Light and Electron Microscopic Analysis of Tattoos Treated by Q-Switched Ruby Laser

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Short-pulse laser exposures can be used to alter pigmented structures in tissue by selective photothermolysis. Potential mechanisms of human tattoo pigment lightening with Q-switched ruby laser were explored by light and electron microscopy. Significant variation existed between and within tattoos. Electron microscopy of untreated tattoos revealed membrane-bound pigment granules, predominantly within fibroblasts and macrophages, and occasionally in mast cells. These granules contained pigment particles ranging from 2 in diameter. Immediately after exposure, dose-related injury was observed in cells containing pigment. Some pigment particles were smaller and lamellated. At fluences \( \geq 3 \) J/cm\(^2\), dermal vacuoles and homogenization of collagen bundles immediately adjacent to extracellular pigment were occasionally observed. A brisk neutrophil infiltrate was apparent by 24 h. Eleven days later, the pigment was again intracellular. Half of the biopsies at 150 d revealed a mild persistent lymphocytic infiltrate. There was no fibrosis except for one case of clinical scarring. These findings confirm that short-pulse radiation can be used to selectively disrupt cells containing tattoo pigments. The physical alteration of pigment granules, redistribution, and elimination appear to account for clinical lightening of the tattoos. J Invest Dermatol 97:131–136, 1991

MATERIALS AND METHODS

Fifty-seven tattoos (35 amateur and 22 professional) were treated with the QSRL (Lasermetrics model 936R31-1) at a wavelength of 694 nm with a pulse duration of 40 nsec. All of the tattoos were dark blue or black although some (<10%) also had very small areas of red, green, yellow, and turquoise. Each tattoo received up to five treatments at a fixed, but randomly assigned, exposure dose (1.5–4.0 J/cm\(^2\)) at a mean interval of 3 weeks. The exposure field diameter was 5 mm. Tattoos were exposed to multiple contiguous pulses in an attempt to cover the tattooed area. The clinical results are reported elsewhere [3].

Under local anesthesia (1% lidocaine without epinephrine), 33 punch biopsies (3 or 4 mm) were obtained from 13 healthy Caucasian volunteers who had given their informed consent. Biopsies were taken before treatment, immediately after, and at 1, 5, 7, 11, 30, 90, and 150 d after the first treatment. Specimens were preserved in formalin and stained with hematoxylin and eosin. In 17 cases, a 1-mm portion was processed for electron microscopy by initial fixation in 4% glutaraldehyde in 0.1 M cacodylate buffer and post-fixation in 2% osmium tetroxide also in cacodylate buffer. After dehydration and embedding in epon, thin sections were cut on a Reichert Ultracut, left “unstained” with no further contrast enhancement, or stained with uranyl acetate and lead citrate, and examined with a Philips CM10 transmission electron microscope.

LIGHT AND ELECTRON MICROSCOPIC RESULTS

Before Irradiation Before QSRL exposure, the epidermis appeared normal by light and electron microscopy except for the rare presence of tattoo pigment identified by light microscopy. The pigment will absorb light and be a potential source of epidermal injury; however, because the tattoo particles are rare, the contribution to the total epidermal injury will be minimal. The basement membrane was continuous at the epidermal-dermal junction. No scarring, inflammatory reactions, or granulomas were seen in the dermis. There were intracellular pigment granules or pigment-laden phagosomes, existing predominantly in perivascular fibroblasts and occasionally in mast cells and macrophages (Fig 1a). Al-
Figure 1. Light micrographs of a professional blue-black tattoo before, immediately after, and 90 d after ruby laser irradiation at 3 J/cm². a, Before irradiation. Toluidine blue-stained 1-μ section showing pigment-laden cells (arrows) in perivascular locations (×400). b, Before irradiation. Unstained section adjacent to a from central area showing intracellular round granules (magnification ×1000). c, Immediately after irradiation. Toluidine blue stained section shows cellular debris (arrows, magnification ×400). d, Immediately after irradiation. Unstained section adjacent to c from central area showing lacy quality to pigment and loss of density (magnification ×1000). e, 90 d after irradiation. Toluidine blue-stained section showing pigment-laden cells (arrows) in perivascular location (magnification ×400). f, 90 d after irradiation. Unstained section adjacent to e from central area demonstrating presence of pigment in granules. b and d were printed with the same conditions; however, f was exposed for a longer time because the density of the granules is much less (magnification ×1000).
though polymorphous, the majority of these pigment granules were round or oval with a diameter of approximately 0.5 – 4.0 μ (Fig 1b). They were surrounded by a single membrane although in some cases the limiting membrane could not be clearly traced. The granules were both perinuclear and peripheral in thin-cell processes.

By light microscopy, the pigment granule color corresponded to that of the tattoo. The majority were black. One turquoise tattoo was noted to contain polymorphous turquoise granules, generally twice as large as the black ones. In a red tattoo, polymorphous red granules, also twice as large as the black ones, were seen.

In professional tattoos, these pigment granules were deposited regularly at the junction of the papillary and reticular dermis, often reaching mid-dermis. In amateur tattoos, they were more haphazardly arranged, extending from papillary dermis to subcutaneous fat. Those located more deeply were more tightly packed. In amateur tattoos pigment granules displayed more heterogeneity of size and shape than in professional tattoos. In both types, the histologic picture from site to site within a given tattoo was highly variable with regard to pigment depth and density.

Higher magnification revealed that these pigment granules were composed of three kinds of loosely packed pigment particles, ranging from 2 to 400 nm in diameter (Fig 2). The most common pigment particle was approximately 40 nm in diameter. It was round and of moderate electron density. The second kind of particle was much less common, also round, but slightly more electron dense and 2 – 4 nm in diameter. Observed even less frequently were the largest particles, which were up to 400 nm in diameter, oval, and very electron dense. Only the largest particles had a crystalline structure. In addition to these three kinds of pigment particles, the pigment granules occasionally contained melanosomes as well as amorphous cellular material. Except for the pigment granules, the ultrastructure of the cells containing the tattoo pigment was normal.

Immediately After Irradiation Immediately after QSR L exposure, fluence-related injury to cells containing either tattoo pigment or melanin was observed. Generally, cells without pigment were not altered. In the epidermis, vacuolization of melanocytes and keratinocytes was seen at all doses, with rupture of melanosomes, as previously described following QSR L pulses [6,7]. With doses of 3 – 4 J/cm², sub-epidermal blisters were occasionally noted. At 4 J/cm², epidermal necrosis was detected.

Figure 2. Transmission electron micrograph shows unstained tattoo particles within a non-irradiated blue-black professional tattoo. Note variability in size of mid-sized particles and single large crystalline particle (arrow). Bar, 0.1 μm.

By light microscopy, the pigment granules were less discrete with a lacy quality (Fig 1c,d). They were brown or gray. Most of the superficial granules appeared altered, whereas fewer of the deep ones were altered.

Tattoo pigment particles, whether altered or unaltered, were extracellular and adjacent to cellular debris or within damaged cells (Fig 3). Particles that appear similar to each of the three types of pre-irradiation pigment particles could still be found. In addition, round, lamellated, electron-lucent (25 – 40 nm diameter) particles were identified in all tattoos, whether amateur or professional (Fig 4). These altered pigment particles were found throughout the depth of the dermis.

Figure 3. Transmission electron micrograph shows a severely damaged fibroblast in the deep dermis immediately after laser irradiation of a blue-black professional tattoo at 3 J/cm². Nuclear (N) and membrane (arrowheads) damage is noted as well as disruption of the pigment granules (arrows). Bar, 1 μm.

Figure 4. Transmission electron micrograph shows unstained pigment particles immediately after irradiation of a blue-black professional tattoo at 4 J/cm². Some particles (arrows) show a changed appearance, lamellated and less opaque compared to the remaining unchanged particles. Bar, 0.1 μm.
In general, blood vessels (Fig 5), hair follicles, sweat glands, sebaceous glands, nerves, and lymphatics appeared normal. When surrounded by large amounts of pigment or when irradiated at 4 J/cm², occasional superficial blood vessels showed cytoplasmic vacuolization and nuclear chromatin condensation. Rarely, at fluences of 3 J/cm² or higher, there was homogenization of collagen fibers lying close to altered pigment particles and cellular debris (Fig 6). In some biopsies there were numerous round or oval dermal vacuoles ranging from 25 – 60 microns in diameter, sometimes lined by cellular debris or altered tattoo (Figs 5 and 7). In biopsies taken 1 h after exposure, there was mild edema of the connective tissue and dilated lymphatics. A mild polymorphonuclear cellular infiltrate with occasional eosinophils was noted.

Short-Term Biopsies By 24 h, the neutrophilic infiltrate was prominent, becoming mild again by day 5. Epidermal hyperplasia was also seen at day 5. Many of the pigment granules were already repackaged in fibroblasts and occasional mast cells and macrophages, but some granules were still extracellular along with debris. In one case, unstrained sections of the scale crust that formed by day 7 revealed tattoo pigment in the material being shed.

By day 11, there were many perivascular and periadnexal lymphocytes and macrophages. The lacy appearance of the pigment granules by light microscopy was no longer present. The granules had a fine stippled quality and were gray or brown in color. Indeed, all of the altered and unaltered pigment particles were no longer extracellular but rather intracellular in phagolysosomes (0.5 – 4.0 μm in diameter) in the same types of perivascular cells, namely fibroblasts, macrophages, and occasional mast cells. The granules were surrounded by a single membrane once again. There was minimal cellular debris.

Long-Term Biopsies (30 – 150 D) From day 30 – 150, biopsies revealed a similar picture. The epidermis, sweat glands, sebaceous glands, and hair follicles were normal. Occasionally, there was duplication of the basement membrane surrounding capillaries in tattoos treated at 4 J/cm². Only one patient showed clinical scarring. In that case, light microscopy revealed a parallel array of dense fibrosis to a depth of 0.8 mm. Otherwise, no fibrosis was seen in biopsies at 150 d. Very seldom was there a slight increase (less than 20%) in the number of fibroblasts. In half of the biopsies at 30 – 150 d, a mild lymphocytic infiltrate persisted around vessels and appendages. There were enlarged fibroblasts with dilated rough endoplasmic reticulum and macrophages filled with polymorphous, membrane-bound pigment granules whose diameters now measured approximately 0.2 – 1.0 μm (Fig 8). The reason for persistence of the mild increase in fibroblasts and the presence of a mild inflammatory cell infiltrate is not clear; however, it may be speculated that the altered tattoo particles are providing continuing inflammatory stimulus. Both perinuclear and peripheral pigment granules were observed. The appearance of the granules by light microscopy was still that of a gray or brown stippled quality (Fig 1e,f). By electron microscopy, all four types of pigment particles were identified: the three types noted prior to irradiation and the lamellated post-irradiation type. The majority of the pigment particles were of the
Figure 8. Transmission electron micrograph of a fibroblast in the upper dermis shows a variety of granules containing amorphous remnants of tattoo particles. Biopsy is from a blue-black amateur tattoo approximately 6 months after five irradiations with 2 J/cm². Bar, 1 μm.

lamellated type. These altered particles could be seen even at the depth of the subcutaneous fat. The pigment granules remained polymorphous. However, in a given tattoo, there were more lamellated particles in granules that lay deep (Fig 9) than in those that were superficial (Fig 10). In the deep dermis, for example, most pigment particles in a given granule were lamellated. Pigment particle packing was loose with distinctly clear areas noted within the granule. Granule diameters approximated 1 μ and overall electron density was minimal. In mid-dermis, the granules were more electron dense and their contents were amorphous with fewer distinct particles visible. Those granules located in papillary dermal cells were very densely packed, measuring 0.2-0.4 μ in diameter. Discrete particles were very rarely detected. The pigment granule contents appeared homogenized with no discrete particles detectable.

Interestingly, the clinic-histologic correlation was poor. Tattoos responding well clinically often had much residual pigment; tattoos responding poorly clinically sometimes had little residual pigment. Although the residual pigment often lay deep, there was no apparent correlation between depth, quantity, or proportion of altered versus unaltered pigment with clinical lightening.

**DISCUSSION**

It is interesting to speculate on the mechanisms leading to clinical lightening in treated tattoos. Photon absorption by tattoo pigment within cells appears to be the initiating event. The temperatures that occur transiently within the pigment granule may be sufficient for pyrolytic chemical alterations of the pigment particle. During the 40-nsec laser pulse, temperature exceeding 1000°C may occur. Gasous products of pyrolysis and/or pores created by superheated steam or drossed gas may account for the lamellated appearance of the granules after laser exposure. There is clear evidence of mechanical changes in both the tattoo pigment granules and the cells containing them. The reduction in pigment particle size and fragmentation of pigment-containing cells probably results from rapid thermal expansion, shock waves, and potentially localized cavitation. In addition, localized thermal denaturation of cellular proteins, such as enzymes, and extracellular proteins, such as collagen, may play some role in the damage and initiation of its repair. There is a clear gradient in the changes in the pigment granules and particles at different depths, which is consistent with decreasing energy densities at increasing depths. Selective death of pigment-containing cells ensues, and pigment is released. Similar events occur in epidermal cells containing melanin pigment. The inflammatory response that follows engulfs the debris and tattoo pigment. It is possible that this process of inflammation and phagocytosis reduces the overall amount of tattoo pigment, and that some pigment is eliminated during desquamation of epidermis during repair.

It is clear that there is also limited fluence-dependent thermal damage to collagen immediately surrounding pigment-containing
cells. Following high radiant exposures, the presence of petechiae, capillary damage immediately after irradiation, and basement membrane duplication at later times suggests a degree of fluorescence-dependent vascular damage as well. Clinically, sometimes there are petechiae in the tattoo after irradiation, but not in exposed normal skin, suggesting that vascular injury is secondary to the destructive events occurring in tattoo particles. Whether this ultrastructural damage results from photoacoustic, photochemical, or photothermal effects remains speculative. The dermal vacuoles are related to the treatment and may represent the destruction of pigment-containing cells secondary to photothermally induced, rapid-phase changes. At higher doses, some pigment may be lost in the scale crust, which is shed.

The occurrence of the lamellated pigment particles after irradiation and their apparent persistence in the long-term biopsies merit further investigation. Although the identity of the precursor of this lamellated form is not firmly established, it is likely to be the 40-nm particle because of its size and frequency of appearance. A possible explanation for its lamellation is that there is a sudden release of gaseous products, which may occur with pyrolysis or evolution of bound or dissolved gases within the particles. Alternatively, stress-induced deformation can occur.

The lack of clinicopathologic correlation between the quantity/ location of tattoo pigment and clinical appearance of the lesions was surprising. In contrast to findings reported after argon and tunable dye laser treatment of tattoos [8], fibrosis, which conceivably might obscure pigment by scarring, does not appear to play a major role in clinical lightening. However, it is conceivable that subtle alterations in dermal scattering coefficients may occur, with the result being obscuring of deeper pigment. A redistribution of pigment particles, without their removal, may also be implicated in lightening of the tattoos. The finding of amorphous cellular debris and melanosomes within the pigment granules suggests that they may be lysosomal in nature. The diameter of the granules is smaller in the long-term biopsies (0.2–1.0 micron) than in the control biopsies (0.5–4.0 microns). Conceivably, this repackaging difference could affect clinical lightening. Optical scattering by particles approximately equal to the wavelength of light (0.5 μm) is in theory stronger than that for either much larger or much smaller ones. In addition, the scattering by smaller particles is more isotropic than that for larger particles, which is forward directed. The alteration in size and structure may also change the ability for phagocytosis of the particles. Tattoo histology and ultrastructure reflect the manner in which tattoos are made. Amateur tattoos are generally made using non-sterile or semi-sterile needles and India ink [9]. Other pigments such as cigarette ash, pencil, graphite, or carbon particles, all of which are forms of elemental carbon, may be used. The amount and depth of pigment introduced is poorly controlled [9,10]. Professional tattoo artists often use a fine vibrating needle to deposit pigment just below the epidermal-dermal junction [10]. They use organic dyes often mixed with metallic elements [11]. As we have seen, histology reflects the basic difference in depth rendered by the two tattooing techniques. Despite the diverse origins of the tattoo pigment, the light and electron microscopic appearances of the materials are remarkably similar. It is striking that neither method leaves any detectable clinical or histologic scarring. Tattoo pigment is relatively inert and normally very little foreign body reaction to the pigment is seen.

The inherent heterogeneity of tattoos makes it difficult to compare responses between different tattoos and may explain the lack of clinico-histologic correlation. Substantial intra-tattoo variability makes it difficult to correlate clinical pigment lightening, even in a given tattoo by quantitative microscopic analysis of sequential biopsies. The different composition of pigment particles in multicolored tattoos may explain in part why professional tattoos respond less favorably.

Fibroblasts, macrophages, and mast cells are capable of active phagocytosis and each may contain tattoo pigment [12,13]. Although the intracellular location of the pigment granules in an untreated tattoo is well described in the literature [12,13], some reports say that tattoo pigment can be extracellular in untreated tattoos [14,15]. In our experience, the pigment in an untreated tattoo is exclusively intracellular. In contrast to earlier reports stating that the pigment granules are only found perinervely [12], we have seen that they are both perinuclear and peripheral in thin-cell processes.

The task of analyzing QSWL-treated tattoos may be facilitated by knowledge of the specific ingredients used in the making of the tattoos. In the case of amateur tattoos, amorphous carbon (e.g., India ink) or graphite are most commonly used. Many professional tattoo pigment suppliers, however, guard their recipes closely. Some investigators have turned to sophisticated techniques to identify tattoo pigment constituents [11]. A systematic study of a series of treated tattoos in an animal model might be helpful, especially if they were done by the same artist with known ingredients.

REFERENCES