Virchows Arch (1996) 428:99–106

ORIGINAL ARTICLE

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Tumour-cell-endothelial interactions: free radicals are mediators of melanoma-induced endothelial cell damage

Received: 14 November 1995 / Accepted: 5 January 1996

Abstract Damage to vascular endothelium may play an important role during metastasis. We used a three-dimensional model of tumour cell extravasation to test the hypothesis that certain types of tumour cells are able to induce vascular endothelial cell injury. Multicellular tumour spheroids (MCTS) of 14 human cancer cell lines and spheroids from two benign cell lines were transferred onto confluent monolayers of human endothelial cells (EC). MCTS from 4 of 7 melanoma cell lines induced damage of the endothelium which was closely associated with tumour cell attachment. Endothelial cell injury became evident morphologically by loss of cell membrane integrity and sensitivity to shear stress. Similar results were obtained with EC derived from human umbilical veins, umbilical arteries and saphenous veins. Addition of the oxygen radical scavenger catalase showed a dose- and time-dependent inhibition (up to 48 h) of EC damage in the case of the melanoma cell lines ST-ML-11, ST-ML-14 and SK-MEL-28. The scavenging enzyme superoxide dismutase proved to be protective (up to 12 h) in ST-ML-12 MCTS. In contrast, allopurinol, deferoxamine mesylate, ibuprofen, nor-dihydroguaretic acid, soybean trypsin inhibitor or aprotinin had no protective effect. None of the non-melanoma cancer cell lines or benign cells induced endothelial cell damage. Endothelial injury has been shown to enhance the process of metastasis. Our results suggest that free-radical-mediated endothelial cell damage may be one of the mechanisms contributing to the devastating metastatic potential of melanoma.

Key words Melanoma · Endothelial cell damage · Free radicals

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Introduction

To metastasize, tumour cells must invade and enter the vasculature, survive within the circulation, bind to and traverse the vascular endothelium and basement membrane, and proliferate in a foreign location [10, 22, 25, 38, 42]. Only specialized subpopulations of tumour cells can perform all of the steps necessary in this complex, highly selective cascade.

During tumour cell arrest and extravasation the vascular endothelium plays a dual role. On one hand, it serves as a site for specific or non-specific tumour cell adhesion [9, 10, 22, 25, 32, 42]. On the other hand the intact endothelial cell lining forms a protective anatomical barrier to extravasation by limiting tumour cell access to the subendothelial basement membrane, a structure to which tumour cells demonstrate preferential adhesion [10, 18, 22, 25, 28]. In vivo, cytotoxic drugs, X-irradiation or hyperoxaemia can cause endothelial cell damage and both animal experiments as well as clinical observations indicate that injury of the vascular endothelium promotes the process of metastasis [10, 20, 22, 42]. However, the molecular mechanisms of the destruction of the integrity of the vascular wall during tumour cell extravasation are still poorly understood. While the importance of specific degradative proteases such as collagenases is well-documented, there is increasing evidence that reactive oxygen species are also involved [1, 20, 38, 42]. Generated by host leucocytes clustered with tumor cells or by radiation or chemotherapy, they are thought to attack the endothelium as well as the vascular basement membrane [12, 20, 22, 29]. Recent observations, moreover, have demonstrated that neoplastic cells produce increased levels of reactive oxygen species and thus may themselves have

the capability to damage vascular endothelial cells (EC) [6, 35–37, 39]. However, whether this capability is restricted to certain types of cancer is unknown.

In vivo, as a result of cell-cell interaction, multicellular clusters of cancer cells also commonly enter the vasculature and have repeatedly been shown to be much more effective in initiating metastases than single circulating tumour cells [10, 21, 22, 42]. Increased mechanical trapping and better tumour cell survival as well as metabolic cooperativity within these tumour cell emboli may account for this phenomenon [10, 31, 42, 43]. Multicellular tumour spheroids (MCTS) provide well-defined in vitro models of tumour cell clusters and have been used in various models of invasion [3, 17, 22]. We have adopted MCTS to simulate the interaction of tumour cell emboli with the vascular endothelium and recently demonstrated that the melanoma cell line ST-ML-12 causes free-radical-mediated damage of human umbilical vein EC [27, 28]. In the present study we have used this model to test the hypothesis that certain types of tumour cell – in particular melanomas – injure vascular endothelium.

Materials and methods

Human melanoma cell lines Hs294T and SK-MEL-28, derived from metastases, were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). The human melanoma cell lines ST-ML-11, ST-ML-12, and ST-ML-14 were established and cloned at the Department of Dermatology, Free University of Berlin, Germany from a cutaneous metastasis, a primary nodular melanoma, and a metastatic pleural effusion respectively, and were kindly provided by Dr. C. Garbe [11]. The metastatic melanoma cell lines SK-MEL-25 and MeWo were a gift from Dr. C. Sorg, Department of Experimental Dermatology, University of Münster, Germany [5].

The human breast carcinoma cell line ZR75.1 (metastatic pleural effusion), the choriocarcinoma cell line BeWo, and the colon carcinoma cell lines CaCo-2 and HRT18 were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The transitional cell carcinoma cell lines RT4 and J82 were provided by Dr. R. Knuechel (Department of Pathology, University of Regensburg, Germany) [23]. The grade III transitional cell carcinoma cell line HOK-1 was established in our laboratory [26]. Skin fibroblasts (Malme 3) were obtained from the ATCC. Cultures of human articular chondrocytes (HAC) were kindly provided by Dr. H. Rixen, Institute of Cytopathology, University of Düsseldorf, Germany.

All cell lines were maintained as monolayers in 25 cm^2 culture flasks (Falcon, Oxnard, Calif., USA) in a 1:2 mixture of Iscove's modified Dulbecco's medium and Ham's F-12 medium (Gibco, Paisley, Scotland) supplemented with 20% heat-inactivated human serum and penicillin/streptomycin (100 IU/ml, 100 mg/ml, Seromed, Berlin, Germany) at 37°C in humid air with 5% CO_2.

MCTS of the various cancer cell lines and spheroids of benign cells were produced using the liquid overlay culture technique as described previously [27]. Briefly, cells were detached using trypsin/versene (0.05% /0.02%, Seromed), washed once in medium, and then plated on agarose-coated (1%, Serva, Heidelberg, Germany) 96-multiwell test plates. To obtain spheroids of approximately the same size (220–250 µm in diameter) cell suspensions were serially diluted and the cells seeded at concentrations of 500–2000 cells/200 µl to initiate spheroid formation. Within 4 days of incubation the cells formed tight aggregates, which were then used for co-cultures.

For endothelial cell culture human umbilical cords and human saphenous veins were obtained form consenting donors. Human umbilical vein endothelial cells (HUVEC), human umbilical arterial endothelial cells (HUAEC) and human saphenous vein endothelial cells (HSVEC) were then washed out from the respective blood vessels after collagenase treatment (0.5%, Worthington, Boehringer, Mannheim, Germany), as described previously [27]. All EC were routinely grown in 75-cm^2 tissue culture flasks (Falcon) coated with 0.2% gelatine and maintained in the same medium as the tumour cell lines (see above). For experiments 24-well tissue culture dishes were coated with fibronectin (10 µg/ml, Sigma, St. Louis, Mo., USA) by overnight adsorption of the protein (500 µl/well) at 4°C and rinsed in phosphate-buffered saline (PBS). EC were then harvested with collagenase (0.5%), seeded, and grown to confluency in the fibronectin-coated wells. EC phenotype was demonstrated by their cobblestone morphology as well as by demonstration of factor-VIII-related antigen using a specific monoclonal antibody (Dakopatts, Glostrup, Denmark). EC used in experiments were either at passage 1 or 2.

To establish co-cultures MCTS were harvested with glass pipettes from the agarose-coated dishes, sedimented through a column of 10 ml fresh medium, and then transferred onto confluent EC monolayers (1 MCTS/well) in a volume of 1 ml in 24-well tissue culture dishes. Co-cultures were run in triplicate under the various culture conditions and used to analyse cell membrane integrity and shear stress sensitivity after defined periods from 1.5–72 h. Melanoma-endothelial cell cultures were also used for ultrastructural analyses.

To modulate the co-culture conditions catalase from bovine liver (200–8000 U/ml; Serva), superoxide dismutase from bovine erythrocytes (0.3–1 mg/ml; SOD), deferoxamine mesylate (10 mM), allopurinol (10 mM), ibuprofen (0.1 mM), nor-dihydroguaretic acid (1 mM), and the proteinase inhibitors soybean trypsin inhibitor (1–5 mg/ml) and aprotinin (12 TIU/ml) (all from Sigma) were repeatedly included in the co-cultures.

Cell membrane integrity was tested by using a combination of the supravital fluorescent dye acridine orange (AO) and the fluorescent dye ethidium bromide (EB) as described previously [27, 28]. Briefly, a stock solution of 0.3 mg/ml 3,6-*bis* (dimethylamino) acridine (Sigma) and 1 mg/ml 2,7-diamino-10-ethyl-9-phenylphenanthridinum bromide (Sigma) was prepared in ethanol/Gey's balanced salt solution (GBSS; 1% v/v ethanol) and stored frozen at –20 °C. After removal of the co-culture medium final dilutions (1:100 in GBSS) of the dye were carefully added (0.5 ml) to the co-cultures for 2 min at room temperature. Thereafter the AO-EB assay solution was removed and the specimen examined in a fluorescence inversion microscope (Zeiss IM 35) at an excitation wave length of 490 nm. AO readily penetrates cell membranes of viable cells and intercalates with double-stranded nuclei acids, resulting in a green nuclear and cytoplasmic fluorescence. EB only enters cell with damaged cell membranes and intercalates with double-stranded nucleic acids exhibiting a red-orange fluorescence of cell nuclei.

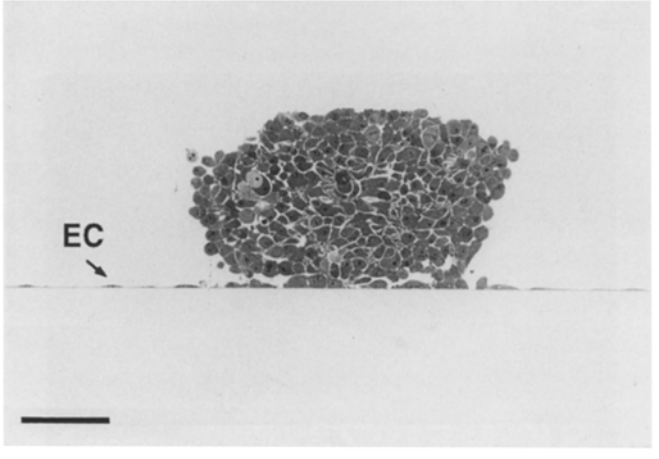
Shear stress was applied as described previously by removing the co-culture medium and rinsing the co-cultures three times with fresh medium. Quantification of damaged/intact EC was performed as described previously [28]. Briefly, colour prints of the co-cultures were used to count the viable EC per culture area (4.5 mm^2). The percentage of damaged cells was then determined by the formula: $A–B/A\times100$, where A equals the number of viable cells in controls (HUVEC alone) and B the number of viable cells found under experimental conditions. In every experiment MCTS of the cell line ST-ML-12 were used as a positive control to assure appropriate function of the membrane integrity assay.

For electron microscopy co-cultures were carried out on fibronectin-coated 12-mm glass coverslips placed in 24-well tissue culture dishes. Specimens were then fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5, for 30 min at 4°C, rinsed three times in cacodylate buffer, and postfixed with 1% OsO_4 in 0.1 M cacodylate. Samples for scanning electron microscopy were then dehydrated, critical point-dried, coated with 20 nm gold in a sputter coater and viewed in a Philips SEM 515 scanning electron microscope at 15 or 30 kV. Transmission electron microscopy was

conducted in samples of co-cultures as well as in multiple samples of MCTS which were directly harvested from agarose coated dishes. They were dehydrated in graded ethanols and embedded in Epon. Semi-thin sections (1 μm) were stained with methylene-blue-azure II. Ultra-thin sections were mounted on copper grids, stained with lead citrate and uranyl acetate, and viewed in a Philips 400 transmission electron microscope.

Results

MCTS cultured from 14 different human tumour cell lines were confronted with EC monolayers. Every cell line was tested in at least two independent experiments using EC from different donors. Depending on the various cell lines the attachment of the tumour cell clusters to the endothelium was noticed within 45–90 min after their inoculation onto the EC monolayers (Fig. 1). When assayed for their potential to induce vascular EC injury MCTS from 4 of 7 melanoma cell lines tested (ST-ML-11, ST-ML-12, ST-ML-14, and SK-MEL-28) induced extensive EC damage. In all of these cell lines this injury became apparent morphologically within a localized circular area around the site of tumour cell attachment. This was demonstrated by a loss of the integrity of the EC membranes when analysed with AO-EB (Fig. 2). Concomitantly the damaged EC became sensitive to shear stress. Rinsing of the co-cultures resulted in EC detachment and exposure of the subendothelial matrix within this perispheroidal halo (Fig. 3). The extent of the endothelial damage – the diameter of the halo – was almost fully developed immediately after the attachment of the MCTS and did not significantly increase during the further co-culture period (up to 72 h of incubation). However, slight differences in the size of the halos were noticed between the different cell lines, the diameters being smaller in co-cultures with the cell line SK-MEL-28. Occasionally, a few EC in the area of injury remained intact for a short period of time. However, in all experiments with injurious melanoma cell lines a small rim of endothelium in the immediate vicinity of the attached tumour cell clusters remained intact for up to 12 h before showing evidence of cellular damage. This phenomenon was noticed in assays for membrane integrity of EC as well as for sensitivity to shear stress (Figs. 2, 3). In separate experiments melanoma MCTS were mechanically disaggregated to obtain tumour cell clusters of smaller size. These experiments revealed that also very small emboli (diameter of approx. 50 μm) induced an EC injury and that the size of the halo of damaged EC correlated with the size of the inoculated melanoma cell clusters (Fig. 4). In contrast, single cell suspensions prepared of the 4 injurious melanoma cell lines even when inoculated in high concentrations (1×10^5 cells/well) did not elicit any EC injury.



melanoma cell line ST-ML-12 attached to a monolayer of HUVEC 6 h after inoculation into the endothelial cell (*EC*) culture. Note the raised spheroid on the flat EC layer (*arrow*). Light microscopy, methylene-blue-azure II (*bar* 60 μm)

The endothelial cell damage induced by injurious melanoma cell lines was similarly observed using either HUVEC, HUAEC or HSVEC for confrontation. In contrast with melanoma, none of the other tumour cell lines induced alterations of the endothelium. Furthermore, spheroids of skin fibroblasts and human articular chondrocytes were used in additional control co-cultures and proved to have no deleterious effect (Table 1).

To elucidate the cytopathic mechanism induced by the melanoma cells, co-cultures with monolayers of HUVEC were modulated by various inhibitors. Each of these substances was added to cultures of EC along with the spheroids for up to 72 h. The ferric iron chelator deferoxamine mesylate, the xanthine oxidase inhibitor allopurinol, the cyclo-oxygenase inhibitor ibuprofen, the lipoxygenase inhibitor nor-dihydroguaretic acid, and the proteinase inhibitor aprotinin had no protective activity. In contrast, the addition of the free radical scavengers catalase or SOD resulted in a complete, dose- and time-dependent suppression of the melanoma-induced EC damage (Fig. 2, Table 1) as analysed by AO-EB as well as studies of shear stress sensitivity. Figure 5 depicts the time course of the protective effect exerted by catalase in co-cultures of HUVEC with the cell lines ST-ML-11, ST-ML-14 and SK-MEL-28. Considerable differences were noticed between the various cell lines with regard to the dose of catalase necessary for protection. In co-cultures of ST-ML-11 and ST-ML-14 a dose of 2000 U/ml afforded EC protection for up to 12 and 24 h, respectively, whereas higher doses (4000–8000 U/ml) were required to protect the endothelium in co-cultures with SK-MEL-28 (Fig. 5). Moreover, the time span of inhibition of endothelial injury appeared to be significantly shorter (6 and 12 h respectively) in the latter cell line. After the waning of the protective effect of the scavenging enzymes, EC damage became apparent by the progressive formation of an expanding halo of injured endothelium.

In some co-cultures of the cell lines ST-ML-14 and SK-MEL-28 the protease inhibitor soybean trypsin inhibitor also showed a mild inhibitory activity resulting – especially in earlier time periods – in slightly smaller diameters of the halos of damaged endothelium. However, even at higher doses (5 mg/ml) this inhibitor was never

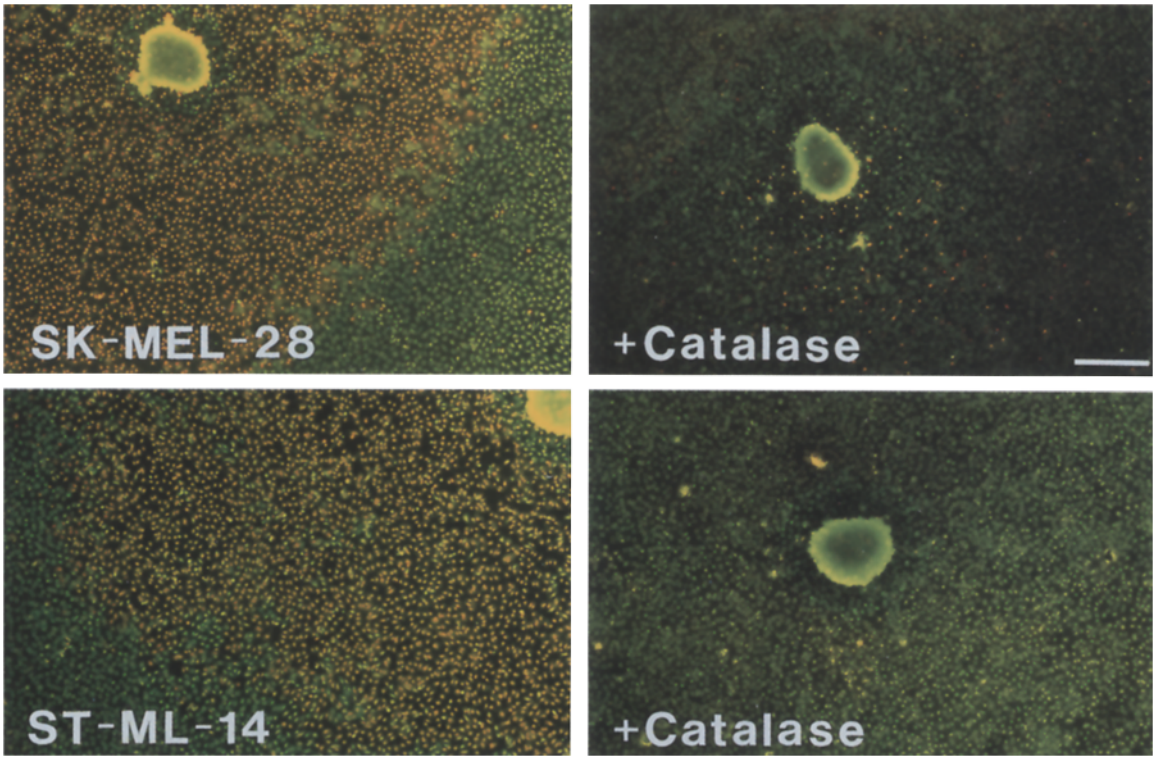


Fig. 2 Damage to EC by the melanoma cell lines SK-MEL-28 and ST-ML-14. Figure parts on the *left* show co-cultures of the melanoma cell lines 6 h after the attachment of the MCTS to HUVEC. A halo of injured endothelium is demonstrated using the membrane integrity assay AO-EB. Note the initial survival of a small rim of endothelium in the immediate vicinity of the tumour cell clusters. The *right panel* depicts the respective co-cultures treated with catalase (4000 U/ml). In both cell lines catalase completely suppressed the EC damage in a dose- and time-dependent manner. Light microscopy, AO-EB (*bar* 220 μm)

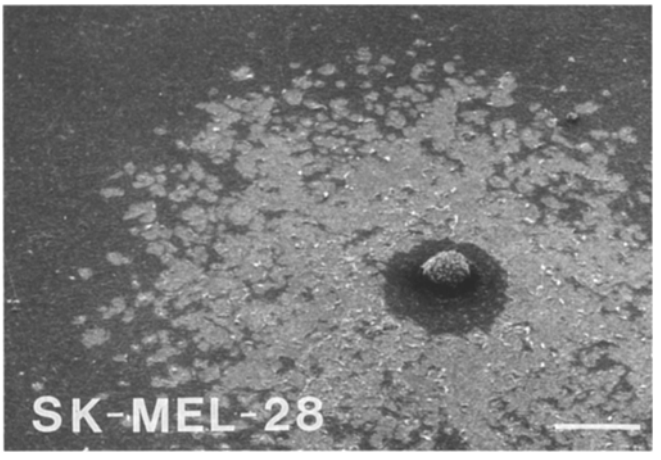


Fig. 3 Shear stress sensitivity of damaged EC. Scanning electron micrographs of a co-culture of the melanoma cell line SK-MEL-28 with HUVEC 6 h after the attachment of the tumour cell cluster. Rinsing of the co-culture resulted in detachment of damaged EC and denudation of subendothelial matrix. Note a small rim of stress-resistant intact EC in the immediate vicinity of the tumour cell cluster corresponding to EC with intact membranes as determined by AO-EB in Fig. 2. Scanning electron microscopy (*bar* 500 μm)

able to completely suppress the cytopathic effect of the MCTS. In additional experiments both MCTS and EC were also preincubated with the scavengers for 24 h, rinsed with PBS to remove the scavengers and then co-cultured in normal medium. No inhibition of endothelial injury could be obtained under these conditions.

Several ultrastructural investigations of melanomas have reported abnormalities of the specific organelle, the melanosome. Recently, it has been proposed that defects of the limiting melanosomal membrane may result in an efflux of toxic oxygen species produced in the course of melanogenesis [4, 8, 15, 16]. Comparative ultrastructural analyses of the melanoma MCTS showed considerable

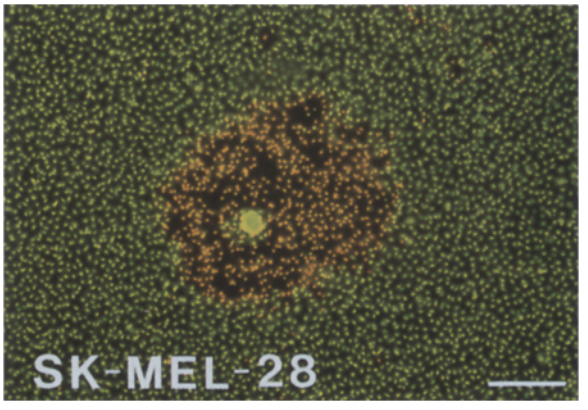


Fig. 4 Damage to endothelium by fragments of SK-MEL-28-MCTS. Even small tumour cell clusters induced EC injury. The diameters of the halos of damaged EC correlated with the size of the attached tumour cell emboli. Light microscopy, AO-EB (*bar* 220 μm)

Table 1 Endothelial cell damage induced by human cancer cell lines. The cell line ST-ML-12 was tested previously [27] (+ appearance of a halo of damaged EC, – no endothelial cell injury noticed, *ND* not done)

Cell line	Endothelial cell injury	Protective agent
Melanoma		
ST-ML-11 (metastasis)[a]	+	Catalase (2000–8000 U/ml)
ST-ML-12 (primary tumor)[a]	+	Superoxide dismutase (0.3–1 mg/ml)
ST-ML-14 (metastasis)[b]	+	Catalase (2000–8000 U/ml)
SK-MEL-25 (metastasis)[c]	–	ND
SK-MEL-28 (metastasis)[b]	+	Catalase (4000–8000 U/ml)
Hs294T (metastasis)[b]	–	ND
MeWo (metastasis)[d]	–	ND
Carcinoma		
CaCo-2 (colon, grade I)	–	ND
HRT-18 (colon, grade III)	–	ND
ZR75.1 (breast, metastasis)	–	ND
BeWo (choriocarcinoma, metastasis)	–	ND
RT4 (bladder, grade I)	–	ND
J82 (bladder, grade II)	–	ND
HOK-1 (bladder, grade III)	–	ND
Benign cells		
HAC (chondrocytes)	–	ND
MALME-3 (fibroblasts)	–	ND

[a] Pigmented
[b] Hardly any pigmentation
[c] Strong pigmentation
[d] No pigmentation

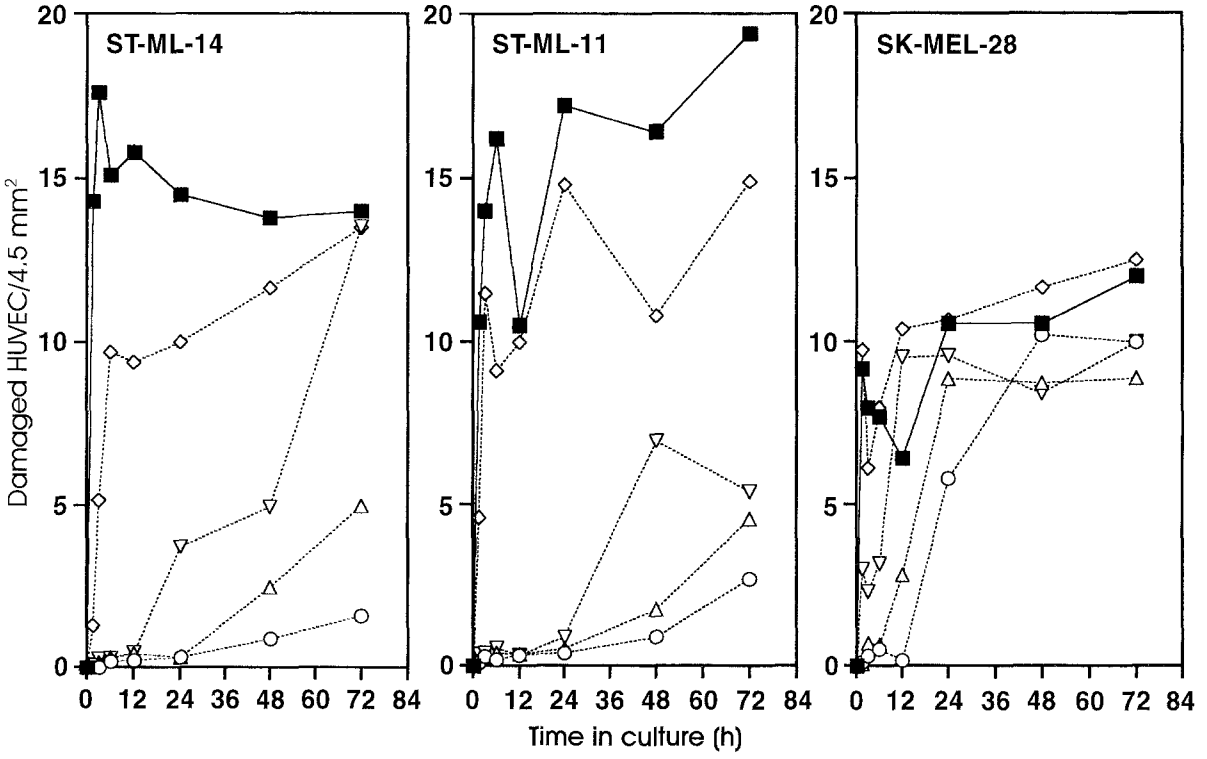


Fig. 5 Suppression of melanoma-induced EC damage by catalase. The EC damage was completely abrogated by catalase in a dose- and time-dependent manner. Time course of the endothelial damage. At the indicated time points triplicate co-cultures were stained with AO-EB and the percentage of damaged HUVEC determined per unit area. Data points represent the means of two representative independent experiments (■ co-culture in normal medium, ◇ 200, ▽ 2000, △ 4000, ○ 8000 U/ml catalase)

variation of the melanosomal structure and the degree of pigmentation, the latter being strongest in the cell line SK-MEL-25 and absent in the cell line MeWo (Table 1). Every cell line contained melanosomes exhibiting a variety of shapes. This pleomorphism of the melanosomes was accompanied by considerable derangement of the internal organization with filaments, showing an angulated or zigzag outline and forming irregular coils and spirals. However, although we could find occasional filaments which were not unequivocally limited by a melanosomal membrane in the cell line SK-MEL-28, this observation was not made in the other melanoma cell lines (Fig. 6).

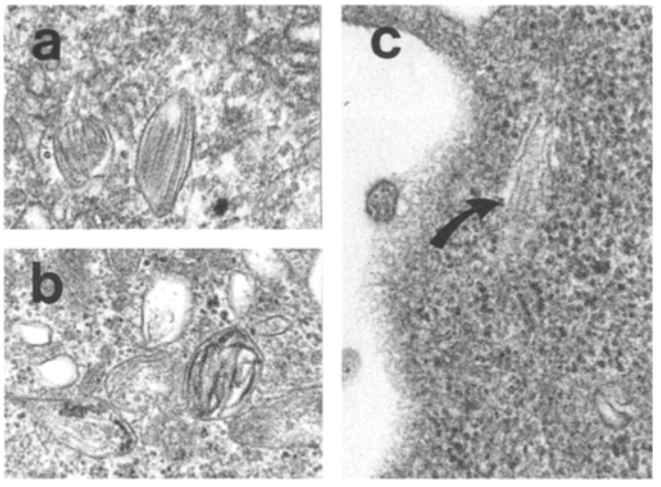


Fig. 6a–c Transmission electron micrographs of melanosomal abnormalities. Besides normal melanosomes (**a**) pleomorphic melanosomes with frequent derangement of the internal filaments were observed (**b**). Only in the cell line SK-MEL-28 occasional melanosomes with ill-defined, apparently defective limiting membranes (*arrow*) were noticed (**c**). Transmission electron microscopy (**a**×16 000, **b**×12 500, **c**×20 000)

Discussion

Abnormal, uncontrolled free radical reactions have been invoked in the initial molecular events for a variety of pathological processes and increasing evidence suggests that they play a substantial role in tumour cell invasion [1, 12, 20, 22, 29, 35–37, 42]. The escape from the hostile environment of the blood stream – the step of tumour cell extravasation – is a critically important event during haematogenous metastasis [10, 22, 42]. Previous studies have demonstrated that intravascular coinoculation of tumour cells with neutrophils or macrophages – which may in vivo be impacted within tumour cell clusters – results in a significant enhancement of experimental metastases formation [12, 20, 29]. A perturbation of the integrity of the capillary endothelium by leucocyte-derived radicals was thus suggested as an important mechanism in fostering tumour cell penetration of the vascular wall. However, other data stress the importance of the release of tumour-cell-derived radicals. Neoplastic cells can produce increased amounts of reactive oxygen species which may even be as large as those produced by stimulated neutrophils and it has been suggested that these radicals may enhance destructive tumour cell growth [39]. This hypothesis is supported by the observation that oxygen radical scavenging enzymes are able to inhibit tumour cell invasiveness in vitro [1, 22]. Moreover, it was recently reported that Walker 256 carcinosarcoma cells via free radicals are able to damage vascular endothelium directly and that oxygen radicals are also produced by these cells during metastasis in vivo [35, 37].

In the present study 4 of 7 melanoma cell lines disrupted the integrity of vascular endothelium by a free-radical-dependent mechanism. In all melanoma cell lines tested the injury became apparent morphologically by a loss of cell membrane integrity and concomitantly by a sensitivity to shear stress of damaged EC within a highly localized perispheroid zone. In all damaging cell lines both features of damage could be dose- and time-dependently prevented by the addition of the oxygen radical scavenging enzymes SOD or catalase.

Interestingly the EC injury was only observed when the melanoma cells were applied in the form of spheroids or smaller tumour cell clumps. The size of the halos of damaged EC correlated with the size of the attached tumour cell clusters. This indicates that toxic, i.e. high, radical levels are only generated in the microenvironment of clusters but not single melanoma cells. In addition, intercellular communication and metabolic cooperation of the melanoma cells within the clusters may contribute to this phenomenon, supporting the hypothesis that cooperation between tumor cells may foster their invasive behaviour [17, 22, 31].

The characteristic time-dependent development of this halo of damaged endothelium, i.e. the initial survival of a few EC layers in the immediate vicinity of the attached MCTS, was also observed in all damaging cell lines. Although no explanation for this phenomenon is available at this time, we believe that it is due to the particular geometrical configuration of the culture model – a raised spheroid on the flat endothelial cell layer – and particular diffusion and reaction conditions of the radicals. This hypothesis is supported by fact that a similar phenomenon occurred in the immediate environment of dust particles which have occasionally been noted within the area of EC injury [27, 28].

The EC used in the present model were derived from human umbilical veins, umbilical arteries and saphenous veins, respectively. Although we believe that the damaging melanoma cells are able to induce similar damage in EC obtained from the capillary microvasculature, this remains to be tested.

Differences between the melanoma cell lines were noticed with regard to the effectiveness of the different radical scavenging agents. Since superoxide is the sole substrate for SOD and hydrogen peroxide for catalase, respectively, it is suggestive that superoxide is the predominant injurious reactive oxygen species in co-cultures of the cell line ST-ML-12 and hydrogen peroxide in co-cultures of the cell lines ST-ML-11, ST-ML-14 and SK-MEL-28. Deferoxamine mesylate, allopurinol, ibuprofen, nor-dihydroguaretic acid and the proteinase inhibitor aprotinin did not display any protective effect. Soybean trypsin inhibitor, however, was slightly inhibitory in the case of ST-ML-14 and SK-MEL-28 in some experiments. Studies by Varani and coworkers [40] indicate that the killing of EC by activated neutrophils is a result of the synergistic interaction between reactive oxygen products and proteases. A similar interaction might be operative in the melanoma cell lines. Thus, free radicals could render the EC more sensitive to the action of proteases and also activate latent degradative enzymes which in vivo might furthermore support the destruction of the subendothelial basement membrane [36].

In contrast to melanomas, none of the non-melanoma cancer cell lines induced EC alterations. Melanoma is one of the most aggressive forms of cancer, metastasizing to any site of the body. Our results therefore tempt us to suggest that an oxygen radical-mediated destruction of the vascular EC lining may contribute to this extraordinary metastatic potential. Compared with other neoplasms melanoma cells generate significant amounts of reactive oxygen species through their unique metabolic pathway – the biosynthesis of melanin [4, 6, 15, 30, 33, 44]. The possibility that toxic damage to cells could occur from the action of these oxidation products has been previously recognized and it has been shown that melanoma-associated free-radical damage can occur in vivo [2, 4, 15, 33]. Consequently the manipulation of free radicals in this tumour has been proposed as a therapeutic targeting strategy for melanoma and both inhibitors or accelerators of free radical chain reactions have indeed been shown to inhibit growth and metastasis formation of melanomas in vivo [13, 30, 33, 41]. In these studies an interference with melanoma cell metabolism and proliferation as well as immunomodulatory effects have been proposed as important mechanisms to inhibit growth and metastasis. Our data suggest that the anti-melanoma activity of free radical scavenging enzymes may also be due to an interference with the invasive behaviour of the melanoma cells.

Melanoma cells are thought to have an exceptionally efficient defence system against oxygen free radicals. The most important mechanisms to restrict and control the intracellular concentration of radicals are compartmentalization within the melanosome and the action of natural anti-oxidants and anti-oxidant enzymes, such as SOD, catalase, glutathione and thioredoxin [4, 15, 44]. It has been proposed that defective melanosomal compartmentalization and consequently leakage of melanin-associated radicals may lead to cytotoxic phenomena in melanomas. Although we found occasional melanofilaments which were not unequivocally lined by a limiting melanosomal membrane in the cell line SK-MEL-28 analyses of all other melanoma cell lines tested did not reveal defective melanosomal membranes and are thus not supportive of the above-mentioned hypothesis. However, in patients with metastatic melanoma high levels of potentially toxic precursors of melanin are released into the blood [15]. An aberrant cellular secretion of such metabolites by neoplastic melanocytes could thus contribute to the cytotoxicity induced by melanomas.

Increasing evidence suggests that a dysfunction or derangement of cellular anti-oxidant enzyme activities plays a crucial role in the growth and invasiveness of melanoma. Melanoma cells are frequently depleted in activities of SOD and catalase and this is even more pronounced in metastatic cell clones [14, 19]. Recently, the human manganese SOD gene has been localized to chromosome 6q25 [24]. This region is frequently lost in melanoma and an intriguing study has demonstrated that transfection of manganese SOD cDNA into melanomas suppresses their tumourigenicity in experimental animals [7]. However, melanomas contain abnormally elevated amounts of thioredoxin activity, which are possibly elevated in these tumours to compensate the decrease/loss of other antioxidant enzymes [34]. An analysis of the potential correlation of anti-oxidant enzyme activities with the melanoma-induced endothelial cell cytotoxicity demonstrated in our study thus seems to be of particular interest.

The present panel of injurious and non-injurious cell lines may provide a useful model to elucidate the pathomechanims of the unique metastatic propensity of human melanoma.

Acknowledgements The excellent technical assistance of Mr. P. Kaden, Mrs. I. Hermanns, Mrs. D. Ruchatz, Mrs. Inge Jehardt and Mrs. U. Gessenich is gratefully acknowledged. We thank Mrs. R. Dillenberg for her invaluable help with the ultrastructural studies and Drs. H. Feichtinger, S. Dirnhofer and M. Vetterlein for critically reviewing the manuscript. F.A.O. gratefully acknowledges the support of Prof. Dr. G. Mikuz (Head, Department of Pathology, University of Innsbruck). This work was supported by BRITE EURAM grant P-2217 and DFG grant SFB-106.

References

1. Akedo H, Shinkai K, Mukai M, Komatsu K (1989) Potentiation and inhibition of tumur cell invasion by host cells and mediators. Invasion Metastasis 9: 134–148
2. Augustin AJ, Boker T, Breipohl W (1994) Increased lipid peroxides and inflammatory parameters in the retina adjacent to choroidal melanoma. Br J Ophtalmol 78: 130–132
3. Bjerkvig R (1992) Spheroid culture in cancer research. CRC Press, Boca Raton, Fla
4. Borovansky J, Mirejovsky P, Riley PA (1991) Possible relationship between abnormal melanosome structure and cytotoxic phenomena in malignant melanoma. Neoplasma 38: 393–400
5. Brueggen J, Sorg C, Macher E (1978) Membrane associated antigens of human malignant melanoma. V. Serological typing of cell lines using antisera from nonhuman primates. Cancer Immunol Immunother 5: 53–62
6. Bustamante J, Guerra L, Bredeston L, Mordoh J, Boveris A (1991) Melanin content and hydroperoxide metabolism in human melanoma cells. Exp Cell Res 196: 172–176
7. Church SL, Grant JW, Ridnour LA, Oberley LW, Swanson PE, Meltzer PS, Trent JM (1993) Increased manganese superoxide dismutase expression suppresses the malignant phenotype of human melanoma cells. Proc Natl Acad Sci USA 90: 3113–3117
8. Curran RC, McCann BG (1976) The ultrastructure of benign pigmented naevi and melanocarcinomas in man. J Pathol 119: 135–146
9. Dejana E, Bertocchi F, Bortolami MC, Regonesi A, Tonta A, Brevario F, Giavazzi R (1988) Interleukin 1 promotes tumor cell adhesion to cultured human endothelial cells. J Clin Invest 82: 1466–1470
10. Fidler IJ (1990) Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. Cancer Res 50: 6130–6138
11. Garbe C, Krasagakis K, Zouboulis C, Schroeder K, Krueger S, Stadler R, Orfanos CE (1990) Antitumor activities of interferon alpha, beta, and gamma and their combinations on human melanoma cells in vitro: changes of proliferation, melanin synthesis and immunophenotype. J Invest Dermatol 95: 231–237S
12. Gorelik E, Wiltraut RH, Brunda MJ, Holden HT, Herberman RB (1982) Augmentation of metastasis formation by thioglycollate-elicited macrophages. Int J Cancer 29: 575–581

13. Hourani BT, Demopoulos HB (1969) Inhibition of S-91 mouse melanoma metastases and growth by D-penicillamine. Lab Invest 21: 434–438
14. Jaworska A, Stojcevic-Lemic N, Nias AH (1993) The effect of paraquat on the radiosensitivity of melanoma cells: the role of superoxide dismutase and catalase. Free Radic Res Commun 18: 139–145
15. Jimbow K, Lee SK, King MG, Hara H, Chen H, Dakour J, Marusyk H (1993) Melanin pigments and melanosomal proteins as differentiation markers unique to normal and neoplastic melanocytes. J Invest Dermatol 100: 259S–268S
16. Klingmueller G, Schmoeckel C (1971) Frei im Cytoplasma liegende Membrananordnungen beim malignen Melanom. Arch Dermatol Forsch 241: 115–121
17. Knuechel R, Feichtinger J, Recktenwald A, Hollweg HG, Franke P, Jakse G, Rammal E, Hofstaedter F (1988) Interactions between bladder tumor cells as tumor spheroids form the cell line J82 and human endothelial cells in vitro. J Urol 139: 640–645
18. Kramer RH, Gonzalez R, Nicolson GL (1980) Metastatic tumor cells adhere preferentially to the extracellular matrix underlaying vascular endothelial cells. Int J Cancer 26: 639–645
19. Kwee JK, Mitidieri E, Affonso OR (1991) Lowered superoxide dismutase in highly metastatic B16 melanoma cells. Cancer Lett 57: 199–202
20. Lafrenie R, Shaughnessy SG, Orr FW (1992) Cancer cell interactions with injured or activated endothelium. Cancer Metastasis Rev 11: 377–388
21. Liotta LA, Kleinerman J, Saidel GM (1974) Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. Cancer Res 34: 997–1004
22. Mareel MM, De Baetselier P, Van Roy FM (1991) Mechanisms of invasion and metastasis. CRC Press, Boca Raton, Fla
23. Masters JRW, Hepburn PJ, Walker L, Highman WJ, Tredosiewicz L, Povey S, Parkar M, Hill BT, Riddle PR, Franks LM (1986) Tissue culture model of transitional cell carcinoma: characterization of twenty-two human urothelial cell lines. Cancer Res 46: 3630–3636
24. Millikin D, Meese E, Vogelstein B, Witkowski C, Trent J (1991) Loss of heterozygosity for loci on the long arm of chromosome 6 in human maligant melanoma. Cancer Res 51: 5449–5453
25. Nicolson GL (1993) Paracrine and autocrine growth mechanisms in tumor metastasis to specific sites with particular emphasis on brain and lung metastasis. Cancer Metastasis Rev 12: 325–343
26. Offner FA, Ott G, Povey S, Knuechel R, Preisler V, Fuezesi L, Klosterhalfen B, Ruebben H, Hofstaedter F, Kirkpatrick CJ (1991) Characterization of the new bladder cancer cell line HOK-1: expression of transitional, squamous and glandular differentiation patterns. Int J Cancer 49: 122–128
27. Offner FA, Wirtz HC, Schiefer J, Bigalke I, Klosterhalfen B, Bittinger F, Mittermayer C, Kirkpatrick CJ (1992) Interaction of human malignant melanoma (ST-ML-12) tumor spheroids with endothelial cell monolayers: damage to endothelium by oxygen-derived free radicals. Am J Pathol 141: 601–610
28. Offner FA, Bigalke I, Schiefer J, Wirtz HC, Klosterhalfen B, Feichtinger H, Kirkpatrick CJ (1993) Interaction of human malignant melanoma tumor spheroids with endothelium and reconstituted basement membrane: modulation by RGDS. Int J Cancer 54: 506–512
29. Orr FW, Warner DJA (1987) Effects of neutrophil-mediated pulmonary endothelial injury on the localization and metastasis of circulating Walker carcinosarcoma cells. Invasion Metastasis 7: 183–196
30. Prezioso JA, FitzGerald GB, Wick MM (1992) Melanoma cytotoxicity of buthionine sulfoximine (BSO) alone and in combination with 3,4-dihydroxybenzylamine and melphalan. J Invest Dermatol 99: 289–293
31. Raz A, Ben-Zè ev (1983) Modulation of the metastatic capability in B16 melanoma by cell shape. Science 221: 1307–1310
32. Rice GE, Bevilacqua MP (1989) An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science 246: 1303–1306
33. Riley PA (1985) Radicals and melanomas. Philos Trans R Soc Lond B 311: 679–689
34. Schallreuter KU, Jänner M, Mensing H, Bretbart EW, Berger J, Wood JM (1991) Thioredoxin reductase activity at the surface of human primary cutaneous melanomas and their surrounding skin. Int J Cancer 48: 15–19
35. Shaughnessy SG, Buchanan MR, Turple S, Richardson M, Orr FW (1989) Walker carcinosarcoma cells damage endothelial cells by the generation of reactive oxygen species. Am J Pathol 134: 787–796
36. Shaughnessy SG, Whaley M, Lafrenie RM, Orr FW (1993) Walker 256 tumor cell degradation of extracellular matrices involves a latent gelatinase activated by reactive oxygen species. Arch Biochem Biophys 304: 314–321
37. Soares FA, Shaughnessy SG, MacLarkey WR, Orr FW (1994) Quantification and morphologic demonstration of reactive oxygen species produced by Walker 256 tumor cells in vitro and during metastasis in vivo. Lab Invest 71: 480–489
38. Stetler-Stevenson WG, Aznavoorian S, Liotta LA (1993) Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 9: 541–573
39. Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 51: 794–798
40. Varani J, Ginsburg I, Schuger L, Gibbs DF, Bromberg J, Johnson KJ, Ryan US, Ward PA (1989) Endothelial cell killing by neutrophils: synergistic interaction of oxygen products and proteases. Am J Pathol 135: 435–438
41. Varga JM, Airoldi L (1983) Inhibition of tranplantable melanoma tumor development in mice by prophylactic administration of Ca-ascorbate. Life Sci 32: 1559–1564
42. Weiss L, Orr FW, Honn KV (1988) Interactions of cancer cells with the microvasculature during metastasis. FASEB J 2: 12–21
43. Weiss L, Nannmark U, Johansson BR, Bagge U (1992) Lethal deformation of cancer cells in the microcirculation: a potential rate regulator of hematogenous metastasis. Int J Cancer 50: 103–107
44. Wood JM, Schallreuter KU (1991) Studies on the reaction between tyrosinase, superoxide anion, hydrogen peroxide and thiols. Biochim Biophys Acta 1074: 378–385