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Selective photosensitizer distribution in vulvar condyloma acuminatum after topical application of 5-aminolevulinic acid

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OBJECTIVE: Our purpose was to determine the feasibility of selective photosensitization of vulvar condylomas by use of topical application of 5-aminolevulinic acid.

STUDY DESIGN: In vivo fluorescence was assessed and biopsy specimens of condylomas were taken for fluorescence microscopy in 24 patients at different times after application of 2.5% 5-aminolevulinic acid ointment or 20% 5-aminolevulinic acid cream.

RESULTS: Both in vivo fluorescence imaging and fluorescence microscopy showed selective fluorescence of condylomas of the labia minora and vestibule only within short time intervals, because fluorescence of poorly keratinized normal epithelium was induced by both 5-aminolevulinic acid formulations. In non-hair-bearing skin, lesional fluorescence remained highly selective. Fluorescence microscopy showed that 90 minutes after drug application peak selectivity in epithelial lesional fluorescence was significantly higher with 2.5% 5-aminolevulinic acid ointment (4.5 ± 0.9) than it was with 20% cream (2.1 ± 0.2).

CONCLUSIONS: Selective fluorescence of vulvar condyloma acuminatum can be induced by nonselective topical 5-aminolevulinic acid application. Studies evaluating selective photodynamic destruction of condylomas are justified. (Am J Obstet Gynecol 1996;174:951-7.)

Key words: 5-Aminolevulinic acid, condyloma acuminatum, fluorescence, photodynamic therapy, vulva

Photodynamic therapy after topical application of photosensitizers has shown high complete response rates in a variety of skin diseases, including superficial basal cell carcinoma, superficial squamous cell carcinoma, actinic keratosis, and psoriasis.[1, 2] In the case of these diseases it has been shown that topically applied 5-aminolevulinic acid readily passes the abnormal layer of keratin and is metabolized to photosensitizing concentrations of porphyrins. 5-Aminolevulinic acid is a precursor of protoporphyrin IX in the biosynthetic pathway for heme. Normally the synthesis of heme regulates the synthesis of 5-aminolevulinic acid through feedback control. The administration of exogenous 5-aminolevulinic acid bypasses this feedback and induces the accumulation of protoporphyrin IX, causing tissue photosensitivity because the step of converting protoporphyrin IX into heme is relatively slow.[3] Because the adjacent normal skin is less permeable, it is not necessary to restrict the topical application of 5-aminolevulinic acid to the lesion itself.

Condyloma acuminatum is caused by human papillomavirus (HPV) infection, an infection that usually involves the entire lower female genital tract. As noted by the Centers for Disease Control, the treatment of condyloma acuminatum has not been well studied, and no treatment is completely satisfactory.[4] The management of a subclinical HPV infection is even more controversial than that for overt condyloma. Because 5-aminolevulinic acid–induced photosensitization is highly restricted to epithelial tissues and tumors[1, 3, 5] and a higher conversion rate to protoporphyrin IX can be expected in proliferative tissues because of higher metabolic activity, we hypothesized that selectivity in photosensitization of condyloma could be achieved by use of appropriate time intervals and drug concentrations. Selectivity of photosensitivity would consequently imply that multicentric overt condyloma and subclinical lesions could be treated simultaneously by photodynamic therapy. This concept could easily be expanded to multicentric vulvar intraepithelial neoplasia, which is associated with HPV infection

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with certain virus types and similarly shows a disturbed epithelial architecture.[6, 7]

We chose a 20% 5-aminolevulinic acid emollient cream because these formulations have been shown to optimize percutaneous 5-aminolevulinic acid penetration in vitro (unpublished data) and induce high protoporphyrin IX conversion in vivo.[1, 2] With the use of the 2.5% 5-aminolevulinic acid ointment we wanted to test a lower 5-aminolevulinic acid concentration that might induce minimal fluorescence in normal tissues and achieve better adherence of the formulation to the skin. Most clinical studies of skin lesions with topical 5-aminolevulinic acid have been performed with cream formulations and occlusive dressings,[1, 2] which can hardly be used on the external genitalia.

To our knowledge, this is the first study investigating the feasibility of 5-aminolevulinic acid–induced photosensitization of typical vulvar condyloma and its selectivity to the lesion by comparing the fluorescence to the surrounding macroscopically normal-appearing skin. Different drug concentrations and time intervals were evaluated by in vivo fluorescence imaging and fluorescence microscopy. Results show that selective photosensitization of vulvar condyloma can be achieved within short intervals.

Material and methods

Twenty-four patients (27 ± 1.6 years old) referred to our institution for carbon dioxide laser treatment of typical vulvar condyloma acuminatum were included in this study. Written informed consent was obtained after approval by the Human Subject Review Committee of the University of California, Irvine. The diagnosis was made by colposcopic inspection of the vulva after application of 4% acetic acid. All acetowhite lesions were mapped on a sketch and photographed. Pregnant or nursing patients were excluded, as were patients with inflammatory changes or colposcopically unclear lesions requiring biopsy.

Two formulations were studied: a 20% 5-aminolevulinic acid hydrochloride emollient cream (oil-in-water emulsion) and a 2.5% 5-aminolevulinic acid hydrochloride ointment. The cream was prepared immediately before application by mixing 1 gm of crystallized 5-aminolevulinic acid (DUSA Pharmaceuticals, Denville, N.J.) with 4 gm of emollient cream (DUSA Pharmaceuticals) for at least 3 minutes with a spatula. The anhydrous ointment (DUSA Pharmaceuticals) consisted of 2.5% 5-aminolevulinic acid hydrochloride in a preformulated stable form.

Before drug application, the vulvar skin was washed with a watery 0.4% chlorhexidine solution to reduce the bacterial flora that could cause autofluorescence or enhance 5-aminolevulinic acid–induced bacterial fluorescence. Before drug application, fluorescence images were acquired to assess autofluorescence, and then 5 gm of the 5-aminolevulinic acid cream or ointment was spread over the entire vulva and perianal skin. The patients were given a nonadhering dressing (Release, Johnson & Johnson Products, Arlington, Tex.) to prevent absorption of the formulation by clothing. Patients who were scheduled for fluorescence evaluation after 24 hours were asked not to shower or wipe off the cream until the next morning.

In vivo fluorescence was evaluated 1, 3, or 6 hours after application of the 2.5% 5-aminolevulinic acid ointment and 1, 3, or 24 hours after application of the 20% 5-aminolevulinic acid cream. Patients were surveyed for discomfort at the drug application site, and the drug was washed off with wet gauze. Fluorescence was activated by light from a model B-100 AP ultraviolet lamp (UVP, San Gabriel, Calif.), which was positioned in front of the vulva at a distance of approximately 50 cm. Fluorescence was monitored with an intensified charge-coupled device camera (model C2400-86 intensifier head, C2400-77 CCD, Hamamatsu Photonics, Hamamatsu City, Japan) displaying the real-time images on the monitor of a Macintosh (Apple Computer Corporation, Cupertino, Calif.) Quadra 840AV computer. Images were acquired by 450 and 650 nm bandpass filters (25 nm bandpass, Corion, Holliston, Mass.) and a built-in frame grabber. To assess excitation light distribution, each defined lesion was first imaged with a 450 nm bandpass filter. After the filter was quickly exchanged without moving the camera position, the fluorescence image was acquired with the 650 nm filter. After the ultraviolet lamp was turned off, images were acquired for each filter to determine dark noise signal. After in vivo fluorescence imaging, which took <10 minutes (including dark noise), biopsy specimens were taken after local anesthesia by 1% lidocaine hydrochloride injection. At least one macroscopically identified typical condyloma of the labia minora or vestibule, together with normal-appearing adjacent skin, was excised with a scalpel. For the sake of simplicity, the stratified squamous epithelium of the hymen, vestibule, and labia minora is referred to as vulvar skin without hair in contrast to hair-bearing vulvar skin. Specimens were immediately placed in molds containing embedding medium for frozen specimens (Tissue-Tek II ornithine carbamyltransferase media, Miles, Elkhart, Ind.), frozen on dry ice, and stored at −70° C for 6 days maximum. The blocks were sectioned in low diffuse light (Cryostat microtome, AO Reichert, Buffalo, N.Y.) to obtain 6 μm slices for fluorescence analysis. Biopsy specimens of typical vulvar condyloma of four patients without drug application were used as controls.

Low-light-level fluorescence microscopy was performed with a slow-scan, thermoelectrically cooled, charge-coupled device camera system (Princeton Instruments, Trenton, N.J.) coupled to a Zeiss Axiovert 10 inverted fluo-

rescence microscope (Carl Zeiss, Oberkochen, Germany). A 10× objective (Zeiss Plan-neofluan numeric aperture 0.3) was used to visualize bright-field and fluorescence images of frozen sections. A 100 W mercury arc lamp filtered through a 405 nm bandpass filter (20 nm band width, Omega Engineering, Stamford, Conn.) provided excitation light. A dichroic filter (Zeiss, FT 420, Carl Zeiss) was used to separate excitation from emission signals and a 635 nm broad bandpass filter (55 nm band width, Omega Engineering) was used to isolate the fluorescence emission. Protoporphyrin IX has a strong absorption peak around 400 nm and a fluorescence peak at 635 nm. Instrument control, image acquisition, and processing were performed with a Macintosh IIfx computer and IPLab software (Signal Analytics, Vienna, Va.). Sample photo degradation was minimized by limiting arc lamp exposure to 2 seconds by electronically synchronizing camera and lamp shutters (Uniblitz, model T132, Vincent Associates, Rochester, N.Y.). To correct for light distribution, background images were acquired from blank slides under conditions identical to those used for sample measurements. Dark noise levels were determined by acquiring images without source illumination. All fluorescence images were corrected for both nonuniform illumination and dark noise contribution with the following algorithm:

$$\text{Corrected fluorescence image} = \frac{\text{Fluorescence image} - \text{Dark noise}}{\text{Background image} - \text{Dark noise}} \times \text{Mean (Background image} - \text{Dark noise)}$$

where mean (Background − Dark noise) is the mean gray-scale value for the dark-noise-corrected background image. Specimens were divided into anatomic layers for comparative analysis. Mean fluorescence intensity of the epithelium without stratum corneum and of the papillary dermis was measured in arbitrary units. Measurements were made on four different sections through the condyloma and on four different sections of the adjacent skin. The mean values for each patient were then calculated. After fluorescence analysis the sections were stained with hematoxylin-eosin and reviewed. Maximal epithelial thickness of the condyloma and adjacent normal skin was measured with the built-in scale of the ocular.

The in vivo fluorescence images were likewise processed with IPLab software and corrected for nonuniform illumination and contributing dark noise by the following algorithm:

$$\text{Corrected fluorescence image} = \frac{\text{Image (650 nm)} - \text{Dark noise (650 nm)}}{\text{Image (450 nm)} - \text{Dark noise (450 nm)}} \times \text{Mean (Image [450 nm]} - \text{Dark noise [450 nm])}$$

Light distribution without fluorescence signal was determined with the 450 nm bandpass filter, and the dark noise was determined for each filter separately. On the corrected fluorescence image the mean fluorescence of the condyloma and of the surrounding macroscopically normal-appearing skin were measured.

For statistical analysis differences in fluorescence intensities and ratios between fluorescence intensity of condyloma and adjacent normal skin were examined at different time points for each drug formulation with the Kruskal-Wallis test. Statistical significance was taken as $p < 0.05$. If a significant overall difference was present, multiple comparisons were performed with the Mann-Whitney test with Bonferroni correction. Data are presented as mean ± SE.

Results

Of the 12 women who had the 20% 5-aminolevulinic acid cream applied, 9 reported a mild burning or stinging at the drug application site after specific questioning for this side effect, whereas no discomfort was reported with the 2.5% 5-aminolevulinic acid ointment. No patient felt it necessary to wash off the cream and all had a temporal decrease in intensity of discomfort. No subject enrolled in the 24-hour group showered or washed the cream off before the next morning.

Autofluorescence was detected by in vivo imaging in only two patients at low fluorescence intensity, and it was located primarily in the folds surrounding the glans of the clitoris. At longer time intervals drug-induced fluorescence of the non-hair-bearing vulvar and perianal skin was visible, whereas no fluorescence of the hair-bearing skin of the labia majora, mons pubis, or perineum could be detected. Weak fluorescence of condyloma could be detected with high selectivity after a 1-hour interval because fluorescence of surrounding skin, including the vestibule, was weak at that time point (see Fig. 1, *A*). Three hours or more after drug application the contrast in fluorescence between condyloma and surrounding skin decreased for non-hair-bearing skin (Fig. 1, *B*), whereas condyloma of vulvar skin with hair remained clearly depicted. The decrease in the ratio (condyloma vs surrounding skin) from 1 to 6 hours for the 2.5% 5-aminolevulinic acid ointment and from 1 to 3 hours for the 20% 5-aminolevulinic acid cream in Fig. 2 was significant ($p = 0.021$ and $p = 0.021$, respectively). No significant difference between the two drug formulations at 1 or 3 hours was evident after Bonferroni correction ($p = 0.04$ and $p = 0.99$, respectively). All typical condyloma and lesions mapped as acetowhite lesions showed fluorescence. Moreover, low-intensity fluorescence in the skin folds surrounding the glans of the clitoris could be detected in some patients even after a 1-hour interval.

Fluorescence microscopy revealed that after short intervals peak fluorescence was localized in the basal epithelial layer (Fig. 3, *A*). Three and six hours after drug

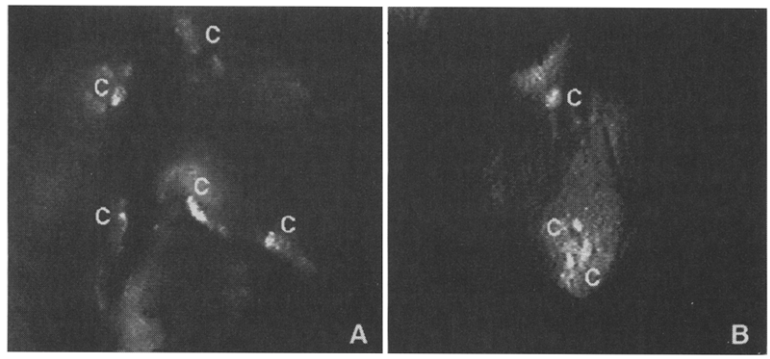

Fig. 1. In vivo fluorescence imaging with intensified charge-coupled device camera and 650 nm band-pass filter. **A,** At 1 hour after 2.5% 5-aminolevulinic acid ointment application, condylomas on frenulum of clitoris, left labium minus, and hairless side of right labium majus are fluorescing *(C)*. On prepuce of clitoris there is fluorescence of lower intensity, which could not be clearly attributed to typical condyloma or acetowhite epithelium. **B,** At 3 hours after drug application, whole vestibule and labia minora are fluorescing. Still, typical condylomas of fourchette and right labium minus are depicted *(C)*.

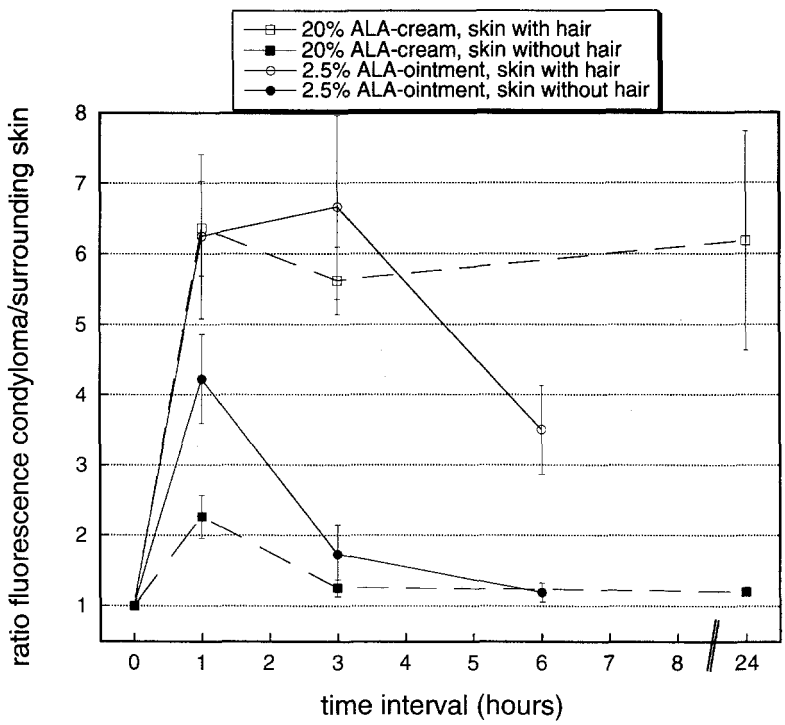

Fig. 2. Ratio of condyloma fluorescence to fluorescence of surrounding normal skin in fluorescence imaging. *Points* represent mean ± SE, four patients per *point*. *ALA*, 5-Aminolevulinic acid.

application the whole epithelium was fluorescing uniformly, whereas after 24 hours fluorescence had shifted to the granular and horny layer (Fig. 3, *B*). The ratios between the mean fluorescence of the epithelium and the underlying papillary dermis for the 2.5% 5-aminolevulinic acid ointment were 4.0 ± 0.4, 4.0 ± 0.5, and 4.9 ± 0.5 at 1.5, 3, and 6 hours, respectively, and for the 20% 5-aminolevulinic acid cream the ratios were 4.6 ± 0.3, 4.5 ± 0.6, and 3.5 ± 0.4 for 1.5, 3, and 24 hours, respectively. After 90 minutes only the 2.5% 5-aminolevulinic acid ointment showed a further significant temporal increase in overall fluorescence of the condyloma epithelium (Fig. 4). The 20% 5-aminolevulinic acid cream–induced fluorescence was significantly higher than that induced by 2.5% 5-aminolevulinic acid ointment only at the 3-hour time point ($p = 0.014$). For the 2.5% 5-aminolevulinic acid ointment the decrease in the ratio between the epithelial fluorescence of the condyloma versus the adjacent skin was significant when the periods 90 minutes to 6 hours and 3 hours to 6 hours were

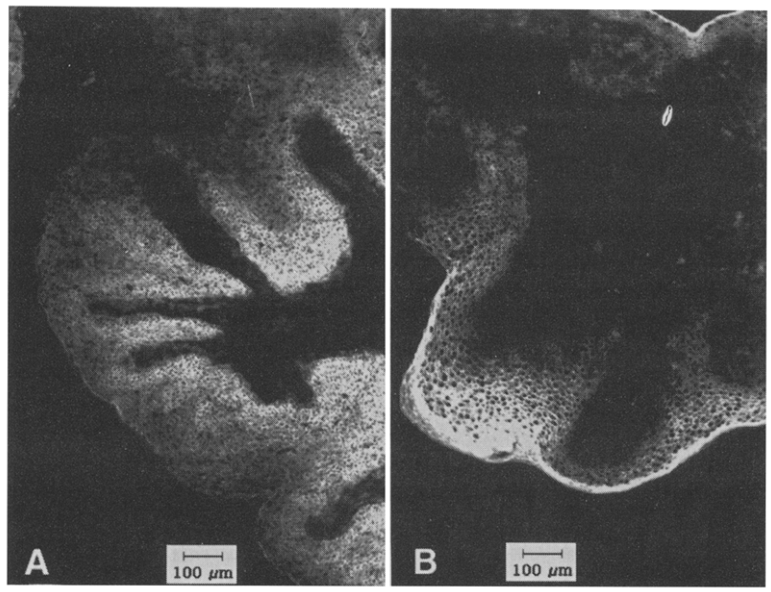

Fig. 3. Fluorescence photomicrograph showing fluorescence of condyloma acuminatum after 20% 5-aminolevulinic acid cream application. **A,** At 90 minutes after drug application, fluorescence is primarily in basal epithelial layer. **B,** At 24 hours after drug application, fluorescence has shifted to superficial epidermal layers.

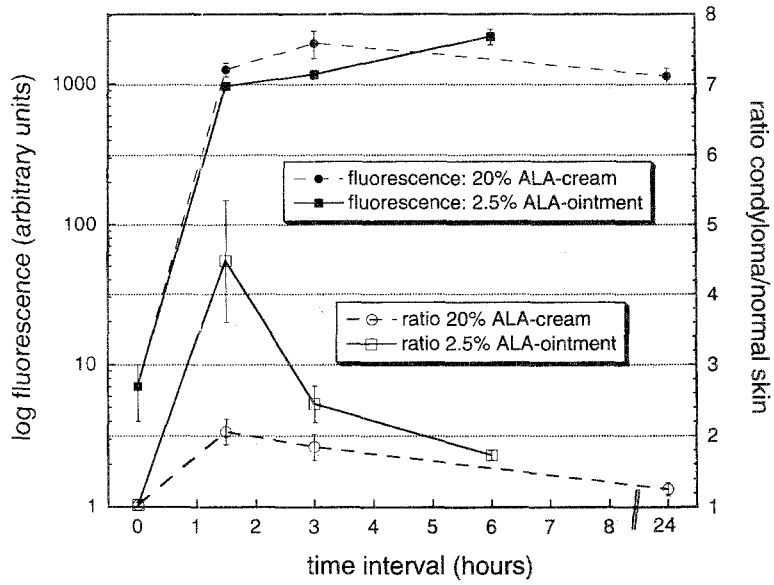

Fig. 4. Fluorescence microscopy results: Mean fluorescence of epithelium of condyloma on *left y* axis and ratio of epithelial condyloma fluorescence to adjacent skin on *right y* axis. Four patients per *point; points* represent mean ± SE. *ALA,* 5-Aminolevulinic acid.

compared but not 90 minutes to 3 hours. For the 20% 5-aminolevulinic acid cream only the ratios at 90 minutes were significantly different from those at 24 hours. In addition, 90-minute ratios with the 2.5% 5-aminolevulinic acid ointment were significantly higher ($p = 0.014$) than those with the 20% 5-aminolevulinic acid cream. The maximal epithelial thickness of the condyloma was 0.54 ± 0.04 and 0.28 ± 0.01 mm for the adjacent normal skin.

Comment

Photodynamic therapy could provide a simple, safe alternative to more radical surgical procedures for treatment of condyloma acuminatum. Furthermore, if HPV-

infected microscopic hyperproliferative intraepithelial cell nests accumulate the photosensitizer selectively, nonvisible hyperproliferations could be destroyed photochemically, which might influence the recurrence rate of this disease.

At all time intervals 5-aminolevulinic acid–induced fluorescence was more than twofold higher in the epidermis than in the papillary dermis. This feature may limit photodynamic destruction to epithelial tissue and may minimize scarring. This selectivity of 5-aminolevulinic acid–induced fluorescence to epithelial tissues is not limited to topical drug application because the same selectivity could be observed in animal and human studies that used systemic 5-aminolevulinic acid administration.[1, 8-10]

Our observation that epidermal fluorescence showed a temporal shift from the basal layer at short time intervals to the superficial layer at 24 hours suggests that the metabolically active dividing cells convert 5-aminolevulinic acid faster to protoporphyrin IX than to the more differentiated cells. After 24 hours the photosensitizer was cleared from the basal layer. Hence photodynamic therapy at this time interval may lead to incomplete epidermal destruction. Because this critical shift in photosensitizer localization can only be detected by fluorescence microscopy, the in vivo fluorescence imaging results at long intervals are of questionable significance for photodynamic therapy.

Ninety minutes after drug application the 2.5% 5-aminolevulinic acid formulation resulted in higher epithelial selectivity of condyloma fluorescence than the 20% cream did. Because the absolute fluorescence did not differ significantly between the two drug formulations, the higher selectivity is due to a lower fluorescence of the surrounding normal skin. Even at 6 hours, where absolute fluorescence of the condyloma was maximal, fluorescence of normal skin reduced the ratio of condyloma/epithelium to adjacent skin signal.

In vivo fluorescence imaging showed that the macroscopically normal-appearing epithelium of the labia minora, vestibule, and hymen converted 5-aminolevulinic acid readily to fluorescing porphyrins. Toward the hymen the epidermis on the medial aspects of the labia minora becomes thinner. It is widely believed that the epidermis changes into an epithelium of mucous membrane because the epithelial covering is much less cornified than true skin and the vestibule and hymen are covered with nonkeratinized squamous epithelium.[11] As noted by others,[1] a thick stratum corneum resists the penetration of 5-aminolevulinic acid, whereas 5-aminolevulinic acid seems to penetrate nonkeratinized stratified squamous epithelium and induces fluorescence. Therefore the higher mean fluorescence of condyloma compared with adjacent epithelium at short time points might not be solely explained by impaired 5-aminolevulinic acid penetration into normal skin or enhanced penetration into the condyloma as a result of disrupted epithelial architecture. It is more likely that selectivity in fluorescence results from higher metabolic activity of the condyloma/epithelium, leading to faster conversion of 5-aminolevulinic acid to protoporphyrin IX.

A 6-hour time interval was chosen for the 2.5% 5-aminolevulinic acid formulation to see if selectivity could be increased by inducing protoporphyrin IX accumulation in the condyloma over a longer time period, whereas normal skin fluorescence is minimized by low drug concentration. Obviously, such a low drug concentration is also sufficient to induce significant fluorescence in poorly keratinized normal skin. For the highly concentrated 5-aminolevulinic acid cream, we speculated that a 24-hour interval could enhance selectivity by inducing maximal fluorescence in the condyloma, whereas the photosensitizer in the normal epithelium might already be decreasing. Results show that clearance of protoporphyrin IX in condyloma and adjacent skin does not differ significantly and starts in the basal epithelial layer.

In this study selectivity of condyloma fluorescence was only assessed by comparing the fluorescence of the condyloma to that of the immediately surrounding skin and not by histologic evaluation of all fluorescent skin areas. Hence the specificity of fluorescence imaging for the diagnosis of condyloma could not be established. Also, in patients with typical condyloma acuminatum a significant portion of acetowhite lesions may not be induced by HPV. However, the sensitivity of fluorescence detection of condyloma was high because all mapped acetowhite areas and macroscopically typical condylomas showed fluorescence. The clinical significance of the low-intensity fluorescence detected in some skin folds after a 1-hour interval remains unanswered because no biopsy specimens were taken from these areas for histologic classification of the tissue or localization of the photosensitizer. Fluorescence imaging cannot distinguish between 5-aminolevulinic acid–induced porphyrins of bacterial origin on the epithelial surface and epithelial porphyrin production in the basal layer. Furthermore, the correlation between fluorescence signal and actual photodynamic destruction is unknown.

During ultraviolet illumination for in vivo fluorescence evaluation and illumination for the biopsy procedure, photodegradation of protoporphyrin IX may have occurred, resulting in lower fluorescence intensity values measured by fluorescence microscopy than actually occurred. Measurements of protoporphyrin IX photodegradation during photodynamic therapy of basal cell carcinomas in humans after topically applied 5-aminolevulinic acid have given a bleaching fluence in the range of 30 to 50 J/cm^2.[12] Because in all our patients light exposure times were within a few minutes and the ultraviolet lamp was positioned at a distance of 50 cm, resulting in a relatively low fluence rate on the skin,

photobleaching of protoporphyrin IX is not thought to be significant.

We conclude that vulvar condyloma acuminatum can selectively accumulate protoporphyrin IX photosensitizer by use of topical 5-aminolevulinic acid formulations at short time intervals. Consequently, these results suggest that the feasibility of selective photodynamic destruction of vulvar condyloma is reasonable and should be examined.

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