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Rapid communication

Protection of melanoma cells against superoxide radicals by melanins

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Summary. Human melanoma cells transplanted into immunocompetent mice by the 6-day subrenal capsule technique are characterized by high resistance against immunological attack. This resistance is suggested to be the consequence of scavenging of superoxide free radicals by melanin. Scavenging of superoxide radicals by the melanoma cells was clearly demonstrated using electron spin resonance techniques. From comparison with synthetic melanins it is concluded that the scavenger effect can be attributed mainly to low-molecular-mass melanins synthesized in the melanoma cells whereas high-molecular-mass melanins are practically ineffective.

Key words: Melanoma – Superoxide radicals – Melanin – Electron spin resonance

Introduction

The performance of the 6-day subrenal capsule assay (Bogden et al. 1978) with human tumors, using immunocompetent mice, shows that various tumors are replaced on day 6 after transplantation by leucocytic infiltrations or connective tissue. An exception is the human melanoma. Jaworskaja (personal communication), Aamdal et al. (1985), and Atassi et al. (1985) have observed that after 6 days the infiltrations of leucocytes are very small.

Macrophages, granulocytes and other effector cells kill tumor cells by releasing toxic superoxide radicals ($O_2^{\cdot}$) (Bellavite 1988). Melanoma cells are characterized by a high content of melanin pigment. Isolated melanin has been shown to be a potent scavenger of $O_2^{\cdot}$ (Korytowsky et al. 1986; Geremia et al. 1984; Sealy et al. 1980), whereas to our knowledge no measurement of $O_2^{\cdot}$ scavenging by melanoma cells has been reported. As a possible interpretation of the high immunological resistance of melanoma cells we suppose that these cells can escape from the attack by superoxide radicals by the $O_2^{\cdot}$ scavenging effect of their melanin content. To our knowledge no measurement of $O_2^{\cdot}$ scavenging by melanoma cells has been reported. In order to prove the above-mentioned hypothesis, the reaction of superoxide radicals with different melanin preparations, mouse melanoma tissues and, for comparison, normal muscle tissues of the same animal was studied by means of electron spin resonance (ESR) techniques.

Abbreviations. ESR, electron spin resonance; DMSO, dimethylsulfoxide; TEMPOL, 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl

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Materials and methods

Transplantation of human tumor

The basic technique for the 6-day subrenal capsule assay has been described in detail by Bogden et al. (1978). The human tumor was cut into pieces of 1 mm^3, and one piece was implanted under the renal capsule of normal immunocompetent mice ($B6D2F_1$/Bln). On the sixth day, the animals were sacrificed and the tumor-bearing kidneys removed and fixed in 5% formaldehyde. Paraffin sections were cut through the largest part of the xenograft and stained with hematoxylin and eosin.

Preparation of the melanoma and muscle tissue of mice

Bl6 melanoma was transplanted into C57Bl/6/Bln mice, minced with a homogenizer and centrifuged at 35000 rpm. The sediment was washed with physiological NaCl solution and centrifuged at 3000 rpm. The preparation was boiled with 10% HCl for 1 h, treated with ethanol and ether and dried under vacuum. The muscle tissue was obtained from the hind thigh of C57Bl/6/Bln mice. The preparation was carried out analogously to that of melanoma tissue.

Preparation of synthetic melanin by autoxidation of L-*3,4-dihydroxyphenylalanine*

Method (a). L-3,4-Dihydroxyphenylalanine (2 g; Merck) and NaOH (3 g) were dissolved in 15 ml H_2O. The solution was aerated for 48 h and then treated with 20% HCl. The precipitated melanin was

dialyzed against water and dried under vacuum. The material is soluble in dimethylsulfoxide (DMSO).

Method (b). L-3,4-Dihydroxyphenylalanine (1 g) was dissolved in 1000 ml 0.67 *M* sodium phosphate buffer (pH 8.0) and the solution was aerated for 48 h. Acidification with conc. HCl precipitated melanin at pH 2.0. The melanin pellet was washed with water and ethanol and than dried under vacuum. The product was insoluble in DMSO.

Preparation of $O_2^{\cdot-}$ radicals and ESR

Solutions of $O_2^{\cdot-}$ radicals were prepared by dissolving KO_2 (Fluka) in dry DMSO in the presence of 18-crown-6-ether (Merck). Varying amounts (0.06–16 mg) of lyophilized tissue or, for comparison, synthetic melanin was added to 360 µl $O_2^{\cdot-}$ solution (3.5 m*M*). ESR spectra were recorded in cylindrical quartz cuvettes using a finger cryostat at 77 K on a VARIAN E3 spectrometer after 5 min incubation at room temperature followed by rapid freezing to 77 K. The concentration of $O_2^{\cdot-}$ was evaluated by computer-aided double integration of ESR spectra and comparison with standards of free radicals of known concentration. Reductive behaviour of melanoma cells against 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (TEMPOL) was studied by ESR at room temperature using ESR tissue cuvettes.

Results

Human melanoma line 2080 exhibits remarkably high viability after transplantation into immunocompetent mice for 6-day subrenal capsule assay. After 6 days the infiltrations of leucocytes are very small and even after

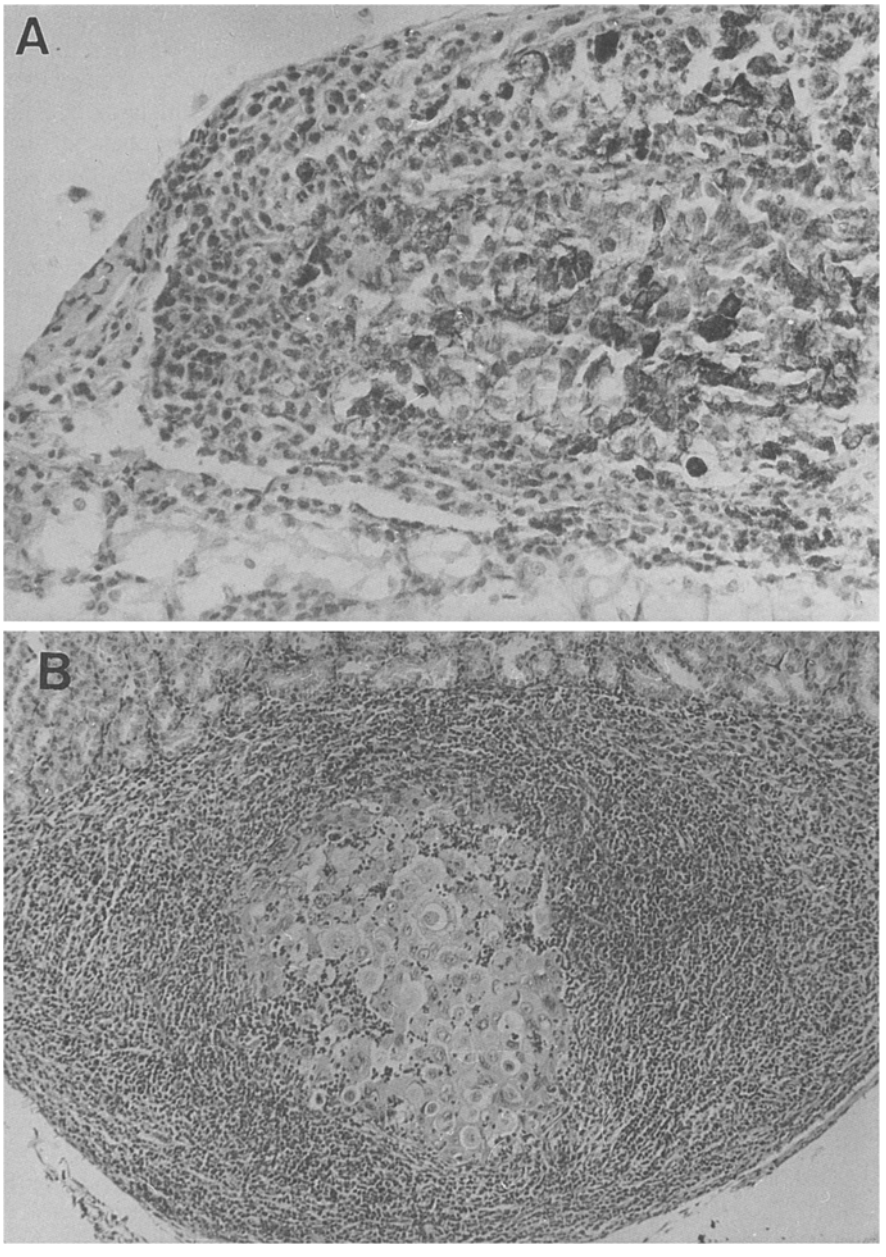

Fig. 1. A Human melanoma line 2080 transplanted by subrenal capsule technique into immunocompetent mice: no replacement of melanoma cells by leucocytes is seen on day 6 (H&E, 32×). **B** Tumor cells of human lung carcinoma line 2045 are replaced on day 6 by leucocytic infiltration and connective tissue (H&E, 32×)

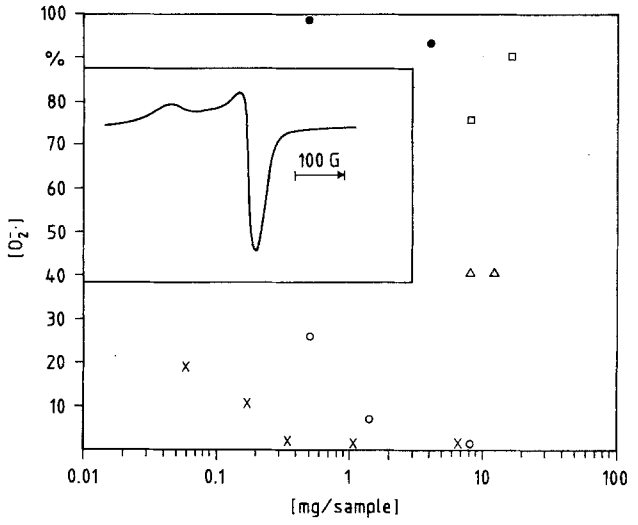

Fig. 2. Relative concentration of superoxide radical (amplitude of ESR signal) after addition of different amounts of – synthetic melanin, dimethylsulfoxide(DMSO)-soluble (×); – synthetic melanin, DMSO-insoluble (□); – melanoma tissue, lyophilized (○); – melanoma tissue, residue after DMSO extraction, lyophilized (●); – muscle tissue, lyophilized (△); to KO_2/DMSO solution. Sample volume: 360 µl; temperature of measurement: 77 K; each point is the mean value of three independent experiments; deviation was within 5%. *Insert:* ESR spectrum of superoxide radicals (O_2^-) from KO_2/DMSO without addition of lyophilized tissues or melanins recorded at 77 K (control). Spectrometer settings: power: 5 mW, modulation: 1 G, gain: 1.25×10^4

8–12 days the tumor content in the transplant was high (50%–75%) as depicted in Fig. 1 A. In comparison, tumor cells of the human lung carcinoma line 2045 are replaced on day 6 by leucocytic infiltration and connective tissue (Fig. 1 B).

Using a spectroscopically relevant system for the study of this behaviour ESR spectra of O_2^- radicals in DMSO, after addition of different amounts (mass) of lyophilized tissues, were recorded. O_2^- is scavenged by the HCl preparation from melanoma tissue, whereas the HCl preparation from muscle tissue has no effect; different effects of DMSO-soluble and DMSO-insoluble synthetic melanins on the ESR spectra of superoxide radicals have been found (Fig. 2). O_2^- is scavenged only by DMSO-soluble melanins, but not by DMSO-insoluble synthetic melanins. The scavenging effect is rather strong, even with the lower dose of 1.1 mg DMSO-soluble melanin.

Quantitative results on the relative concentrations of superoxide radicals (deduced from ESR signals) after addition of the various melanins and tissues to KO_2/DMSO solution are summarized in Fig. 2. The residue of DMSO extraction of melanoma tissue shows no scavenging effect on O_2^- DMSO-soluble melanin as well as lyophilized melanoma tissue decreases O_2^- to about 1% of the initial concentration (possibly even less, since the residual ESR signal originates from melanin). This finding suggests that the reaction of the melanoma cells with O_2^- is mainly due to DMSO-soluble melanin.

Reaction of synthetic melanins with O_2^- may occur via reduction or oxidation (Korytowski et al. 1985). Therefore reductive behaviour of the melanoma cells against a stable free radical (TEMPOL) was measured. Reduction of TEMPOL was observed to be very strong in the supernatant of homogenized melanoma tissue (99% reduction within 5 min compared to 12% in the case of muscle tissue), whereas reduction is slower in intact melanoma cells (18% reduction after 5 min corresponding to a half-life of 11 min), and much slower in melanoma tissue after removal of DMSO-soluble melanin (half-life: 46 min).

Discussion and conclusions

We have shown that a preparation from melanoma tissue provokes a strong scavenging of O_2^- radicals, and exhibits significant reductive behaviour against stable nitroxide free radicals as compared with the corresponding muscle tissue. The observed scavenging of O_2^- by melanoma tissue is explained mainly by reaction with low-molecular-mass melanins (soluble in DMSO), whereas high-molecular-mass melanins (insoluble in DMSO) are rather ineffective. Enzymatic cell protectants, like superoxide dismutase or catalase, are unlikely be involved in this scavenger effect because of the inactivation by hydrochloric acid during the preparation of the tissue samples. It may be assumed that within the melanoma cells the high-molecular-mass melanins in the melanosomes are unable to scavenge superoxide radicals, whereas the intermediate products in the melanin biosynthesis pathway are preferentially active in quenching these radicals. Intermediates of melanin are generated at sites where active tyrosinase is located: in the free cytoplasma and bound on particles. Because of the decreased barrier function of melanoma cell membranes, these cells are also surrounded by soluble melanin intermediates. Therefore, these soluble melanins can play an important role in the resistance of melanomas against macrophages, granulocytes and other effector cells, and in the high metastastic capacity of this type of tumor cells. If the attack produced by phagocytes (Bellavite 1988) contributes to the cytotoxic effect of these cells, then the melanoma cells may be protected by the very strong radical-scavenging effect of the soluble melanin intermediates. The observed resistance of the human melanoma 2080 and other melanomas against the immunological attack demonstrated in the 6-day subrenal capsule assay, as also reported by Jaworskaja, Atassi et al. (1985) and Aamdal et al. (1985) using other human melanomas, can be interpreted now by our results.

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