in the nude mouse [16–18]. As described for the murine B-16 melanoma, we also found a good correlation between the expression of plasminogen activator production and tumor formation in the nude mouse [18, 19]. Here it was investigated whether angiogenic activity production by human melanoma cell lines *in vitro* is correlated with the extent of tumor growth in the nude mouse. No definite correlation was found between these two parameters.

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MATERIALS AND METHODS

Cells and culture media

Human melanoma cell lines (for origin of cells see [17]) and subcultured primary cultures of human adult and embryonic fibroblasts were

maintained as monolayer cultures in plastic flasks in Eagle's Minimum Essential Medium with Earle's salts (MEM-E) containing 10–15% fetal calf serum (FCS) supplemented with penicillin/streptomycin (100 μg/ml), sodium pyruvate (1 mM), L-glutamine (2 mM) and non-essential amino acids (1% v/v) (all supplements were purchased from Seromed, Munich, F.R.G.). Subclones of SK-Mel 25 were obtained as described elsewhere [16, 18]. Cells used for *in vitro* TAA production and for tumor formation were of the same passage number.

Production of tumor angiogenic activity (TAA)

Semi-confluent (50% confluency) cell cultures were washed three times with serum-free MEM-E and then incubated in serum-free MEM-E supplemented as described above plus 10 mM HEPES buffer. After 6 hr incubation at 37°C in 5% CO_2 the supernatants were removed, spun at 36,000 *g* for 30 min (Sorvall RC-5B, SS-34, DuPont, CT, U.S.A.), desalted on Sephadex G-25 coarse (Pharmacia, Freiburg, F.R.G.) and concentrated by flash evaporation at 20°C. Supernatants were concentrated to contain the equivalent of 10^7 cells. Physiological salt concentration was recovered by adding 10-fold concentrated phosphate-buffered saline (PBS). Before storage at –80°C supernatants were sterilized by membrane filtration. Cell counts were determined in a hemacytometer after trypsinization of cultures.

Bioassays of TAA on the chorioallantoic membrane of the chick embryo

Angiogenic activity was determined using the chorioallantoic membrane (CAM) of the chick embryo as decribed previously [14, 20]. On days 6–8 a 1-cm^2 window was cut into the egg shell by using the false air sac technique [21]. The window was sealed with a glass cover slide and silicone grease. On day 10 the CAM was punctured twice with a 27-gauge needle and a glass fiber disc (6 mm in diameter) SM 134 (Sartorius, Göttingen, F.R.G.) impregnated with 50 μl of the supernatant was placed onto the punctured area. Four days later the CAM was fixed in 4% (v/v) phosphate-buffered formaldehyde (Merck, Darmstadt, F.R.G.) for 30 min and excised. The vascular response under the filter disc was judged and the titer end-point was determined, e.g. the cell equivalent needed for a strong vascular response (starting concentration 1×10^7 cells/50 μl). A strong vascular response was defined as a dense corona of new capillaries growing over the borderline of the filter disc toward its center (Fig. 1). Thereby at least half of the circumference of the disc had to be crossed by new capillaries. Cell lines which did not evoke such a response at 10^7 cells equivalent were defined as TAA-negative. In order to minimize unspecific neovascularization we found it necessary during the preparation of filter discs to avoid contact with metal and to wash them in glass-distilled water. As controls filter discs alone or impregnated with PBS, MEM-E or with concentrated supernatants of human adult and embryonic fibroblasts were used.

Growth of melanoma lines in the nude mouse

Athymic, 3- to 5-week-old male nude mice of NMRI background (Zentralinstitut für Versuchstierzucht, Hannover, F.R.G.) were kept under a laminar air flow hood in autoclaved cages and bedding, receiving sterilized food and acidified water. They were inoculated s.c. on the dorsal site between the shoulder blades with viable cells (5×10^6), regenerated after trypsinization in MEM-E spinner plus 10% FCS and injected after 2 washings in 0.1 ml MEM-E. Tumor growth was monitored up to 60 days and the tumor mass was estimated in approximation according to Attia and Weiss [22].

Histological examination of vascularization and necrosis of tumors grown in the nude mouse

Sixty days after s.c. injection (see above) the tumors were excised, preventing bleeding by cauterization, fixed in 4% (v/v) phosphate-buffered formaldehyde (Merck, Darmstadt, F.R.G.) and embedded in paraffin. Five-micrometer paraffin-embedded sections were stained with hematoxylin and eosin. Ten random sections from peripheral to central parts of each tumor were used to determine the degree of tumor vascularization and necrosis. Blood vessels filled with red blood cells and showing a typical inner layer of endothelial cells were counted. For calculation of tumor vascularization and necrosis a statistical method according to Algire and Chalkley [2] was used. Briefly, this was done by projecting an ocular grid onto the histological sections and counting the visual contacts (hits) between the points where grid lines crossed and the structures of interests, i.e. blood vessels and necrotic areas. At least 50 random fields per section were observed in this way at 250–630× magnification and the average of vascular and necrotic hits were expressed as a percentage of hits or total crossing points of the grid.

RESULTS

TAA production by various human melanoma cell lines

Serum-free, concentrated supernatants of several established human melanoma lines were assayed for induction of a vascular response on the chick chorioallantois membrane. The results are

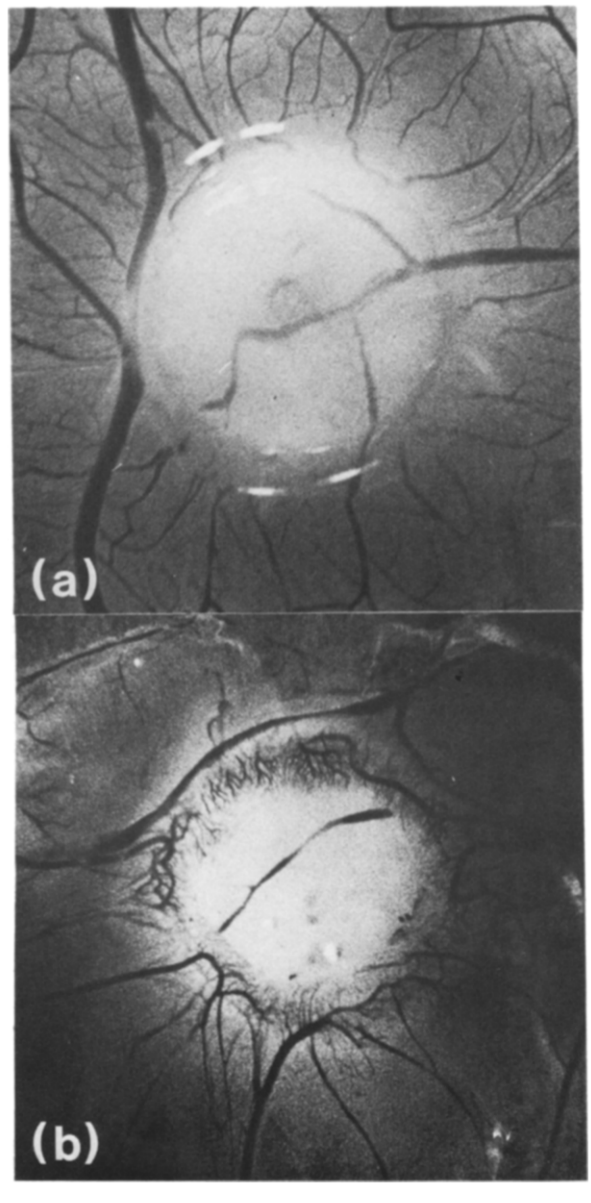

Fig. 1. *(A) Negative vascular response* 4 days *after the application of supernatant equivalent to* 10^7 *human adult fibroblasts* (× 6). *(B) Positive vascular response to an equivalent of* 10^4 *cells of line A*-375 (× 6).

shown in Table 1. Angiogenic activity ranged from no detectable response in four cell lines to an at least 1000-fold higher activity in the two cell lines A-375 and SK-Mel 25. Four sublines derived from single-cell clones of SK-Mel 25 were also included in this study. While the parental cell line SK-Mel 25 released high amounts of TAA, the sublines S-4 and S-5 were negative and the sublines S-7 and S-13 produced activity nearly as high as the parental line. Two other melanoma lines (Mel 67 and RPMI 5966) were also completely negative at the highest test concentration similarly to primary cultures of human adult and embryonic fibroblasts (data not shown).

Table 1. TAA production by human melanoma lines

Cell line	TAA cell equivalent* 10^7	10^6	10^5	10^4	10^3
A-375	6/15	5/15	4/15	2/15†	0/15
SK-Mel 25	3/15	5/15	4/15	2/15	0/15
S-7	2/15	3/15	5/15	0/15	0/15
S-13	4/15	5/15	3/15	0/15	0/15
Mel 2a	6/15	5/15	2/15	0/15	0/15
MeWo	4/15	4/15	2/15	0/15	0/15
Mel 57	5/15	3/15	0/15	0/15	0/15
Mel 67	0/15	0/15	0/15	0/15	0/15
RPMI 5966	0/15	0/15	0/15	0/15	0/15
S-4	0/15	0/15	0/15	0/15	0/15
S-5	0/15	0/15	0/15	0/15	0/15

*Cell equivalent: minimum No. of cells needed for a strong vascular response (underlined).
†Summary of 3 experiments with 5 CAMs/titer step; No. of CAMs with a strong vascular response/No. of CAMs tested.

TAA production and tumor growth in the nude mouse

Since the notion is widely held that vascularization of solid tumors is a prerequisite for tumor growth beyond the size of a few millimeters in diameter [1] we asked whether a high production of TAA by melanoma cells would correlate with the extent of tumor growth in the nude mouse. The results are shown in Table 2. Again a great heterogeneity among melanoma lines is seen. Five out of eleven melanoma lines produced TAA *in vitro* and grew in the nude mouse. In contrast, three lines (RPMI 5966, Mel 67, S-5) which released no detectable amount of TAA produced tumors in the nude mouse.

Because of this lack of a clear correlation the question was asked whether there was any correlation between TAA production *in vitro* and the degree of vascularization and/or necrosis in the tumors. To determine these parameters paraffin-embedded, formaldehyde-fixed and hematoxilin- and eosin-stained sections were used. The prerequisite for a good representation of blood vessels in the tumor was a fast and exact

Table 2. Comparison of various human melanoma lines with regard to TAA production in vitro, growth in the nude mouse, and vascularization and necrosis of tumors grown in the nude mouse

Cell line	A-375	SK-Mel 25	S-7	S-13	Mel 2a	MeWo	Mel 57	RPMI 5966	Mel 67	S-5	S-4
TAA production*	1:1000	1:1000	1:100	1:100	1:100	1:100	1:10	0	0	0	0
Tumorigenicity†	5/5	0/5	0/5	2/5	5/5	5/5	4/5	3/5	3/5	2/5	0/5
Tumor mass (mm^3)‡	1850	—	—	25	250	1630	120	110	40	20	—
	(1440–1980)			(20–30)	(200–300)	(1060–2110)	(70–180)	(70–140)	(30–54)	(16–24)	
Vascularization (vol. %)§	0.6	—	—	0	2.8	1.4	1.9	0.9	1.2	0	—
	(0.55–0.7)				(2.5–3.9)	(1.2–1.5)	(1.5–2.1)	(0.7–1.1)	(1.0–1.3)		
Necrosis (vol. %)§	36.9	—	—	57.3	0	3.3	1.1	2.8	2.5	18.3	—
	(25.2–43.6)			(45.4–60.9)		(2.9–3.7)	(0.9–1.7)	(2.0–3.2)	(1.9–3.1)	(9.8–25.1)	

*End-point titer of supernatant equivalent to 10^7 cells (0: negative at an equivalent of 10^7 cells).
†No. of tumors grown per total No. of mice injected.
‡Tumor mass after 60 days estimated according to Attia and Weiss [22]; range in parentheses.
§Determined according to Algire and Chalkley [2]; range in parentheses.

cauterization of blood vessels during its excision such that most of the blood vessels were filled with red blood cells and could easily be recognized. The results are shown in Table 2. Four cell lines showing TAA production *in vitro* also formed vascularized tumors in the nude mouse. Two other melanoma lines (RPMI 5966 and Mel 67) which were TAA-negative produced moderately vascularized tumors. The two sublines S-5 and S-13 both formed avascular tumors, even though one of these (S-13) produced a rather large amount of TAA *in vitro*. The overall vascularization in all tumors ranged from 0.6 to 2.8 vol.%. In 7 out of 8 different tumors necrotic areas were found ranging from 1.1 to 57.5 vol.%. Three tumors (RPMI 5966, Mel 67, S-5) which were TAA-negative showed moderate to negative vascularization and some areas of necrosis. No necrosis was found in tumors of Mel 2a, which displayed the highest degree of vascularization (2.8 vol.%). Again definite correlation between TAA production *in vitro* and vascularization of the tumor *in vivo* could not be recognized.

DISCUSSION

Tumor growth and formation of metastases is a multistep process whose mechanisms are poorly understood. On a cellular level it requires the phenotypic expression of properties like invasion of surrounding tissue, growth in target organs and resistance to local or systemic growth-regulating and rejection mechanisms. It is not clear at present whether a highly metastatic phenotype has to express the various properties simultaneously or in sequence. It is also not clear whether all required biochemical and biological properties have to be expressed by the tumor cell itself or whether some auxiliary functions are provided by certain inflammatory cells. In very few instances a molecular equivalent has been correlated to a biological property of a tumor phenotype, e.g. expression of plasminogen activator production by murine melanoma cells has been found to be very closely linked with the property of cells to form tumors in the syngeneic host [23]. A similar close correlation has been described in human malignant melanoma cells for the production of plasminogen activator and tumor growth in the nude mouse [18]. No correlation between plasminogen activator production and tumorigenicity or malignancy, however, was found for a large number of non-melanoma tumor cells [24–26]. In the present study we investigated a possible relation between tumor angiogenic factor production *in vitro* and the extent of tumor growth in the nude mouse by human melanoma lines. A great heterogeneity in the capacity of tumor cells to produce angiogenic activity and to grow in the nude mouse was seen, yet no definite correlation between these two parameters could be recognized. As an assay for TAA we used the vascular response of the chick chorioallantoic membrane. In contrast to Folkman [20] and Klagsburn *et al.* [14], the appearance of a 'red spoke wheel' converging to the borderline of the disc was not considered to be a sufficient criterion for a strong vascular response. Rather, we chose to define a strong positive response according to other authors [27] when the new capillaries grew beneath the filter disc and/or crossing its borderline (Fig. 1b). This strong positive vascular response could clearly be distinguished from the slight neovascularization due to the fiber disc which was occasionally found in controls. The tendency of this assay to be false-negative rather than false-positive and the strict standards imposed may be held responsible for the low yield of strong vascular responses (from 2 out of 15 to 6 out of 15), as shown in Table 1.

Because of these rather unexpected results it was of interest to examine the tumors histologically with regard to their vascularization and/or extent of necrosis. Again no correlation between the investigated parameters could be recognized. Despite of high TAA production *in vitro*, the line A-375 was vascularized mainly in the outer areas of the tumor, while the inner tumor mass was largely necrotic. A possible explanation could be that the tumor grew faster than the capillaries or that blood vessels in the central part of the tumor were compressed by the growing tumor [1]. On the other hand, it is difficult to understand why the slowly growing tumor of S-13 was highly necrotic and not vascularized even in the outer areas while producing high amounts of TAA *in vitro*. Furthermore, the lines RPMI 5966 and Mel 67, which were TAA-negative, displayed medium range vascularization and rather low degrees of necrosis. In view of these inconsistencies it is obvious that TAA production *in vitro* is not a reliable criterion for either the extent of tumor growth or for vascularization of a tumor *in vivo*.

The following assumptions can be made in order to explain these observations: first, the predominant tumor phenotype growing in tissue culture medium is not identical with the predominant phenotype in the nude mouse. In previous studies we showed that tissue culture lines are heterogeneous and subclones can be isolated with different serological, biochemical and biological properties [18]. Such subclones of SK-Mel 25 have been included in this study; second, apart from the possibility that different environmental conditions select for different subpopulations, it could also be possible that the

tumor phenotype itself changes into a different functional state under appropriate environmental signals. Evidence for the plasticity of tumor phenotypes have been described [23]. It was also shown that despite the fact that most tumors are monoclonal in origin, their clonal descendants might be phenotypically different compared to their parental cells, thus providing a biological basis for tumor progression [18, 28]; and third, it is possible that those tumor cells that are not capable themselves of producing TAA may induce inflammatory cells to substitute for the lack of TAA. Likely candidates for auxiliary cells might be mast cells, lymphocytes and macrophages within the inflammatory infiltrate [5, 10, 29]. It has been shown that thioglycollate-induced peritoneal macrophages from the BALB/c mouse are also capable of inducing angiogenesis [30]. Since we observed that the tumors formed in the nude mouse were surrounded by a fibrous capsule containing a mononuclear cell infiltrate, we are now investigating this possibility in more detail. So far thioglycollate-induced peritoneal macrophages from nude mice were also found to release high amounts of angiogenic activity into the culture supernatant (unpublished observations). A comparison of the angiogenic activity derived from murine macrophages with that from human melanoma cells by high-performance liquid chromatography showed an identity with regard to the molecular weight (unpublished observations). This might be an indication that tumor angiogenesis can be achieved not only by the tumor cells themselves but also by inflammatory cell infiltrates using the same or similar molecular mechanisms, which is in line with the observations or interpretations of other authors [5, 10, 31].

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