Virchows Arch (1997) 431:63–71 © Springer-Verlag 1997

ORIGINAL ARTICLE

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Light and electron microscopic investigation of the process of healing of the naevus of Ota by Q-switched alexandrite laser irradiation

Received: 11 September 1996 / Accepted: 9 January 1997

Abstract Melanocytes in the naevus of Ota were destroyed by irradiation using the Q-switched alexandrite laser. This laser is highly selective and highly absorbed by melanosomes. Other cells and tissue components of the dermis remained almost intact. Melanosomes were vaporized or fragmented to subelectron microscopical size, or degenerated. If the irradiated energy was sufficient, melanocytes vanished and large vacuoles several times the size of dermal melanocytes formed at the sites. If it was too weak, dermal melanocytes were also vaporized, but vacuoles formed within them. Nuclei were no longer discernible. Following irradiation macrophages infiltrated the irradiated areas and scavenged degenerated melanosomes and cellular debris. Thus, discoloration of the skin was markedly reduced. Although a few melanocytes and melanophages remained, pigmentation cleared to a satisfactory level. Melanocytes and keratinocytes were also injured in the epidermis; however, the epidermis recovered completely. No scarring was observed.

Key words Naevus of Ota · Q-switched alexandrite laser · Light microscopy · Electron microscopy

Introduction

The naevus of Ota is a congenital or acquired, usually unilateral melanocytic lesion of the facial skin and ocular mucous membrane. The pigmented lesion shows a bluish discoloration and involves the area of the trigeminal nerve [3] (Fig. 1a, b). It was originally described by Ota and Tanino in 1939 [13], and the electron microscopical structure of the naevus of Ota has been investigated mainly by Japanese investigators [9, 20], as the naevus of Ota is common in Japan [8]. For patients suffering from disfigurement, cosmetic surgery such as skin transplantation and microsurgery have been proposed. Cryotherapy has recently prevailed; however, scarring commonly results from this therapy. Thus, therapy for selective destruction of the dermal melanocytes is desired.

Based on the theory of selective photothermolysis by laser irradiation [2], the selective destruction of melanosomes of epidermal melanocytes by a xenon fluoride (XeF) excimer laser was first confirmed by light and electron microscopy in 1983 [12]. The successful removal of tattoos using the Q-switched ruby laser (694 nm, 30 ns) irradiation, with short pulsation to avoid scarring, was reported in 1983 [15]. Several basic comparative investigations concerning the destruction of melanosomes and other chromogranules by different lasers with different outputs durations of irradiation are found in the literature [4, 10, 12, 14]. The studies reported were, however, limited to melanocytes or melanosomes in the epidermis. Q-switched alexandrite laser irradiation (760 nm, 50–100 ns) was first used for the naevus of Ota [1] and tattoos [16] in 1995. In addition to a short pulsation, the energy of the Q-switched laser is greater than that of non-Q-switched lasers, making irradiation for the short time possible.

Systemic morphological studies on the process of the destruction and healing of the naevus by both light and electron microscopy are few. The naevus of Ota can be healed almost completely by means of Q-switched alexandrite laser irradiation (755 nm, 100 ns), as shown in Fig. 1a and b. In the present study, this process was investigated in detail. Using light and electron microscopes, we investigated the alterations in melanocytes and recovery of injured tissue, using sequential biopsy specimens obtained after irradiation. Morphometry was also performed, for quantitative evaluation of the alterations in melanocytes and macrophages in the dermis and any alterations in epidermal melanocytes and keratinocytes after irradiation.

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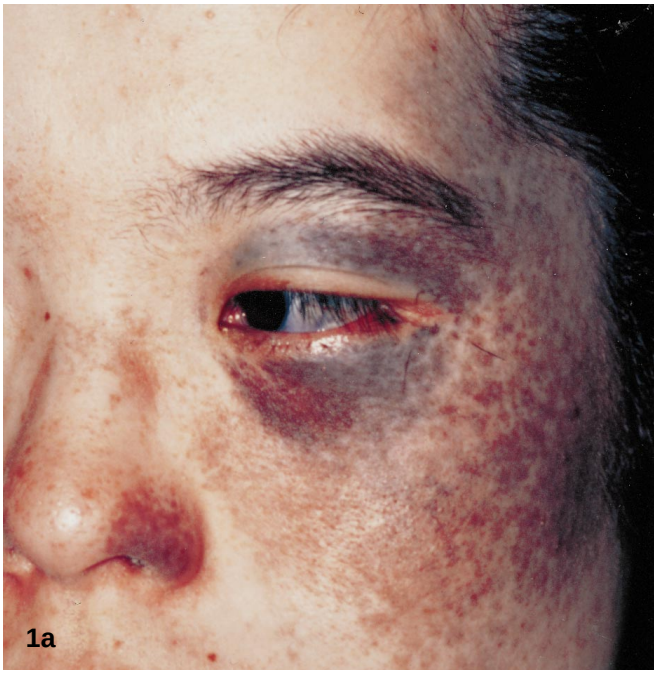
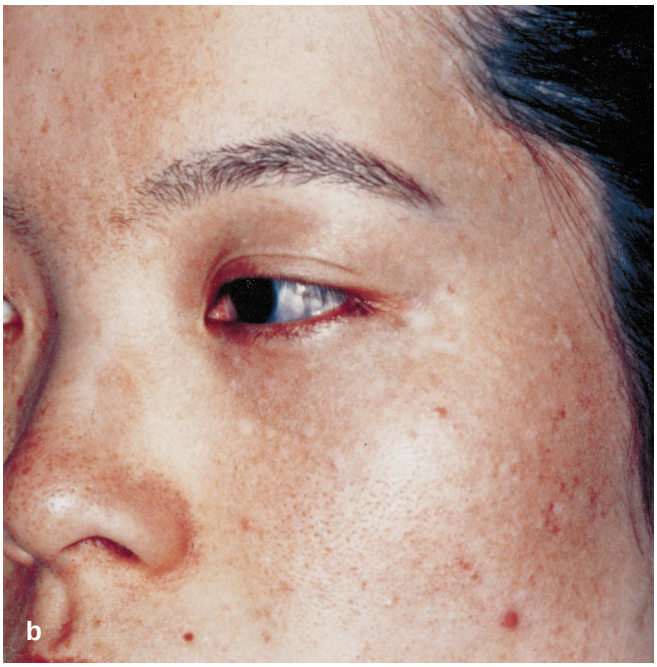
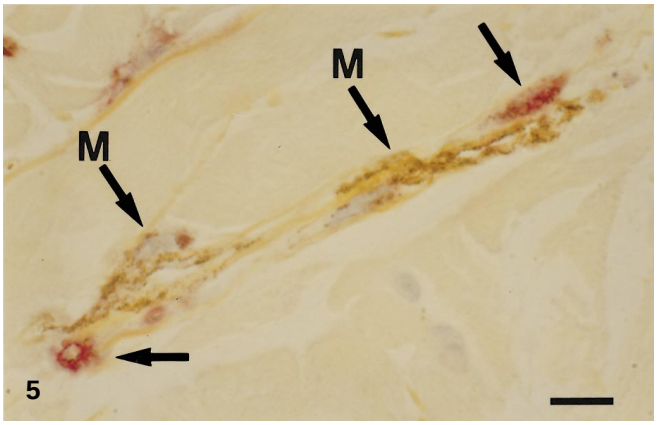






Materials and methods

Fourteen Japanese patients with the naevus of Ota (4 male and 10 female patients) were treated with laser irradiation. None of the patients had had any previous treatment. With the patients' consent, sequential biopsy was performed using a disposable trepan (diameter 4 mm; DISPOPUNCH, SFM, Germany) twice to four times before, and immediately (within approximately 5 min), 4 days and 3 months after, irradiation in each. A total of 36 skin biopsy specimens of naevi were obtained, and biopsy was performed an average of 2.6 times in each patient (Table 1). Laser irradiation was performed with the Q-switched alexandrite laser beam (PLTL-1, Candela Laser, Wayland, Mass.) at a wavelength of 755 nm, energy density of 8.0 J/cm^2, duration of 100 ns and spot diameter of 3.0 mm under local anaesthesia of 1% lidocaine admixed with adrenaline at 1/100,000.

Specimens were bisected: one piece was fixed in 10% neutral buffered formalin solution for light microscopy and the other in 2.4% glutaraldehyde solution for electron microscopy. The fixed tissues were dehydrated in a series of graded alcohol concentrations and embedded in paraffin for light microscopy, and then postfixed in osmium tetroxide, dehydrated and embedded in epon for electron microscopy. Paraffin sections were cut 3 µm thick and stained with haematoxylin and eosin and by the Fontana-Masson method for melanin pigment. Immunohistochemistry was performed using CD68 monoclonal antibodies (KP1, Dako, Denmark), a marker for human macrophages, by the streptavidin-biotin method (SAB-AP kit, Nichirei, Tokyo). Coloration was performed by the alkaline phosphatase-antialkaline phosphatase (APAAP) method using fast red violet LB (Sigma, St. Louis, Mo.). Haematoxylin was used for counterstaining. For electron microscopy, ultrathin sections were double stained with uranyl acetate and lead citrate.

Using a 40× objective lens and an eyepiece on which a square was printed, melanocytes, macrophages containing no melanin pigment and melanophages were counted in sections stained with CD68 antibodies. The square field of the eyepiece corresponded to a real area of 0.0576 mm^2 in a section. Six fields were randomly chosen for observation in each case. Fields having cutaneous appendages were excluded. Melanocytes, macrophages and melanophages were identified in light microscopy by the following criteria: melanocytes, CD68-negative cells containing melanin pigment; macrophages, CD68-positive cells; melanophages, CD68-positive cells containing melanin pigment. Macrophages and melanophages were counted in sections from all specimens. Melanocytes were counted in sections from the specimens obtained before and 3 months after irradiation. Results were expressed by the number of cells per square millimetre and the ratio of the number of melanophages in all macrophages. Areas of vacuoles in the dermis and basal layer immediately after irradiation and also of melanosomes in the dermal and epidermal melanocytes and in the basal cells were measured by light and electron micrographs using an image analyser (KS 300, Zeiss, Jena, Germany).

The results of morphometry were compared by means of the Mann-Whitney U-test at the critical value of $P<0.05$.

Results

Prior to irradiation, relatively large, dendritic melanocytes were sparsely scattered (Figs. 2a, 3a). Dermal

Fig. 1a, b Naevus of Ota of patient 6. **a** On 14 July 1994, prior to laster irradiation. **b** On 14 May 1996 after six laser irradiation treatments

Fig. 5 Macrophages, 4 days after irradiation. Macrophages (*arrows*) attach to the degenerated melanocytes (*M*). Immunohistochemical stain for CD68, with haematoxylin counterstain, *bar* 10 µm

Table 1 Patients and dates of biopsy (+ biopsy was performed, – biopsy was not performed)

Patient no.	Age (years)	Sex	Before irradiation	After irradiation		
				Immediately	4 days	3 months
1	5	f	+	+	–	–
2	15	f	+	+	+	+
3	17	f	+	+	–	–
4	19	f	+	+	–	+
5	21	m	+	–	+	–
6	22	f	+	–	–	+
7	27	f	+	+	–	–
8	29	f	+	+	–	–
9	31	f	+	+	+	+
10	34	f	+	–	+	–
11	37	m	+	–	+	+
12	44	f	+	+	–	–
13	46	m	+	+	+	–
14	55	m	+	+	+	–

melanocytes were distributed to an average depth of 2 mm (range, 1.80–2.45 mm) from the surface of skin. Melanosomes were at stages III and IV according to the classification of Fitzpatrick et al. [5] (Fig. 3b). In addition, a few melanophages (Fig. 3c) were found on electron microscopy to be intermingled in the naevus tissue.

Immediately after irradiation, a number of extracellular, tense, round vacuoles measuring 1,259±1,337 μm^2 (mean±standard deviation, *n*=452 in 9 patients, maximum 9,384 μm^2, in area were present in 9 of the 10 patients who were examined immediately after irradiation (Fig. 2b). In 1 patient (no. 8) no extracellular vacuoles formed. All vacuoles were intracellular in this case. Extracellular vacuoles were distributed to an average depth of 1.18 mm (range, 0.55–2.00 mm), and intracellular vacuoles to an average depth of 1.60 mm (range, 1.23–2.05 mm). Melanin pigment granules were found at the periphery of the vacuoles (Fig. 4a). Electron microscopy confirmed that they were the fragmented and degenerated melanosomes (Fig. 4d). Residual melanocytes became vacuolated and irregular in shape and were degenerated (Figs. 4b, c). There were three ways in which the melanosomes were altered: by fragmentation, vacuolation and fragmentation with vacuolation. The incidence of central vacuolation in melanosomes was 23.3% of irradiated dermal melanocytes. The alterations that were found in melanocytes were also present in melanophages (Fig. 4e).

Four days after irradiation, vacuoles disappeared (Fig. 2c) and the remaining melanocytes and numerous fragmented melanin pigment granules became scattered. Immunohistochemical and electron microscopical observation revealed that macrophages accumulated around the destroyed melanocytes and phagocytosed the fragmented and degenerated melanosomes (Figs. 5, 6a, b).

Three months after irradiation, a few melanocytes and more melanophages were found (Fig. 2d). Melanocytes at this stage were similar to those before irradiation in both light and electron microscopic features, but melanophages were different. Phagosomes containing the degenerating melanosomes were more numerous than before irradiation or immediately and up to 4 days after, although the melanosomes were more digested and reduced in size in phagosomes.

Table 2 Number of melanocytes in the naevi before and 3 months after irradiation

Patient no.	Number of melanocytes (per mm^2)		Reduction rate (%)
	Before irradiation	3 Months after irradiation	
2	249	52	79
4	365	31	92
6	404	26	94
9	435	49	89
11	623	35	94
Mean±SD	415±136	39±12	90±6

From 4 days to 3 months, a few lymphocytes and plasma cells had occasionally infiltrated around the vessels.

Numerical changes of melanocytes, macrophages and melanophages during the investigation are shown in Table 2 and Fig. 7 (left and right). Melanocytes were significantly decreased 3 months after irradiation. Macrophages decreased immediately after irradiation and increased significantly 4 days after irradiation; after 3 months they had recovered almost to the pre-irradiation level. The ratio of melanophages in all macrophages was significantly decreased immediately after irradiation and had increased significantly 4 days later. No differences between the ratios before and 3 months after irradiation were observed.

In the epidermis, prior to irradiation the cells containing melanin pigment were melanocytes and keratinocytes. Melanocytes were clear and contained a few melanin pigment granules, while basal cells were rich in melanin pigment (Fig. 8a). Melanosomes were much smaller in epidermal melanocytes and basal cells than in dermal melanocytes; the areas of melanosomes were

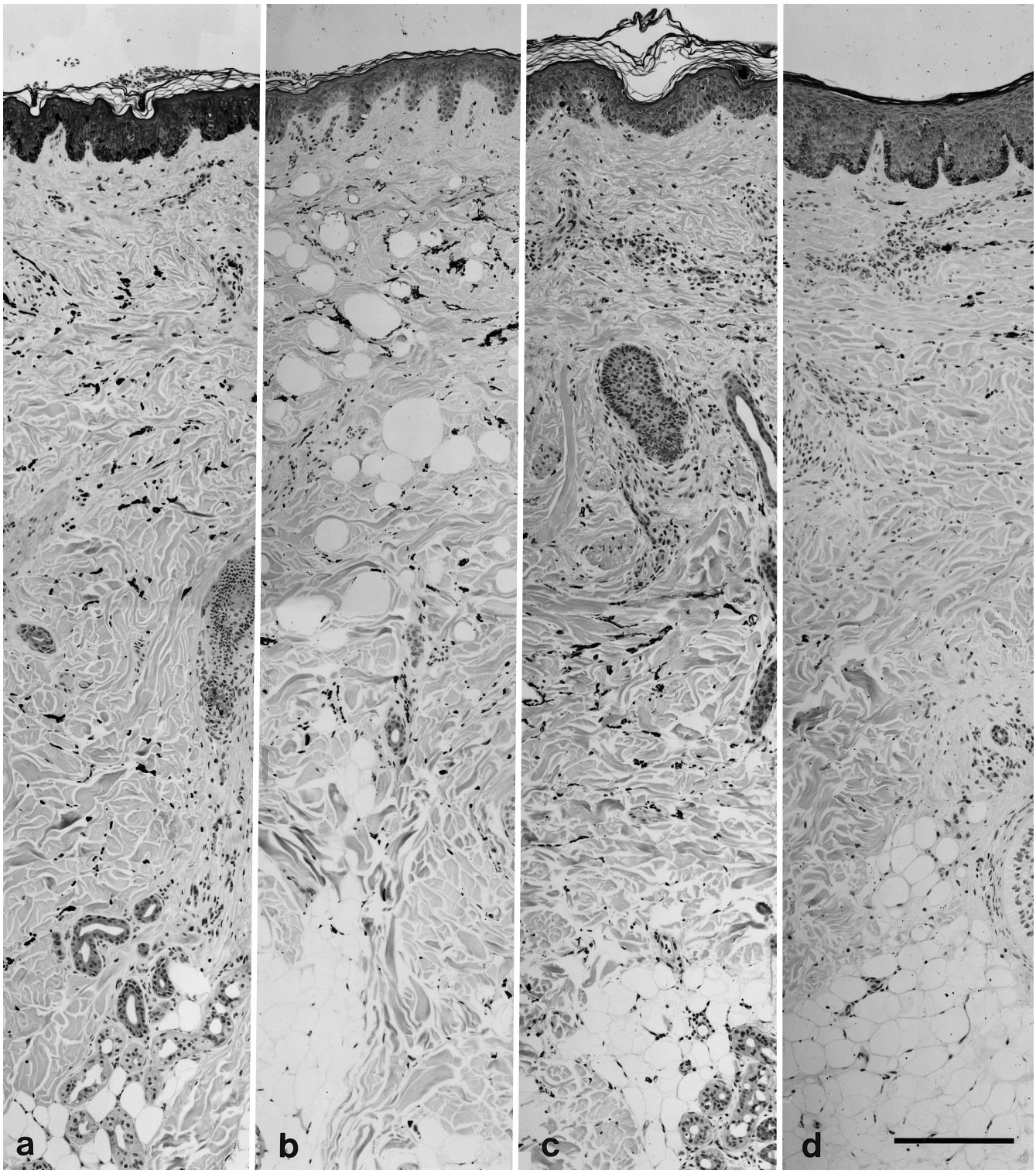


Fig. 2a–d Histological features of the biopsy specimens obtained from naevi of Ota. Fontana-Masson, nuclear fast red counterstain, *bar* 200 µm. **a** Before irradiation in patient 4. Dermal melanocytes are sparsely scattered in the deeper dermis. **b** Immediately after irradiation in patient 7. Melanin pigment-containing cells have decreased in number, and large, extracellular, tense round vacuoles are present in the dermis. **c** At 4 days after irradiation in patient 3. Vacuoles have disappeared from the dermis. A few melanin pigment-containing cells remain, as in **b**. A hyperkeratotic change containing melanin pigment is seen in the epidermis. **d** At 3 months after irradiation in patient 9. The number of melanin pigment-containing cells has declined further. No scarring. Cutaneous discoloration is becoming inconspicuous

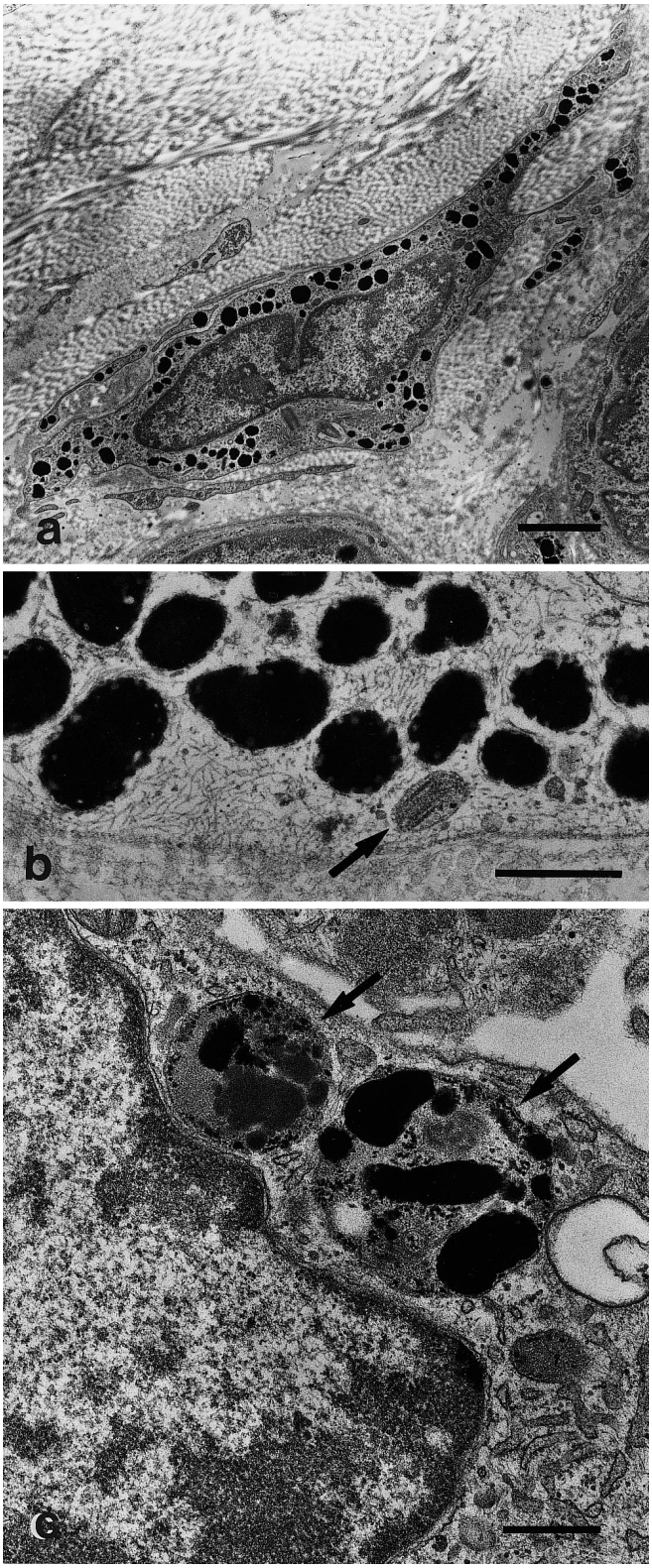


Fig. 3 Electron microscopic features of dermal melanocytes and melanosomes (**a, b**) and **c** of a melanophage before irradiation. **a** A dendritic dermal melanocyte filled with melanosomes. *Bar* 2 μm. **b** Melanosomes at stages III (*arrow*) and IV are seen. *Bar* 0.5 μm. **c** A melanophage found in the naevus tissue. *Arrows* indicate the phagosomes containing fragmented and degenerating melanosomes. *Bar* 0.5 μm

0.013±0.008 μm^2 (447 melanosomes in 11 cells from 9 patients) in epidermal melanocytes, 0.018±0.012 μm^2 (561 melanosomes in 10 cells from 8 patients) in basal cells, and 0.101±0.058 μm^2 (971 melanosomes in 13 cells from 10 patients) in the dermal melanocytes. The ratio of the total area of melanosomes in epidermal melanocytes and basal cells was 7.53±3.74% (12 cells from 8 patients) or 8.75±2.55% (13 cells from 6 patients), respectively, while that in dermal melanocytes was 35.30±12.25% (20 cells from 9 patients).

Immediately after irradiation, vacuolated cells were sparsely dispersed in the basal layer (Fig. 8b), although the vacuoles were smaller than those observed in the dermis, measuring 25.2±17.9 μm^2 in area. The main alterations to irradiated melanocytes were expansion of intercellular space and intracellular vacuoles (Figs. 9a, b). Injury to melanocytes was relatively limited, and some melanocytes were considered to be viable from their morphological features (Fig. 9a).

Four days after irradiation, keratinized debris and melanin pigment had increased in the thickened stratum corneum (Fig. 8c). Cells containing melanin pigment had decreased in the basal layer (Fig. 8c).

Three months after irradiation, the number and size of melanocytes and basal cells were similar to those found before irradiation (Fig. 8d).

Discussion

The theoretical basis of laser therapy is ‘‘selective photothermolysis’’, and one of the biologically, most interesting targets is a melanocyte [2]. Laser irradiation is widely used for benign cutaneous pigmented lesions and tattoos [16, 18]. In this study, tissular, cellular and ultrastructural alterations of the naevus of Ota induced by Q-switched alexandrite laser irradiation and subsequent repair have been investigated using sequential biopsy specimens.

Although several publications have the light microscopic features of the alteration of naevus cells induced by laser irradiation, they have described only those features related to the healed stage [6, 7, 18]. No reports of sequential observation of the time-course have been published. The present study has shown large tense vacuolation (Fig. 4a) immediately after irradiation, although irregularly shaped vacuoles have been reported as an epidermal change in guinea pig skin after ruby laser irradiation [14] and a change of melanocytes in the naevus of Ota [17, 19] by Q-switched ruby laser. This discrepancy between tense, round withered, irregularly shaped vacuolations was thought to result from the interval between irradiation and biopsy. In the present study, all biopsy specimens were obtained within approximately 5 min after irradiation, while Polla et al. [14] obtained specimens from 5 to 30 min after irradiation. The characteristic of early changes due to laser irradiation is though to be the tense round vacuoles, followed by deformation.

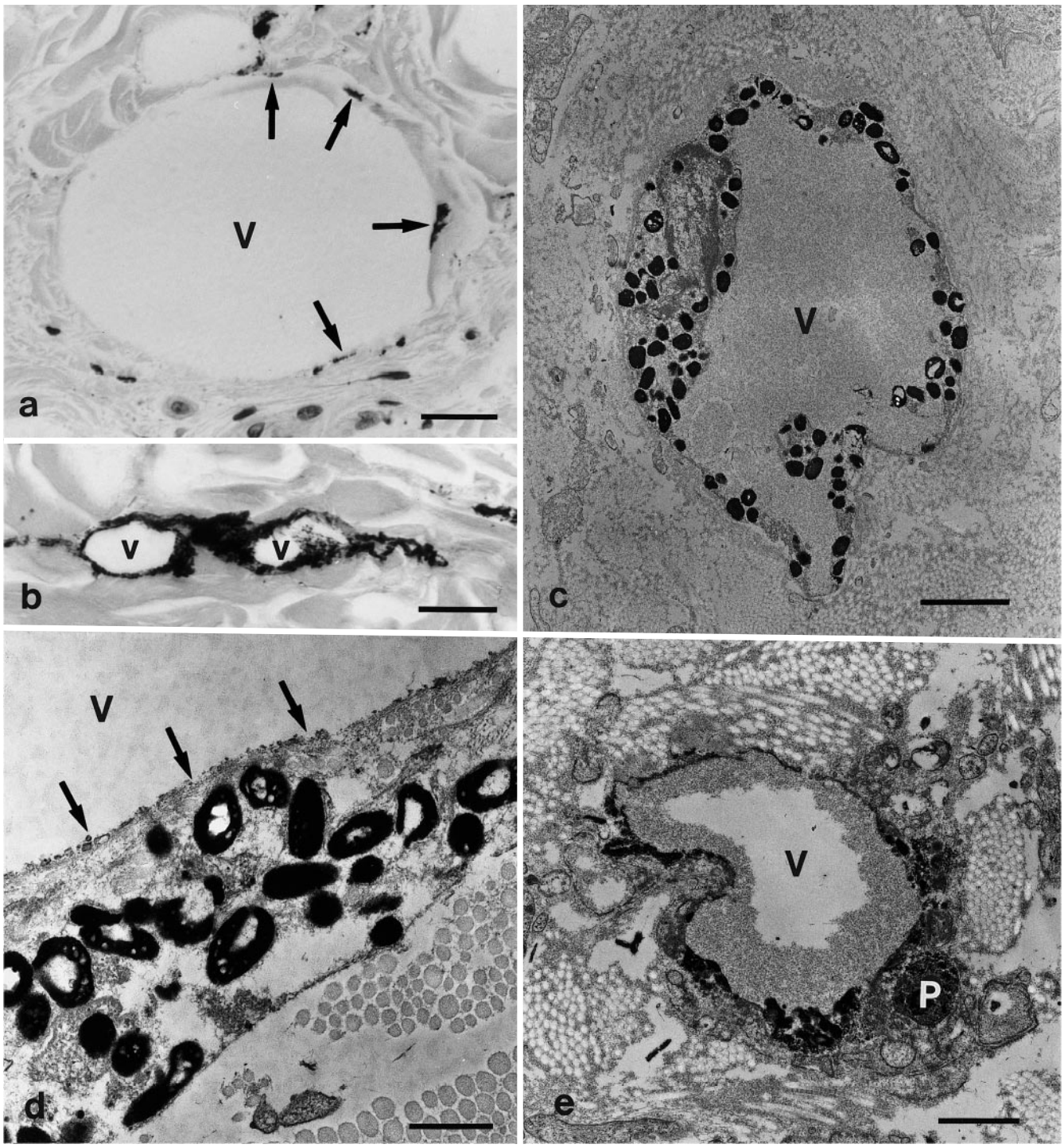


Fig. 4a–e Changes in the naevus immediately (within approximately 5 min) after irradiation. **a** An extracellular, large, tense, round vacuole (*V*) resulting from the vaporization of a dermal melanocyte or melanophage induced by absorption of the laser. No cellular vestige can be found, although a few melanosomes or their fragments (*arrows*) are scattered at the rim of the vacuole. Fontana-Masson stain, nuclear fast red counterstain, *bar* 20 µm. **b** Two vacuolated melanocytes. An intracellular large vacuole (*V*) can be observed in each cell. *Bar* 20 µm. **c** An electron microscopic feature of a vacuolated dermal melanocyte. A vacuole (*V*) occupies almost the whole cell. Central vacuoles are observed in some of the remaining melanosomes at the cellular rim. The nucleus (*N*) is deformed and degenerated. Both cellular and nuclear membranes are unrecognizable. This dermal melanocyte is not viable. *Bar* 2 µm. **d** An electron microscopic feature of the rim of an extracellular, tense, round vacuole. A central vacuole forms in several melanosomes as an effect of laser irradiation. Fragmented melanosomes can be seen on the inner margin (*arrows*) of the vacuole (*V*). *Bar* 0.5 µm. **e** An electron microscopic feature of a melanophage. A large vacuole (*V*) occupies almost the whole cell. Degenerating melanosomes are pushed to the periphery of the cell. Many phagosomes containing pigment granules are deformed. A phagosome (*P*) is well preserved. *Bar* 1.0 µm

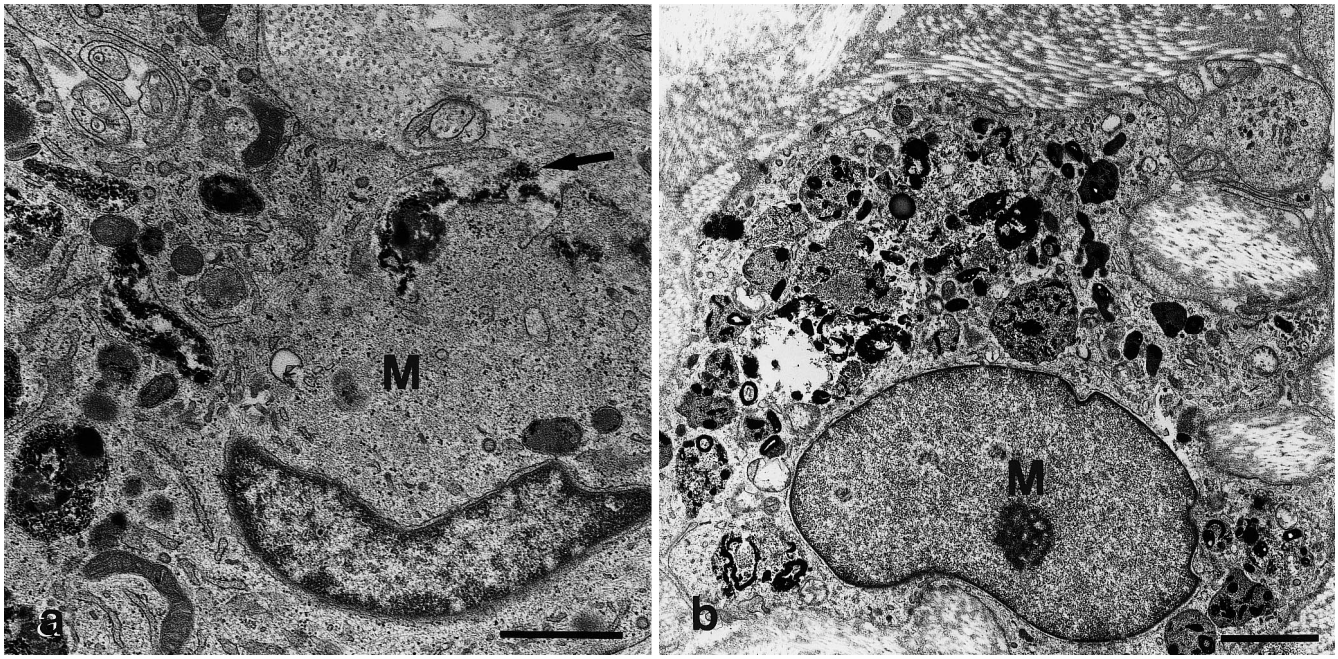
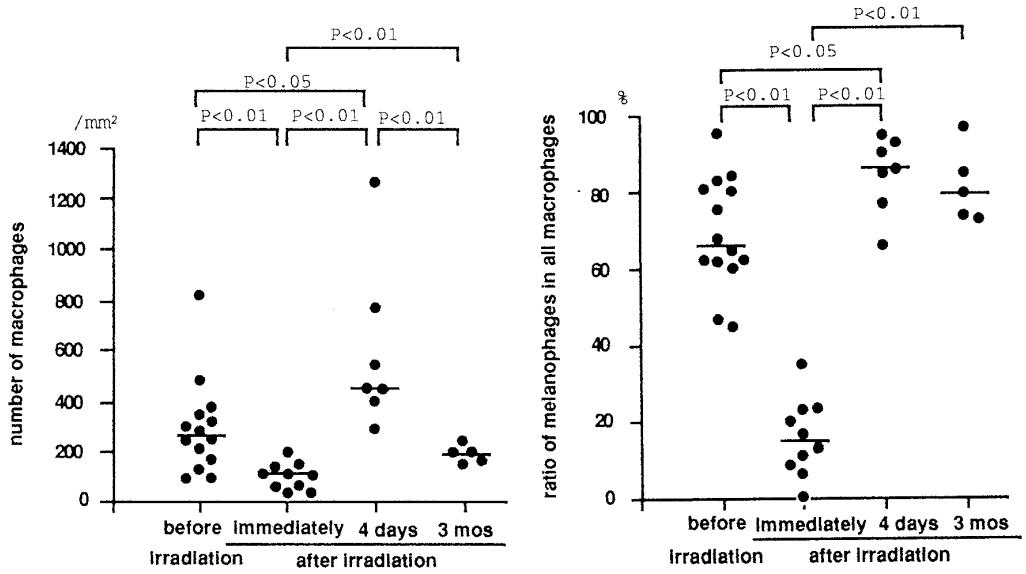


Fig. 6a A macrophage (*M*) phagocytosing the degenerated melanosomes 4 days after irradiation. *Arrows* indicate pinocytotic activity. *Bar* 2.0 μm. **b** A macrophage (*M*) 3 months after irradiation. Numerous phagosomes containing degenerative melanosomes can be observed. *Bar* 1.0 μm

The sequential change we observed may be presented as follows: the large tense vacuolation, fragmentation and degeneration occur first by vaporization of melanosomes and melanin pigment granules contained in both melanocytes and melanophages. The cells and tissue components that contain no melanin pigment remain intact and show only contact pressure from vacuoles. The vacuoles soon disappear, and macrophages gather around damaged melanosomes and melanocytes and phagocytose them. Some fragments of melanosomes are considered to be eliminated by lymphatics without phagocytosis by macrophages. A few melanocytes and more melanophages persist in the dermis, which becomes clear almost to the same level as unaffected skin. In tattoos of the skin, microscopic examination has also demonstrated persistence of tattoo ink granules in the clinically clean area irradiated by laser irradiation [11]. Neither keloid nor scar tissue formed.

Alteration of the epidermis is an inevitable side effect of laser therapy for naevus of Ota situated in the deeper reticular layer of the dermis. Epidermal melanocytes and basal cells are situated closer to the laser source than the naevus, and the laser reaches the naevus by passing through the basal layer, so that epidermal melanocytes

Fig. 7 *Left* number of macrophages; *right* ratio of number of melanophages in all macrophages. *Bars* median values



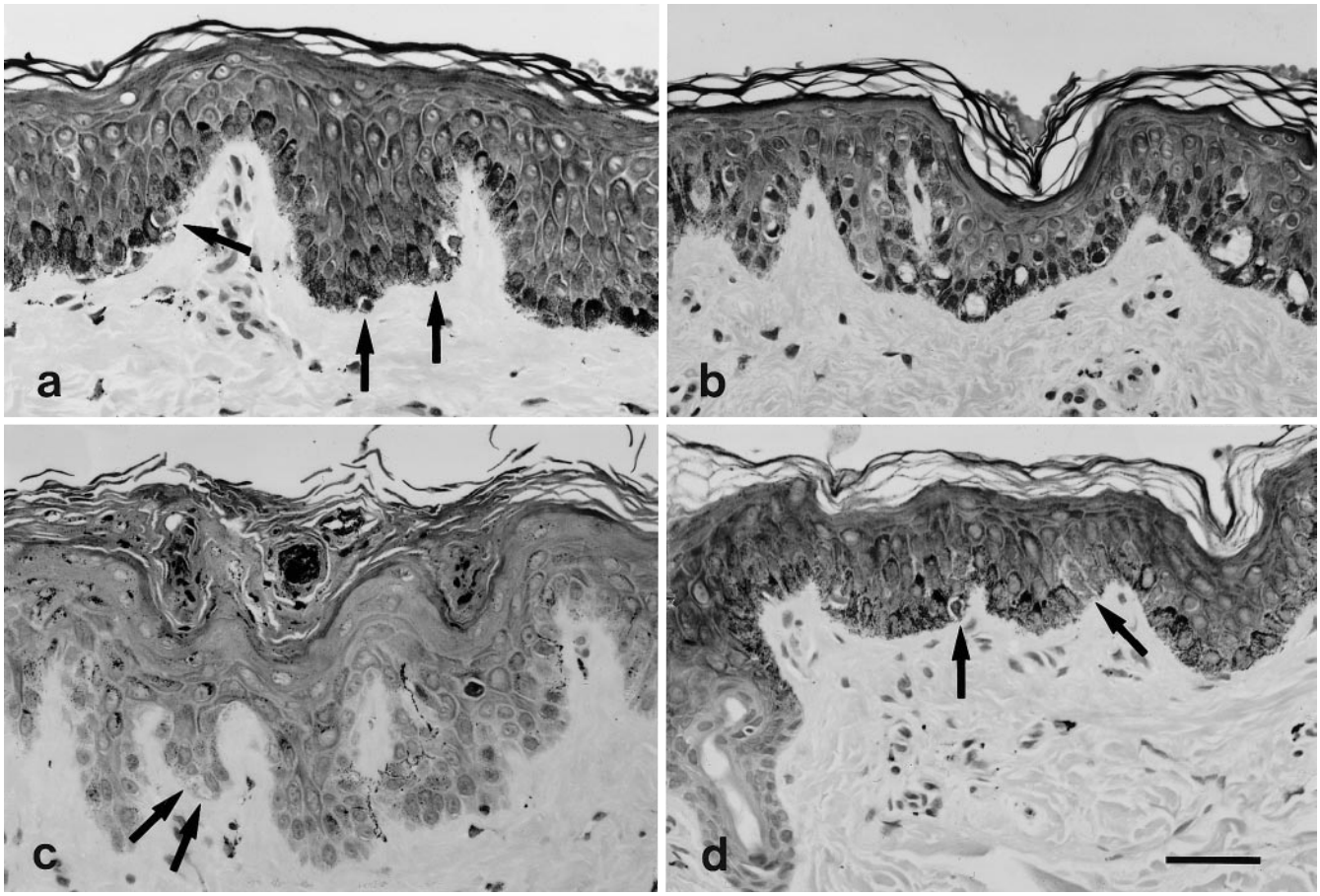


Fig. 8a–d Epidermis covering the naevus. *Arrows* indicate melanocytes. Fontana-Masson, nuclear fast red counterstain, *bar* 20 μm. **a** Before irradiation. **b** Immediately after irradiation. Vacuoles are present in the basal layer. **c** Four days later. Melanin pigment granules have been displaced to the higher level and accumulated in the hyperkeratinizing stratum corneum. **d** Three months after irradiation. Near-recovery to the same status as before irradiation

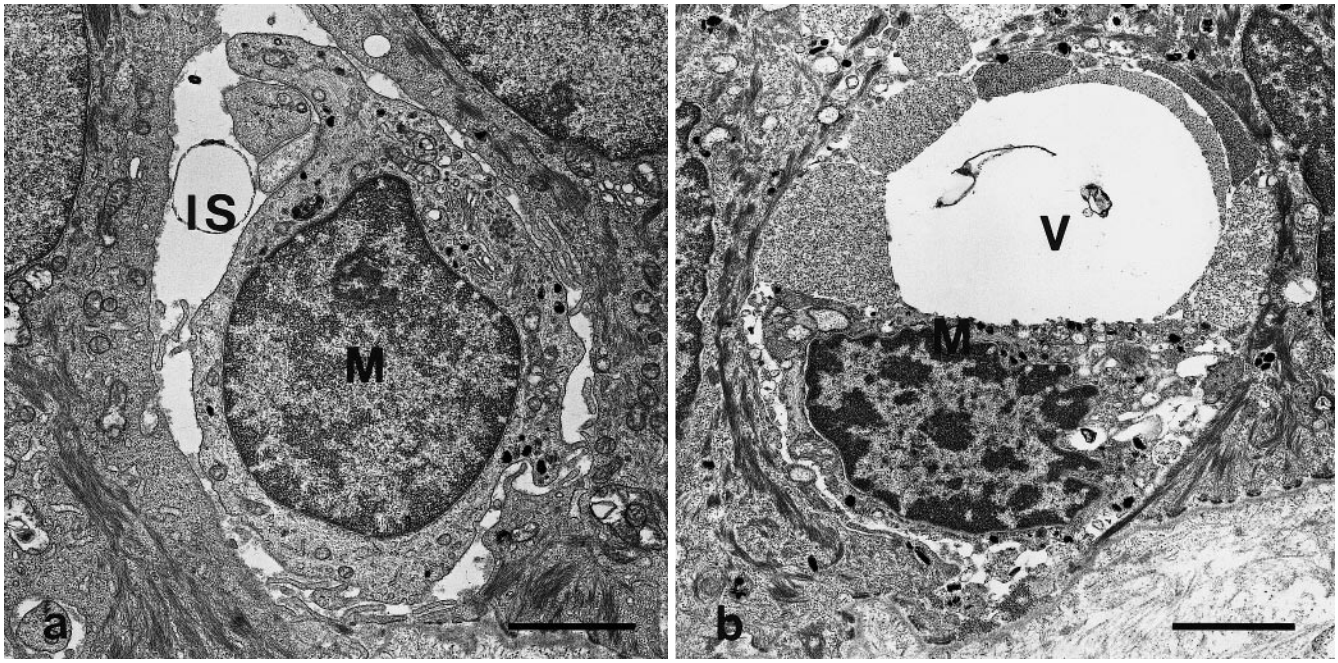


Fig. 9a, b Epidermal melanocytes (*M*) immediately after irradiation. **a** Intercellular space (*IS*) expands, and the processes become shortened and inconspicuous. However, the intracellular structure is well preserved. *Bar* 2.0 μm. **b** A large vacuole (*V*) is present. Although a nucleus is well preserved, the cell membrane disappears. This cell is considered not viable. *Bar* 2.0 μm

and basal cells cannot avoid exposure. However, injury to melanocytes and basal cells of the epidermis was insignificant, and some melanocytes and basal cells remained viable. It is reasonable to assume that the absorption of the laser by epidermal melanocytes and basal cells is low because their melanosomes and the area ratio of the melanosomes are small.

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