Original Article

Non-invasive measurement of melanin-derived radicals in living mouse tail using X-band EPR

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The aim of this experiment is to measure *in vivo* generation of melanin-derived radicals non-invasively, as a quantifiable index of radio-biological effect. Melanin-derived radicals in a living intact mouse tail tip were non-invasively measured in very simple way using an X-band electron paramagnetic resonance spectrometer. Colored mouse strains, C57BL/6NCr, BDF1, and C3H/He, have clear EPR signal corresponding to melanin-derived radicals in the tail tip; however, albino mouse strains, BALB/cCr, ddY, ICR, have no EPR signals. An X-ray fraction of 2 Gy/day (1 Gy/min) was repeatedly irradiated to a C3H/He mouse tail skin every Monday to Friday for 4 weeks. In comparison to before starting irradiation, the C3H/He mouse tail skin became darker, like a suntan. The melanin-derived radicals in C3H/He mouse tail skin were increased in association with X-ray fractions. Melanin-derived radicals in mouse tail skin can be readily and chronologically measurable by using X-band EPR spectrometer, and can be a marker for a radiobiological effect in the skin.

***Key Words*: melanin-derived radicals, electron paramagnetic resonance, non-invasive measurement, radio-biological effect, X-ray irradiation**

Exposing the skin to ultraviolet (UV) radiation causes cutaneous responses such as sunburn/suntan.[1–4] As well as the suntan of the skin by UV, suntan caused by radiation is known as a side effect of radiation therapy.[5] The generation of free radicals in skin is believed to be a trigger of cutaneous effects, and melanin in skin acts as a photo-protector through body coloration and a scavenger against reactive oxygen species (ROS).[6–9] Chemical studies on melanin have been rendered exceptionally difficult due to its insolubility and resistance to hydrolysis.

Melanin is the black or brownish pigment which appears to occur at all phyletic levels of biological organization and is ordinarily considered to be an insoluble, nitrogen-containing polymer.[10] Melanin includes eumelanin and pheomelanin, and the exact chemical structures of the two different types of melanin have not been identified yet, probably because of the complication of polymerization and modifications of polymerization.[11–13] Melanin is, therefore, not a single compound but class of polymer compounds, and is formed by several chemical structural components.

Melanin contains endogenous free radicals, which are class of plural free radical components stabilized on the complicated polymer structure of melanin, can be directly measured using the electron paramagnetic resonance (EPR) spectrometer.[10,14] EPR signal from natural melanin, e.g., the primary root of *Glycyrrhiza*, skin or melanoma cell, has been observed and reported.[14–17] In addition, an increase of melanin-derived radicals upon UV irradiation has been reported.[14] Melanin-derived free radicals generated *in vivo* may become an index for free radical generation in skin; however, any trial for non-invasive measurement of endogenous melanin-derived radicals in a living animal has not been reported to the present.

In this paper, a newly developed simple technique for non-invasive measurement of melanin-derived radicals in living intact mouse tail tip is proposed. Daily changes of the amounts of melanin-derived radicals induced by repeated X-ray irradiations were evaluated.

Materials and Methods

Chemicals. L-Tyrosine and melanin (prepared by persulfate oxidation of tyrosine) were purchased from Wako Pure Chem. Ind., Ltd. (Osaka, Japan). All other reagents used were of analytical grade.

Animals. Eight-weeks-old female BALB/cCrSlc, Slc:ddY, Slc:ICR, C57BL/6NCrSlc, Slc:BDF1, and C3H/HeSlc mice, i.e., 3 albino and 3 colored strains, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Mice were housed and habituated for a week. C3H/He Slc mice were housed three per cage. The other mice were housed one per cage. Mice were housed in a room with a 12 h light/dark cycle and were allowed food and water *ad libitum* during the experiment. Experiments were carried out in compliance with the Guidelines for Animal Experiments of the National Institute of Radiological Sciences.

***In vivo* EPR measurement of melanin-derived radicals.** Melanin-derived radicals in mouse tail skin were measured using an X-band EPR spectrometer (JES-TE100, JEOL, Tokyo, Japan), which was equipped with WIN-RAD EPR Data Analyzer System (Radical Research, Inc., Hino, Tokyo). A mouse was anesthetized using 1.5% isoflurane in air (flow rate was 1.5 L/min) using a vaporizer unit (TK-6, Bio Machinery Co., Ltd., Funabashi, Chiba, Japan). The mice were fixed on a special mouse holder using paper adhesive tape, and the 7 cm tail tip was set in the TE-mode EPR cavity, as shown in Fig. 1A. The EPR signal of the melanin-derived radicals was recorded under the following conditions; microwave frequency: 9.45 GHz, microwave power: 8 mW, center magnetic field: 336.40 mT, field sweep width: ±5 mT, sweep rate: 0.67 mT/min, time constant: 1 s, field modulation frequency: 100 kHz, and field modulation width: 0.063 mT. EPR data acquisition was controlled by the WIN-RAD EPR Data Analyzer System. The acquired EPR spectra (4096-point digital data) were

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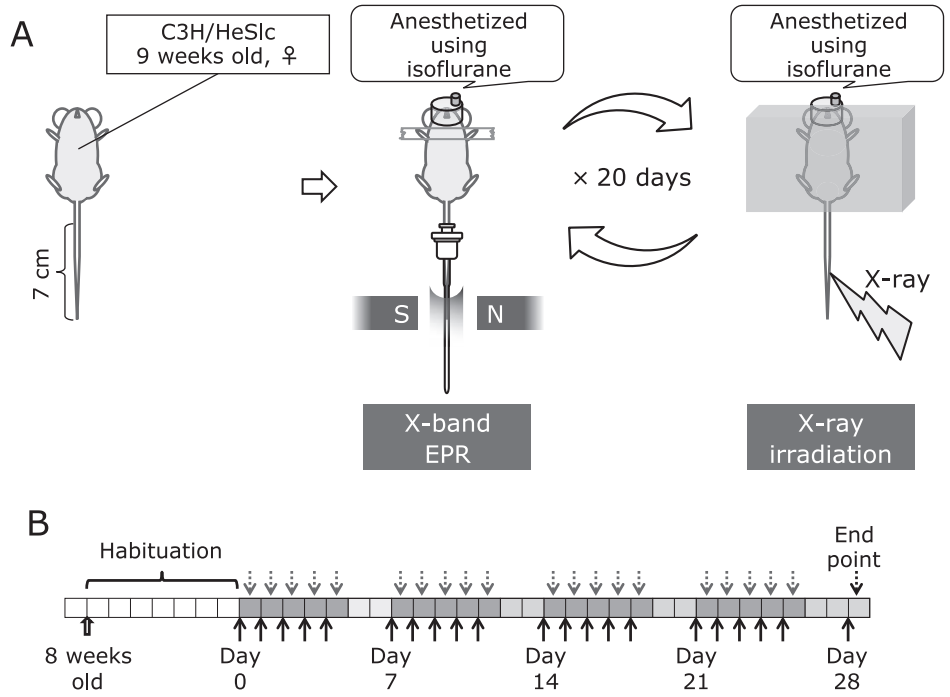

Fig. 1. Outline of the *in vivo* experiments. (A) Schematic drawing of the protocol of repeated EPR measurements and X-ray irradiations. (B) Time line of the experiments. Upward arrows (black line) show the points of EPR measurement for melanin-derived radicals, and the day before starting irradiation is shown as day 0. Downward arrows (gray dots) show the points of X-ray irradiation. The end point of the study was after EPR measurement on day 28, shown as downward arrows (black dots).

analyzed using an in-house line fitting program, and the Gaussian line shape was fitted. The signal height and line width of the fitted Gaussian line were measured and then EPR signal intensity was obtained as follows: (signal height) × (line width)^2.

X-irradiated C3H/HeSlc mice tails were measured once a day for 4 weeks (5 days/week) under the following conditions; center magnetic field: 336.69 mT, field sweep width: ±1.25 mT, data point: 1024-point, and any other conditions were same as above. Since the in-house line fitting program can process only a single line spectrum, the singlet EPR line of melanin-derived radical, 3rd manganese, and 4th manganese was separately swept to analyze the signal intensity for each mouse. The center magnetic field was set as 331.85 mT or 340.46 mT for 3rd or 4th manganese signal, respectively.

***In vitro* EPR measurement of melanin-derived radicals.** An aliquot (3 mg) of commercial melanin powder or L-tyrosine was put in a capillary (Drummond Microcaps: o.d. φ1.44 mm, i.d. φ1.05 mm, Drummond Scientific Company, PA). Both ends of the capillary were plugged with sealing putty (TERUMO CORPORATION, Tokyo Japan). The capillary containing the sample powder was placed in the TE mode cavity, and scanned by the X-band EPR spectrometer. Each single line of melanin-derived radical, 3rd manganese, and 4th manganese was separately scanned. EPR parameters were the same as described above except that the microwave power was 2 mW.

X-ray irradiation. After every EPR measurement, the tail of a C3H/He Slc mouse was irradiated with 2 Gy X-rays (Fig. 1B). The total dose for 20 days was 40 Gy. During irradiation, each mouse was anesthetized using 1.5% isoflurane in medical air (flow rate was 1.5 L/min). The other parts of the body were shielded by a lead block 5 cm thick. X-ray irradiation was performed using TITAN-320 (Shimadzu, Kyoto, Japan). Effective energy was 80 keV under the following conditions: X-ray tube voltage was 200 kV, X-ray tube current was 20 mA, and the thickness and materials of the pre-filter were 0.5 mm copper and 0.5 mm aluminum. The dose rate of X-ray irradiation for mouse tails was 1.0 Gy/min when the distance between the X-ray tube and the tail of the mouse was 50 cm.

A commercial melanin was irradiated with 10 or 20 Gy X-rays at room temperature using PANTAK-HF320S (Shimadzu, Kyoto, Japan). The dose rate of X-ray irradiation for melanin powder was 1.0 Gy/min and any other conditions were as same as TITAN-320.

Results

Measurement of melanin-derived radicals *in vivo*. Fig. 2A shows the EPR signal obtained in tails of 3 different albino and 3 different colored mice strains. Albino strains have no EPR signal in their tail; however, a broad singlet EPR signal could be obtained in colored mice tail. Lowest panel of Fig. 2A shows the EPR spectrum of 3 mg of L-tyrosine and commercial melanin powder. The commercial melanin powder gave also broad singlet EPR line. Resonance position of the broad singlet EPR lines obtained in colored mice tails and in the commercial melanin powder was compared based on manganese marker. The all broad singlet lines gave a g value of 2.004. The results of the g value agree very well with the results in a previous report.[14] Actual single free radical species was not distinguished, the free radical species in the mouse tail could be said as melanin-derived radicals. Fig. 2B shows comparison of EPR signal intensities of the melanin-derived radicals in unirradiated mouse tails. Since no spectra were obtained from 3 albino strains, the data were not able to digitalize.

Evaluation of melanin-derived radicals in mouse tail by X-ray fractionated irradiation. Next, daily variations of melanin-derived radicals induced by repeated irradiation of X-ray were measured. An increase of melanin-derived radicals in mouse tail skin was observed in association with X-ray fractionated irradiation (Fig. 3A). The mouse tail skin visibly turned brown after 14 days or later (data not shown). Fig. 3B shows the relation-

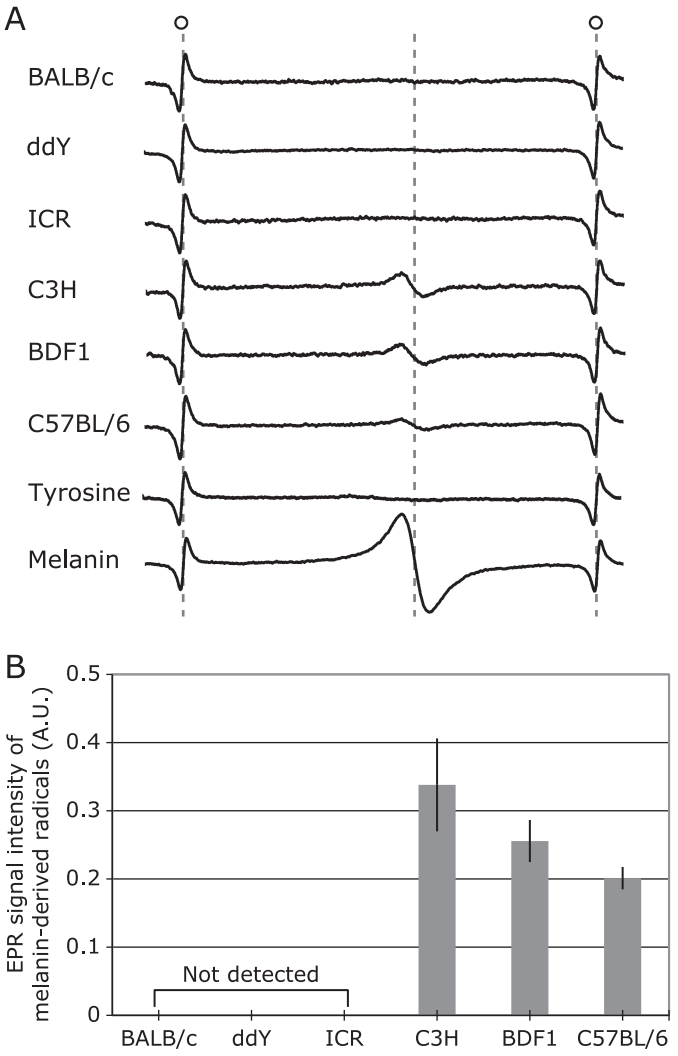

Fig. 2. Comparison of albino and colored mouse strains. (A) X-band EPR signals obtained in unirradiated mouse tail and in commercial melanin powder and L-tyrosin. The positions of EPR signals were adjusted and intensities were normalized using 3rd and 4th EPR lines of manganese marker (open circles). Gray dot lines demonstrate the center of the resonance field of 3rd and 4th manganese signals and melanin-derived radicals of the melanin powder. (B) Comparison of EPR signal intensities in mouse tails. The columns and error bars indicate average and SD of 3 experiments. For 3 albino strains, no EPR signals corresponding to the melanin-derived radicals were detected.

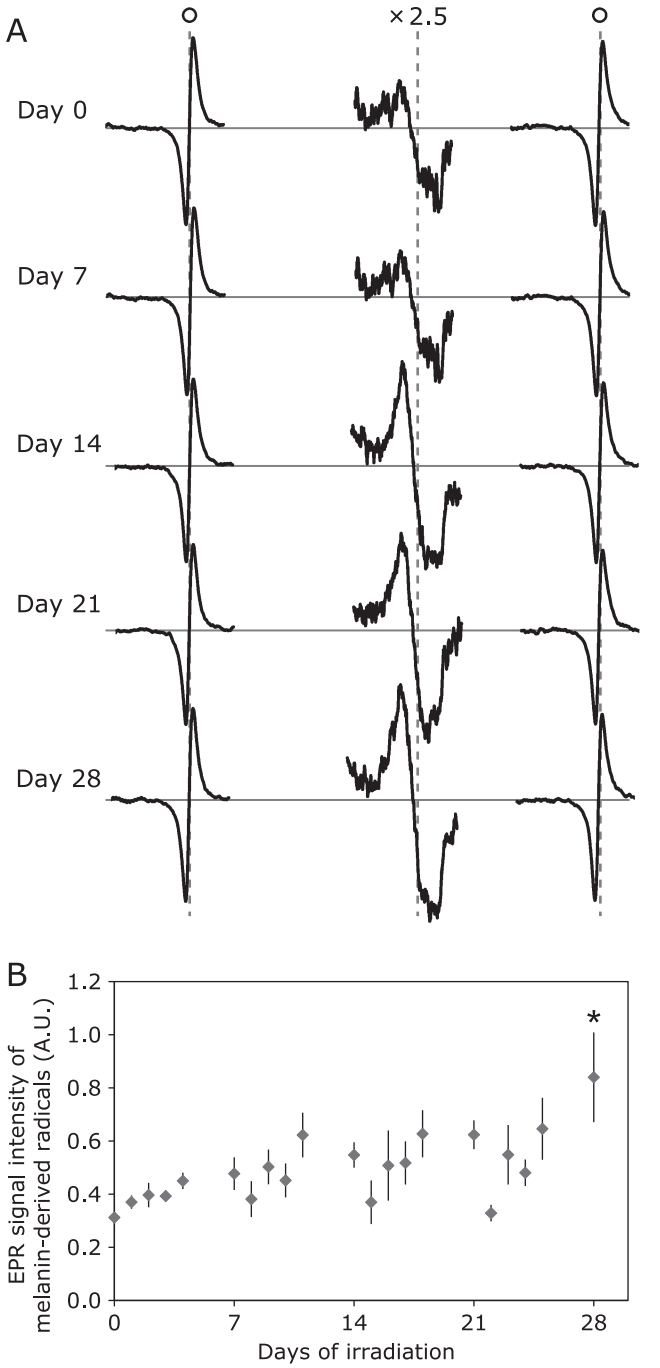

Fig. 3. Time course of melanin-derived radicals in mouse tail during fractionated X-ray irradiation for 4 weeks experimental period. (A) Time course of EPR spectra of melanin radical in mouse tail irradiated fractions of X-ray. Single EPR lines of 3rd and 4th manganese and melanin-derived radicals obtained separately were aligned on the magnetic field axis with reference to the center fields setting. The positions of EPR signals were, then, adjusted and intensities were normalized using 3rd and 4th EPR lines of manganese marker (open circles). Gray dot lines demonstrate the center of resonance field of 3rd and 4th manganese signals and melanin-derived radicals of the melanin powder. The EPR signals of melanin-derived radicals obtained in an irradiated mouse tail were displayed with 2.5 times magnification compare to manganese signals. (B) The signal intensities of melanin-derived radicals in C3H/He mouse tail skin gradually induced with days of X-ray irradiation (n = 6). * indicates significance when the signal intensity of melanin-derived radicals in mouse tail was compared with that of day 0.

ship between the amount of melanin-derived radicals generated in mouse skin and the number of days of irradiation. The level of melanin-derived radicals obtained before starting irradiation is shown as day 0.

Effect of X-ray irradiation on melanin powder. To confirm whether melanin-derived free radical formation is just a result of scavenging ROS or requires some other *in vivo* redox processes, a melanin powder suspension was irradiated with X-rays. The EPR signal intensity obtained in the irradiated melanin sample showed no differences compared to the unirradiated one (Fig. 4).

Discussion

Melanin-derived radicals in tail skin of a living intact mouse were detected in a very simple way. The only instrument required is an X-band EPR spectrometer. Albino mice species, could have no melanin due to no tyrosinase activity, give no such EPR signal.

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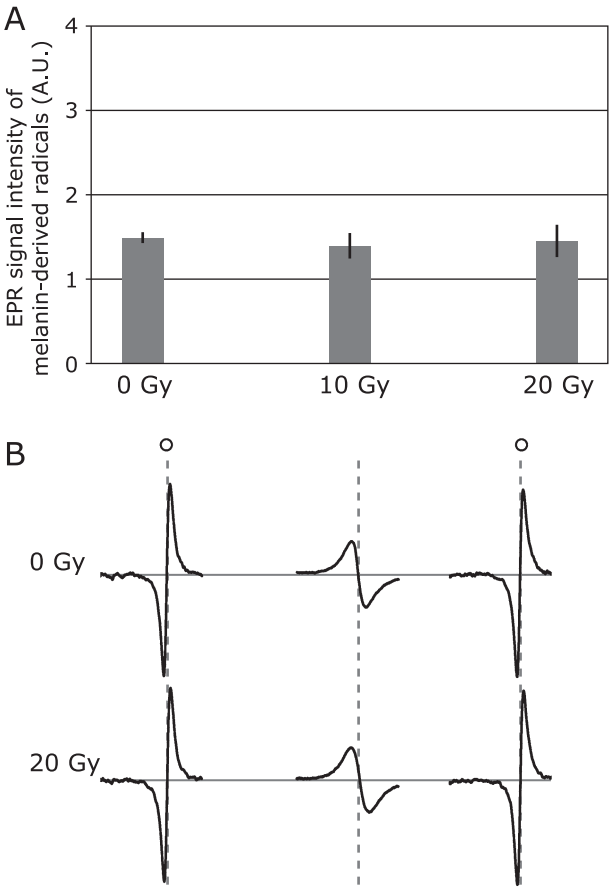

Fig. 4. Effect of X-ray irradiation to commercial melanin powder. (A) The EPR spectra of melanin radical in commercial melanin powder before and after X-ray irradiation. The positions of EPR signals were adjusted and intensities were normalized using manganese marker. (B) Intensity of melanin radical in commercial melanin powder (n = 3). The positions of EPR signals were adjusted and intensities were normalized using manganese marker (open circles). Single EPR lines of 3rd and 4th manganese and melanin-derived radicals obtained separately were aligned on the magnetic field axis with reference to the center fields setting. The positions of EPR signals were, then, adjusted and intensities were normalized using 3rd and 4th EPR lines of manganese marker (open circles). Gray dot lines demonstrate the center of resonance field of 3rd and 4th manganese signals and melanin-derived radicals of the melanin powder.

The EPR signals found in other colored mice tails were in good agreement with the EPR signal obtained in commercial melanin powder with its g value (Fig. 2A). Those results suggest that the EPR signal obtained in the C3H and/or other colored mice tail was considered as melanin-derived radicals. In this paper, however, we did not separate each radical component due to make the EPR measurement simple and sensitive. The melanin-derived radicals in tail skin appeared gradually to increase with increasing days of X-ray irradiation (Fig. 3B). These results suggest the melanin-derived radicals *in vivo* were induced by X-ray irradiation. Fig. 3B, has relatively large error bar. The size of error bars looks to become larger with days of irradiation increased. These data might involve measurement errors and individual variability. In addition, volume/diameter of tail in the EPR cavity might gradually increase with growth of mice for 4 weeks.

As described in the "Results" section, the mouse tails turned brown; however, quantification of the amount of melanin using the tail color was difficult. Amount of melanins in the skin of mouse tail was not measured in this study.

When the X-rays were irradiated directly to the commercial melanin powder suspension, the level of melanin-derived radicals in the commercial melanin powder did not increase (Fig. 4). This result suggests that the induction of melanin-derived radicals probably requires *in vivo* redox processes.

Intrinsic melanin-derived radicals in living mouse tail skin were non-invasively detected for the first time using an X-band EPR spectrometer. To our knowledge, the melanin-derived radicals are the only endogenous intact biological free radicals detected directly and non-invasively. This non-invasive method could measure an individual mouse repeatedly; for example, measurements were available 5 days a week for 4 weeks. The temporal assessment of melanin-derived radicals might be an informative index for oxidative stress and/or a marker of redox status *in vivo*.

Conflict of Interest

No potential conflicts of interest were disclosed.

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