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Phototoxic Damage to Sebaceous Glands and Hair Follicles of Mice After Systemic Administration of 5-Aminolevulinic Acid Correlates with Localized Protoporphyrin IX Fluorescence

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The skin of albino mice given 5-aminolevulinic acid (ALA) by intraperitoneal injection rapidly developed the characteristic red fluorescence of protoporphyrin IX. Fluorescence microscopy of frozen tissue sections revealed intense red fluorescence within the sebaceous glands and a much weaker fluorescence within the epidermis and hair follicles. Little or no fluorescence was detected in the dermis, blood vessels, or cartilage of the ear. Light microscopy of skin taken at intervals after whole-body exposure of ALA-injected mice to photoactivating light revealed destruction of sebaceous cells, focal epidermal necrosis with a transient acute inflammation, and diffuse reactive changes in the keratinocytes. The dermis showed transient secondary edema and inflammation. The location and severity of the phototoxic damage correlated well with the location and intensity of the red fluorescence. The light-exposed skin appeared to recover completely except for a persistent reduction in the number of hair follicles. (Am J Pathol 1990, 136: 891–897)

Hematoporphyrin derivative (HpD) is a mixture of various porphyrin monomers, dimers, and polymers with a characteristic bright red fluorescence and a tendency to accumulate preferentially in malignant tissues. It is being used experimentally in cancer patients to define the exact boundaries of carcinoma *in situ* and to locate foci of malignant tissue that are too small to be detected readily by visual examination under white light.[1–3] Since HpD is also an efficient tissue photosensitizer, it is undergoing clinical evaluation as a photochemotherapeutic agent, a drug that can cause localized destruction of malignant tissue by photodynamic action.[4–6] The main problem encountered during the use of HpD for either purpose is a clinically significant photosensitization of the skin that persists for several weeks to several months after injection.

The administration of exogenous 5-aminolevulinic acid (ALA) to a variety of organisms ranging from bacteria[7] and plants[8] to mice[9,10] and men[11,12] may induce the biosynthesis of an excess of protoporphyrin IX (PpIX) in certain types of cells and tissues. Some tissues that accumulate ALA-induced PpIX emit a characteristic red fluorescence when exposed to appropriate wavelengths of photoactivating light. Those tissues that accumulate a sufficiently high concentration of PpIX may show phototoxic damage after adequate exposure to such light. However, in contrast to HpD, ALA-induced PpIX photosensitizes the skin for less than 24 hours after treatment. A clinical trial in progress has been designed to evaluate the capability of ALA-induced PpIX to accumulate specifically in various malignant skin lesions, and to destroy such lesions through photodynamic action.

We reported previously that mice given systemic ALA develop the characteristic fluorescence of PpIX in the skin.[9,10] The present study was designed to identify the specific types of cells in the skin that show PpIX fluorescence and/or develop phototoxic damage when exposed

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to photoactivating light. This information is required before the initiation of a more comprehensive clinical evaluation of the use of systemic ALA for photodynamic therapy.

Materials and Methods

Animals

Outbred CD1 (albino) female mice 6 to 10 weeks old were housed in plastic containers and allowed food (Purina Mouse Chow, Ralston-Purina, St. Louis, MO) and water *ad libitum*.

Induction of Protoporphyrin IX (PpIX) Biosynthesis

Solutions containing 10 mg of 5-aminolevulinic acid (Sigma A3785, Sigma Chemical Co., St. Louis, MO) per milliliter of isotonic saline (Travenol, Mississanga, ON) were prepared immediately prior to use. Each mouse received 250 mg of ALA per kilogram of body weight by intraperitoneal injection. All mice were kept in the dark for the first 24 hours after injection, unless killed earlier.

Tissue localization of PpIX by Fluorescence

Both ALA-injected and control mice were killed by cervical dislocation 3 hours after injection, a time at which PpIX fluorescence in the skin is near maximal levels.[10] Full-thickness ribbons of tissue were immediately sliced from the ears, placed in OCT medium, and frozen with isopentane. Frozen sections (5 μ) were placed in a light-proof container and then immediately transported to a darkened room for examination. Initial attempts to photograph PpIX fluorescence in the frozen sections failed because the PpIX photobleached too rapidly. The problem was solved by placing an image intensifier (Litton Model M911, Litton, Tempe, AZ) and a relay lens between the microscope and a camera loaded with Kodak Tri-X film (Eastman Kodak, Rochester, NY), and then using very low levels of illumination. The Leitz (Orthoplan/Ploemopak, Wild Leitz, Wetzlar, Germany) epi-illumination fluorescence microscope was equipped with filter block G containing excitation filter BP350-460 (transmits approximately 350 to 460 nm), dichroic mirror RKP510 (reflects below approximately 510 nm), and barrier filter LP520 (blocks wavelengths below approximately 520 nm). An additional barrier filter (Kodak Wratten #70) that was placed between the microscope and the image intensifier blocked most of the background fluorescence of the tissue. Although this filter also absorbed the major emission peak of PpIX in tissue (629 nm) and thus reduced the total intensity of the PpIX fluorescence, it transmitted almost all of the minor emission peak at 697 nm.

Some of the tissue sections that had been photographed for PpIX fluorescence as described above were processed and stained (hematoxylin and eosin [H&E]) for examination by light microscopy, to facilitate identification of both the fluorescent and the nonfluorescent structures.

Tissue Localization of PpIX by Phototoxic Damage

Ten mice given ALA by intraperitoneal injection as described above were exposed for 6 hours immediately after injection to white light from a 100-watt tungsten lamp. The power density (irradiance) measured 2 cm above the floor of the cage (the approximate level of the ears of the mice) by means of a Model 210 Coherent Radiation black body power meter (Coherent Radiation, Auburn, CA) was 21 mW/sq cm. The temperature in the cage never rose above 37°C. Noninjected controls were exposed to the same intensity of light for the same period of time. One ALA-injected mouse was killed for gross and histopathologic studies of the ears immediately after completion of the 6 hours of exposure to the light. The remainder were transferred to a dark room for the next 24 hours. Mice were killed at the following intervals: 12, 24, and 36 hours after injection, and 2, 3, 4, 6, 10, and 55 days after injection. Longitudinal full-thickness sections of the ears were marked with India ink on the inner surface for orientation, fixed in buffered formalin, processed for routine tissue sectioning, and then stained with hematoxylin, phloxine, and saffron. The ALA-injected mice showed a very substantial loss of dorsal body hair approximately 4 days after being exposed to the light. Samples of skin from selected areas on the dorsum were taken for study on days 6, 10, and 55 after treatment.

Results

PpIX Fluorescence

Full-thickness frozen sections of mouse ear were taken 3 hours after injection, close to the time of maximum PpIX fluorescence in the skin.[10] The sebaceous glands showed intense PpIX fluorescence, while in the epidermis the fluo-

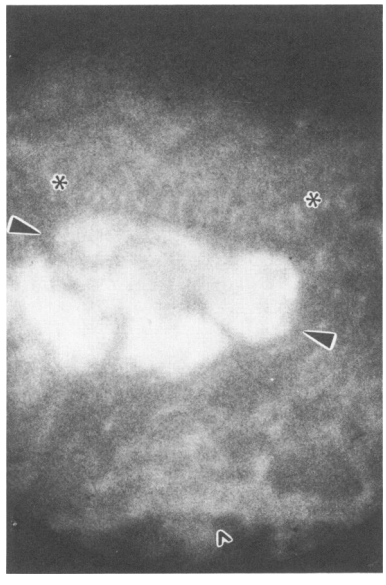

Figure 1. *Unstained frozen section of ear from ALA-injected mouse 3 hours after injection showing intense fluorescence in sebaceous glands (arrow), much weaker fluorescence in epidermis (*), and no fluorescence in dermis or cartilage (^) (×1200).*

rescence was much weaker (Figure 1). Little or no PpIX fluorescence could be detected in either the dermis or the cartilage of the ear. The control ears showed no PpIX fluorescence either visually or by photography. However, ears from both the ALA-injected and the control mice showed a red-orange fluorescence that was confined to the keratin layer. This probably was caused by chlorophyll degradation products that have been shown to be present in both mouse skin and mouse food.[13]

PpIX Phototoxicity

The ALA-injected mice showed changes in both appearance and behavior consistent with the gradual development of PpIX photosensitization. During the first 2 hours of exposure to the white light, the frequency with which the ALA-injected mice rubbed their noses and ears gradually increased, and these exposed and relatively hairless areas of the body developed a dusky erythema. When examined 24 hours after exposure, the ears of the ALA-injected mice showed gross edema as well as erythema. The edema resolved within 36 hours, but the erythema persisted for somewhat longer. By the fourth day from the start of the experiment, many of the ALA-injected mice had lost a significant amount of hair from the dorsum of the body. This hair loss was maximal approximately 10 days after exposure (Figure 2). Most of the hair eventually grew back, but the coat remained somewhat thinner than normal 55 days after exposure. At no time during the experiment was there any detectable change in either the appearance or the behavior of the controls: noninjected mice that had been exposed to the photoactivating light, and mice given ALA but not exposed to the photoactivating light.

Histologic Studies of the Ears

The ears of the ALA-injected mice were taken for histologic study at regular intervals after the exposure of the mice to the white light. Progressive changes were noted in the epidermis, the dermis, and the pilosebaceous units (Figure 3). The damage was of equal intensity on both sides of the cartilage.

Immediately after exposure (6 hours after injection), the only detectable change was a slight loss of organization of the basal layer of the epidermis. Thereafter there was progressive loss of polarity of the keratinocytes (maximal at 4 days), basal vacuolar degeneration (maximal at 12 hours), foci of epidermal necrosis, and acute inflammation (maximal at 24 hours), and increased mitotic activity (maximal at 3 days). Normal keratinocyte orientation was restored by day 10, but reactive nuclear changes persisted for at least 10 days.

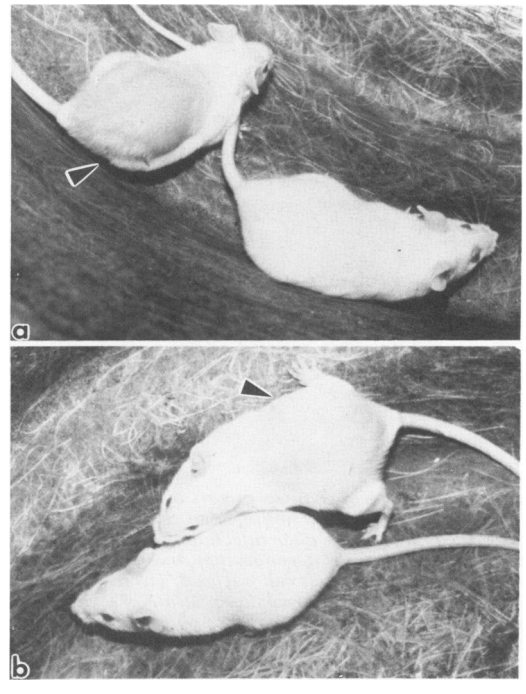

Figure 2. *ALA-injected mouse (arrow), and normal control showing* (a) *hair loss at 10 days followed by* (b) *almost complete recovery at 55 days after exposure to light.*

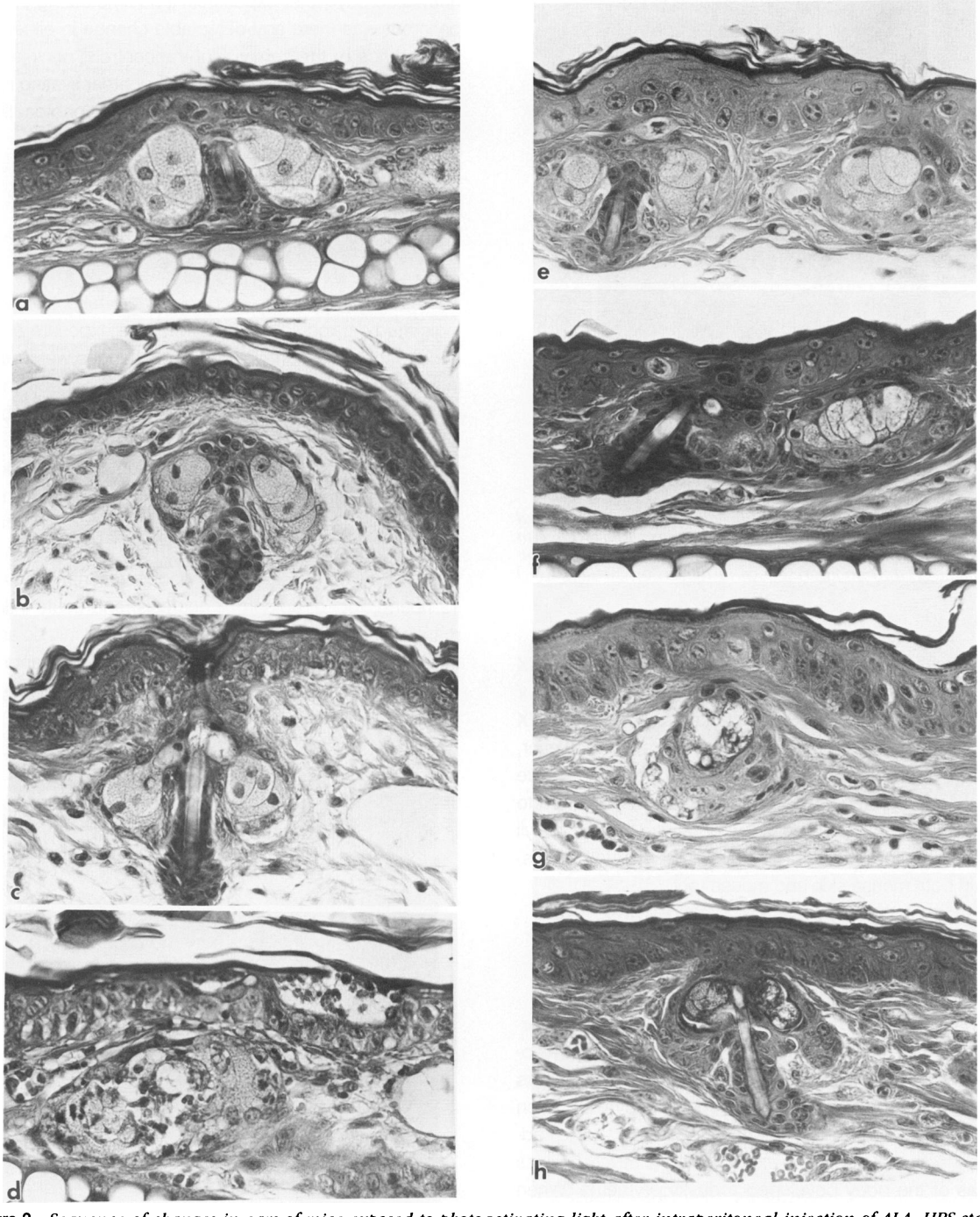

Figure 3. *Sequence of changes in ears of mice exposed to photoactivating light after intraperitoneal injection of ALA. HPS stain, ×500.* **a:** *Light-exposed but noninjected control showing normal arrangement of epidermis and pilosebaceous glands with no light-induced changes. Time after injection of ALA:* **b:** *Six hours. Subtle loss of organization of basal layer.* **c:** *Twelve hours. Irregular keratin, basal vacuolar degeneration and early loss of keratinocyte polarity, with dermal edema and acute inflammation. Early degeneration of sebaceous cell nuclei.* **d:** *Twenty-four hours. Foci of epidermal necrosis, with maximal dermal acute inflammation and edema. Marked neutrophilic infiltrate of sebaceous glands.* **e:** *Thirty-six hours. Increased mitotic activity of keratinocytes.* **f:** *Forty-eight hours. Almost complete resolution of dermal edema and acute inflammation. Pyknosis and necrosis of sebaceous gland cells.* **g:** *Four days. Maximal loss of keratinocyte polarity. Beginning of regenerative changes in sebaceous glands.* **h:** *Six days. Regenerative changes in sebaceous glands and epidermis.* **i:** *Ten days. Normal keratinocyte orientation with persistent reactive changes. Regeneration of sebaceous glands.* **j:** *Fifty-five days. Regeneration of epidermis and pilosebaceous glands to near-normal morphology.*

The dermis showed increasing edema and infiltration by neutrophils and mononuclear cells. These changes were most marked at 24 hours and almost completely resolved by 48 hours.

In the pilosebaceous units, the sebaceous cell nuclei began to show evidence of degeneration by 12 hours after exposure. This progressed to pyknosis and then to cellular necrosis at 48 hours. Some of the glands were markedly infiltrated by neutrophils at 24 hours. There was an accompanying proliferation of the peripheral cuboidal reserve cells, which was maximal at the time of sebaceous cell necrosis. Most of the sebaceous glands had regenerated by day 10, and by day 55 after injection, the ears of the ALA-injected mice appeared to be almost normal.

Histologic Studies in the Areas of Hair Loss

The dorsal skin showed destruction of sebaceous glands, with dilatation of the hair follicles and parakeratosis in the hair shafts at the time of maximal hair loss. Recovery was not complete by day 55; the pilosebaceous units were normal in appearance but showed an obvious decrease in number (Figure 4).

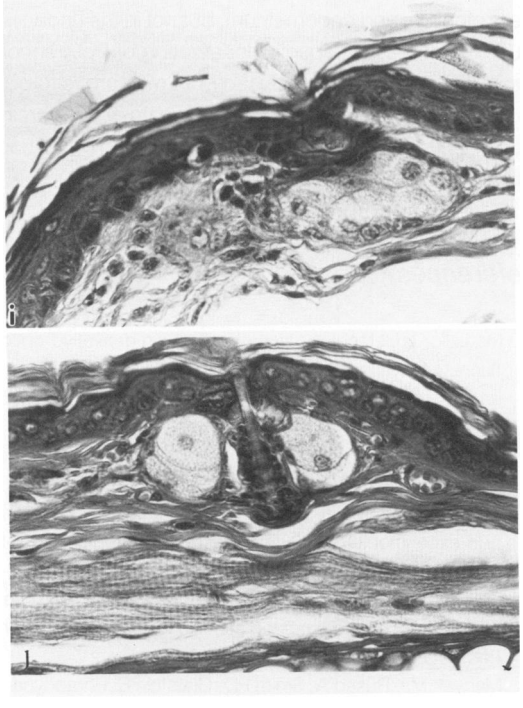

Figure 3. *Continued.*

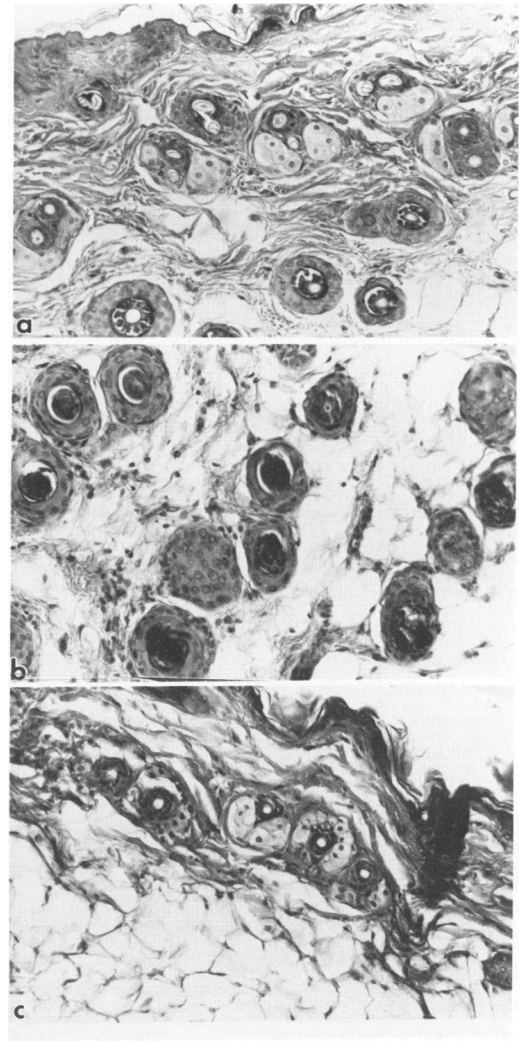

Figure 4. *Dorsal skin of mice exposed to photoactivating light after intraperitoneal injection of ALA. HPS stain, ×200.* **a:** *Light-exposed but noninjected control.* **b:** *Ten days after injection. Destruction of sebaceous glands, dilatation of hair follicles, and parakeratosis of hair shafts.* **c:** *Fifty-five days after injection. Return to normal morphology of pilosebaceous units, but with a decrease in number.*

Discussion

The emission spectrum of the fluorescent material that appears in the skin of mice after the systemic administration of ALA has its major emission peak near 632 nm, and is indistinguishable from the emission spectrum that is obtained after the injection of PpIX into living mice.[10] However, such ALA-induced fluorescence is distinctly different from the *in vivo* emission spectra of uroporphyrin I, uroporphyrin III, heptacarboxylporphyrin I, hexacarboxylporphyrin I, pentacarboxylporphyrin I, coproporphyrin I, or coproporphyrin III, since all of the above porphyrins have

their major *in vivo* emission peaks between 613 and 618 nm (Kennedy and Pottier, unpublished data). Consequently, if porphyrins other than PpIX appear in the skin after the systemic administration of ALA, they must be at concentrations too low to be detected by our *in vivo* spectrophotofluorometric technique.[10]

This study shows a strong correlation between the intensity and location of ALA-induced PpIX fluorescence and the intensity and location of subsequent phototoxic damage to the skin. The sebaceous glands and hair follicles developed very strong PpIX fluorescence a few hours after the intraperitoneal injection of ALA, while the basal layer of the epidermis showed a much weaker fluorescence and the dermis showed essentially none. Exposure of the photosensitized skin to white light caused severe phototoxic damage to the hair follicles but only moderate damage to the basal cell layer. The dermis, which showed little or no PpIX fluorescence, developed only nonspecific signs of damage (edema and transient inflammation). The cartilage showed neither fluorescence nor any evidence of phototoxic damage.

Tissues such as liver may contain a high concentration of PpIX but emit little PpIX fluorescence, because of internal quenching of the fluorescence. However, if there is any nonfluorescing PpIX present in the skin, it must not be a very efficient photosensitizer, since the intensity and location of the phototoxic damage in the skin was directly related to the intensity and location of the observed PpIX fluorescence.

Protoporphyrin IX is the direct precursor of heme in the biosynthetic pathway for heme. Since every nucleated cell must manufacture the heme-containing enzymes required for its own energy metabolism, every cell must have at least a minimal capacity to synthesize PpIX. The biosynthesis of heme normally is regulated so precisely that the intracellular concentrations of PpIX are extremely low. The usual rate-limiting step, the biosynthesis of 5-aminolevulinic acid (ALA), is under negative feedback control. If this control is bypassed by the addition of an excess of exogenous ALA, each cell will begin to produce heme at a rate limited primarily by its own characteristic enzyme activity profile. If the new rate-limiting step in the presence of an excess of ALA is the conversion of PpIX into heme, such a cell will tend to accumulate PpIX. In the absence of significant concentrations of quenching substances, that cell will begin to develop PpIX fluorescence, and also will become photosensitized.

We do not have direct evidence that the PpIX whose fluorescence was observed in the skin actually was synthesized *in situ*. However, we have shown previously that the intradermal injection of ALA produces localized PpIX fluorescence,[9] and a clinical trial now in progress has demonstrated that the topical application of ALA to various types of skin malignancies induces PpIX fluorescence that is limited to those lesions. Consequently, at least some types of cells in the skin must have the capacity to synthesize relatively large amounts of PpIX.

Photodynamic therapy (PDT), also known as photochemotherapy or photoradiation therapy, is a relatively new form of treatment for cancer.[4–6] It normally involves the intravenous administration of a photosensitizer (usually a porphyrin derivative or analogue) and the subsequent exposure of a tumor-containing tissue volume to appropriate wavelengths of light. Unfortunately, the only photosensitizer in common use (HpD) may persist in the skin for several weeks to several months after injection, at a concentration high enough to produce a clinically significant degree of photosensitization. This problem greatly limits the usefulness of photodynamic therapy with HpD. In contrast, the skin photosensitivity caused by ALA-induced PpIX vanishes within 24 hours of treatment, both in mice[10] and in man (clinical trial now in progress). Moreover, ALA is an effective inducer of PpIX either when applied topically or when given orally, routes of administration that rarely are effective with HpD.

We have shown that ALA-induced PpIX photosensitizes the epidermis and epidermal appendages (pilosebaceous units) of mice when the ALA is administered systemically, but does not appear to photosensitize either the vascular structures or the connective tissue elements. In preliminary studies of the bladder and uterus of mice, we found that ALA induced high concentrations of PpIX in the urothelium and endometrium, but not in the underlying muscle. Such tissue-specific localization of PpIX encourages the hope that ALA-induced PpIX may prove to be of as much value in the photodynamic treatment of carcinomas of the bladder and uterus as it appears to be for basal cell and squamous cell carcinomas of the skin.

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