

## DNA INTERSTRAND CROSSLINKS VISUALIZED BY ELECTRON MICROSCOPY IN PUVA-TREATED PSORIASIS

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**Abstract.** An electron microscopic method has been used to visualize crosslinks after total denaturation of DNA isolated from epidermis and dermis in patients with psoriasis treated with 8-methoxypsoralen (8-MOP) and irradiation with ultraviolet light at 360 nm (PUVA treatment). This technique enabled accurate measurement of the number and density of DNA interstrand crosslinks. 30 biopsies were studied and a total of 9503 DNA molecules were scored in the electron microscope. 6 patients were treated topically with 8-MOP and 10 were on systemic treatment and biopsies were obtained immediately after irradiation. 1% of the total DNA molecules examined contained 3 or more crosslinks. The overall frequency of crosslinks was almost identical in the epidermis (1.1%) and in the dermis (0.9%) and, furthermore, virtually the same in patients on topical and systemic PUVA treatment. The total number of crosslinks was of the same magnitude as those previously found by us in normal human skin. Thus, though the formation of interstrand DNA crosslinks can be considered as an indicator of damage of the genetic material, we were unable to demonstrate this as being a consequence of PUVA treatment using 8-MOP.

**Key words:** DNA interstrand crosslinks; EM visualization; 8-methoxypsoralen; Psoriasis; PUVA

8-methoxypsoralen (8-MOP) and related furocoumarines are known to form interstrand crosslinks between pyrimidine bases in the opposite strands of the DNA molecule upon irradiation with ultraviolet light at 360 nm (5, 6).

8-MOP, followed by UVA irradiation at 360 nm (PUVA therapy), is now widely used in the treatment of recalcitrant psoriasis (17, 19, 20), vitiligo (13, 15) and mycosis fungoides (10).

A molecular explanation for this photochemotherapeutic effect is likely to involve the formation of interstrand crosslinks in skin DNA. However this has never been demonstrated by the direct examination and visualization of skin DNA from patients undergoing treatment.

In this study we have isolated DNA from the

epidermal and dermal layers of the skin from patients under photochemotherapy for psoriasis, and evaluated its content of interstrand crosslinks. We have used an electron microscopic method which allowed us to visualize crosslinks after total denaturation of the DNA. This method has previously been used by us to detect psoralen-induced interstrand crosslinks in vitro (1).

Interstrand crosslinks have been demonstrated mainly by different biochemical techniques, including hydroxyl apatite column chromatography (5, 6) alkaline sucrose gradient centrifugation (8), CsCl gradient ultracentrifugation (9, 11), and renaturation kinetic experiments (7, 12, 14).

In contrast to these other methods the electron microscopic technique has the advantage of revealing both the amount and the location of interstrand crosslinks in DNA. Moreover, this technique is sensitive enough to measure the small quantities of DNA in skin biopsies.

## MATERIALS AND METHODS

### *Patients and radiation*

14 patients (5 females and 9 males) with recalcitrant psoriasis were studied (Tables I, II). Ten patients were treated with 8-MOP (Meladinine®, Memphis Chem. Co., Cairo, Egypt) orally 2 hours prior to UVA exposure. The dose was approximately 0.5 mg 8-MOP per kg body-weight. The UVA source consisted of 48 tube lamps (Philips TL/09) with a spectral range from 320 to 400 nm. The light energy reaching the body surface at a distance of 25–30 cm was about 2.5 mW/cm<sup>2</sup>. PUVA therapy was given 2–3 times weekly according to a fixed schedule, starting with 1.4 Joule/cm<sup>2</sup> per exposure and increasing by 0.5 Joule/cm<sup>2</sup> at the subsequent exposures up to a maximum of 4.0 Joule/cm<sup>2</sup>. Six patients were treated with topical application of 8-MOP (Meladinine emulsion, which contained 0.15% (w/v) 8-MOP in ethanol). Two of these (nos. 1, 9) were patients with incomplete clearance of their psoriasis who had been treated systemically with 8-MOP

Table 1. The distribution of DNA interstrand crosslinks in psoriasis patients on oral PUVA treatment  
–, not examined.

| Pat. no. | Age/sex | Joule/cm <sup>2</sup> | No. of crosslinked molecules/<br>No. of non-crosslinked molecules |             |           |             | No. of crosslinked molecules/<br>No. of non-crosslinked molecules (%) |             |           |             |
|----------|---------|-----------------------|-------------------------------------------------------------------|-------------|-----------|-------------|-----------------------------------------------------------------------|-------------|-----------|-------------|
|          |         |                       | Epidermis                                                         |             | Dermis    |             | Epidermis                                                             |             | Dermis    |             |
|          |         |                       | In-volved                                                         | Un-involved | In-volved | Un-involved | In-volved                                                             | Un-involved | In-volved | Un-involved |
| 1        | 42/F    | 227                   | 4/203                                                             | –/–         | –/–       | 0/226       | 2.0                                                                   | –           | –         | 0           |
| 2        | 39/M    | 202                   | 0/199                                                             | 0/197       | 0/172     | 0/207       | 0                                                                     | 0           | 0         | 0           |
| 3        | 80/F    | 81                    | 3/179                                                             | 0/226       | 4/243     | 0/167       | 1.7                                                                   | –           | 1.6       | 0           |
| 4        | 55/M    | 6                     | –/–                                                               | –/–         | 0/198     | 1/199       | –                                                                     | –           | 0         | 0.5         |
| 4        | 55/M    | 58                    | –/–                                                               | –/–         | –/–       | 1/99        | –                                                                     | –           | –         | 1.0         |
| 5        | 71/M    | 135                   | 3/100                                                             | –/–         | 3/100     | –/–         | 3.0                                                                   | –           | 3.0       | –           |
| 6        | 30/M    | 45                    | 1/168                                                             | 0/136       | 0/100     | –/–         | 0.6                                                                   | 0           | 0         | –           |
| 7        | 33/M    | 219                   | 0/250                                                             | 0/234       | 0/387     | 0/223       | 0                                                                     | 0           | 0         | 0           |
| 8        | 82/F    | 3                     | –/–                                                               | 0/102       | –/–       | –/–         | –                                                                     | 0           | –         | –           |
| 9        | 49/M    | 42                    | 2/213                                                             | –/–         | 2/343     | 0/156       | 0.9                                                                   | –           | 0.6       | 0           |
| 10       | 51/F    | 23                    | 0/140                                                             | –/–         | 5/139     | 1/95        | 0                                                                     | –           | 3.6       | 1.0         |

followed by topical treatment of remaining plaques. The skin was irradiated one hour later, using the previously mentioned UVA source.

#### Sampling

Punch biopsies, 2 mm in diameter, were taken from involved psoriasis plaques and uninvolved skin on the lateral side of the thighs of each patient. Ethyl chloride was used for local freezing anesthesia and the skin specimens were immediately frozen and stored in liquid nitrogen. Biopsies were always taken immediately after irradiation. Three patients (nos. 1, 4, 9) had biopsies obtained on more than one occasion after PUVA-therapy (Tables I, II).

#### Methods

Separation of dermis and epidermis, DNA isolation, electron microscopy, and molecular measurements were done as described elsewhere in a study on DNA from normal human skin (2). The general principles are shown in Fig. 1.

## RESULTS

As a control of our ability to detect 8-MOP-induced crosslinks in human skin DNA, we extracted DNA from clinically normal human fresh autopsy skin and added 8-MOP to the extracted DNA and irradiated at 360 nm. We were able to introduce crosslinks and graduate their density in the DNA in vitro.

30 skin biopsies from psoriasis plaque skin and uninvolved skin in 14 patients were analysed. The biopsies were separated into epidermis and dermis.

The DNA molecules were isolated from the skin samples of 10 patients on systemic treatment (Table I) and 6 patients on topical psoralen photochemotherapy (Table II). The molecules were

Table II. The distribution of DNA interstrand crosslinks in psoriasis patients on topical PUVA treatment  
–, not examined

| Pat. no. | Age/sex | Joule/cm <sup>2</sup> | No. of crosslinked molecules/<br>No. of non-crosslinked molecules |             |           |             | No. of crosslinked molecules/<br>No. of non-crosslinked molecules (%) |             |           |             |
|----------|---------|-----------------------|-------------------------------------------------------------------|-------------|-----------|-------------|-----------------------------------------------------------------------|-------------|-----------|-------------|
|          |         |                       | Epidermis                                                         |             | Dermis    |             | Epidermis                                                             |             | Dermis    |             |
|          |         |                       | In-volved                                                         | Un-involved | In-volved | Un-involved | In-volved                                                             | Un-involved | In-volved | Un-involved |
| 11       | 45/M    | 168                   | –/–                                                               | 0/107       | 0/125     | 0/106       | –                                                                     | 0           | 0         | 0           |
| 12       | 51/M    | 36                    | 1/121                                                             | 0/191       | 0/217     | 1/202       | 0.8                                                                   | 0           | 0         | 0.5         |
| 13       | 61/M    | 13                    | 8/211                                                             | 21/612      | 17/370    | 11/241      | 3.8                                                                   | 3.4         | 4.6       | 4.6         |
| 14       | 25/F    | 36                    | –/–                                                               | –/–         | 0/105     | –/–         | –                                                                     | –           | 0         | –           |
| 9        | 49/M    | 78                    | –/–                                                               | –/–         | 2/82      | –/–         | –                                                                     | –           | 2.4       | –           |
| 1        | 42/F    | 263                   | 2/276                                                             | 0/248       | 0/248     | 0/222       | 0.7                                                                   | 0           | 0         | 0           |
| 1        | 42/F    | 299                   | 2/102                                                             | –/–         | 2/216     | –/–         | 2.0                                                                   | –           | 0.9       | –           |

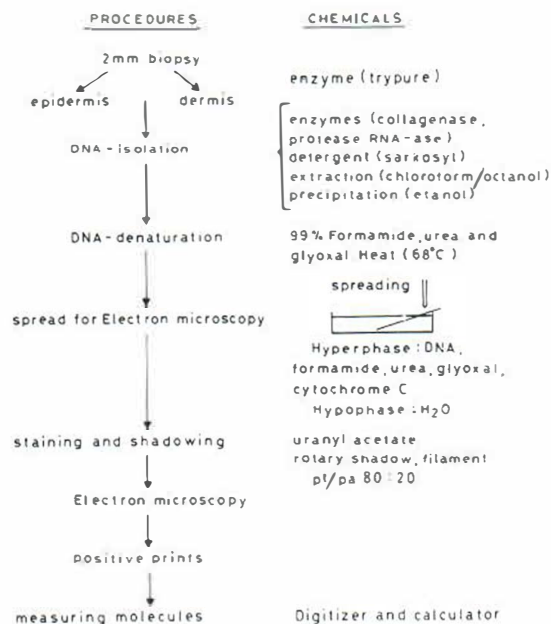


Fig. 1. The frozen biopsies were placed in a buffer containing 1 mg trypsin. After incubation the epidermis was separated from the dermis and throughout the rest of the procedure epidermis and dermis were run in tandem. After isolation of DNA, total denaturation was obtained by heating the DNA to 68°C for one and a half min in 99% formamide, 4 M urea and 30% glyoxal. That total denaturation was obtained was confirmed biochemically by CsCl gradient ultracentrifugation. After cooling to room temperature and addition of a buffer containing 2.3 mg/ml cytochrome C the molecules were spread on a hypophase containing double-distilled water. After staining and shadowing, the molecules were examined in a Philips 201 electron microscope. The molecules were measured from the positive prints at a magnification of 34 900 which was intercurrently checked by use of calibration grids. A calculator and a 9864A digitizer were used for measurements of single- and double-stranded lengths of the DNA molecules. The smallest detectable single-stranded region (bubble) was about 150 basepairs in length. For further details see (2).

spread for electron microscopy under denaturing conditions.

Regarding the whole material, a total of 9503 DNA molecules were scored in the electron microscope. One % of these contained three or more crosslinks. The frequencies of crosslinked molecules were almost identical in the epidermis (1.1%) and in the dermis (0.9%).

All crosslinked molecules were accurately measured for their content of single-stranded and double-stranded regions and the density of cross-

linked regions was indicated as the intramolecular percentage of crosslinking (double-stranded length as a percentage of total molecular length). This percentage appeared to vary considerably, both within each patient and among the patients (Tables III and IV).

Fig. 2 depicts crosslinked molecules.

Few crosslinks were found in epidermis and dermis from uninvolved skin in patients on systemic treatment. In the psoriasis plaques more cross-linked molecules were detected, both in the epidermis and in the dermis (Table V), but the numbers are too small for reliable statistical analysis.

The data for patients on topical treatment are summarized in Table VI. The frequency of cross-linked molecules was of the same order of magnitude in psoriasis plaques as in uninvolved skin areas. As seen in Tables II and IV, one patient (no. 13) had a much higher frequency of crosslinks in skin DNA than any of the other patients. The involved and uninvolved skin of the remaining patients on topical treatment contained virtually the same number of crosslinked DNA molecules as those on systemic treatment.

## DISCUSSION

In the present paper we have evaluated the quantity of interstrand DNA crosslinks by electron microscopy of DNA from uninvolved and involved skin in patients with psoriasis undergoing systemic or topical PUVA treatment.

The frequency of crosslinking was virtually of the same magnitude in skin DNA from patients receiving topical treatment as from those on systemic treatment. We have no explanation for the high number of DNA crosslinks in patient no. 13. When the present data were compared with those previously reported by us in normal untreated human skin (2), we were surprised to find that the frequency of molecules containing crosslinks was not higher in the psoriatic patients undergoing photochemotherapy than those obtained in clinically completely normal skin from healthy volunteers. Our failure to detect more crosslinks in the PUVA-treated patients is not due to faulty technique, as we have previously found good correlation between the number of interstrand DNA crosslinks and irradiation time, irradiation intensity and the concentration of 8-MOP in an *in vitro* system (1). In this

Table III. The intramolecular percentage of crosslinking of the crosslinked molecules from psoriasis patients on systemic PUVA treatment

—, not examined

| Pat. no. | Density of crosslinking (%) |            |                          |            |
|----------|-----------------------------|------------|--------------------------|------------|
|          | Epidermis                   |            | Dermis                   |            |
|          | Involved                    | Uninvolved | Involved                 | Uninvolved |
| 1        | 24.8, 29.4, 38.4, 63.8      | —          | —                        | 0          |
| 2        | 0                           | 0          | 0                        | 0          |
| 3        | 33.8, 9.7, 15.9             | 0          | 11.5, 24.1, 46.4, 46.2   | 0          |
| 4        | —                           | —          | 0                        | 46.8       |
| 4        | —                           | —          | —                        | 1          |
| 5        | 88.1, 34.1, 87.3            | —          | 16.5, 26.0, 28.6         | —          |
| 6        | 47.4                        | 0          | 0                        | —          |
| 7        | 0                           | 0          | 0                        | 0          |
| 8        | —                           | 0          | —                        | —          |
| 9        | 73.1, 55.8                  | —          | 73.0, 52.8               | 0          |
| 10       | 0                           | —          | 0.1, 3.1, 14.9, 54.7, 83 | 34.7       |

Table IV. The intramolecular percentage of crosslinking of the crosslinked molecules from psoriasis patients on topical PUVA treatment

—, not examined

| Pat. no. | Density of crosslinking (%)                    |                                                                                                                         |                                                                                                      |                                                                  |
|----------|------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|------------------------------------------------------------------|
|          | Epidermis                                      |                                                                                                                         | Dermis                                                                                               |                                                                  |
|          | Involved                                       | Uninvolved                                                                                                              | Involved                                                                                             | Uninvolved                                                       |
| 11       | —                                              | 0                                                                                                                       | 0                                                                                                    | 0                                                                |
| 12       | 61.5                                           | 0                                                                                                                       | 0                                                                                                    | 11.8                                                             |
| 13       | 34.3, 77.3, 90.1, 16.3, 10.3, 63.4, 54.4, 36.3 | 23.8, 25.0, 45.7, 3.3, 27.5, 43.0, 30.4, 15.4, 48.9, 9.7, 26.5, 16.1, 1.9, 13.8, 29.2, 18.1, 80.5, 34.5, 6.7, 11.0, 7.8 | 51.3, 25.3, 38.8, 50.6, 17.0, 11.3, 49.7, 39.4, 19.7, 41.3, 23.1, 28.8, 62.6, 36.0, 72.8, 63.3, 73.9 | 89.6, 38.4, 62.8, 52.3, 45.5, 51.0, 48.6, 46.8, 42.3, 50.0, 76.1 |
| 14       | —                                              | 0                                                                                                                       | —                                                                                                    | —                                                                |
| 9        | —                                              | 8.7, 8.8                                                                                                                | —                                                                                                    | —                                                                |
| 1        | 34.8, 40.8                                     | 0                                                                                                                       | 0                                                                                                    | 0                                                                |
| 1        | 38.2, 25.6                                     | 2.0, 0.1                                                                                                                | —                                                                                                    | —                                                                |

Table V. Interstrand DNA crosslinks in epidermal and dermal tissue in patients with psoriasis on oral PUVA treatment

|                 | No. of biopsies | No. of biopsies with crosslinked molecules | No. of non-cross-linked molecules | No. of crosslinked molecules/No. of non-crosslinked molecules (%) |
|-----------------|-----------------|--------------------------------------------|-----------------------------------|-------------------------------------------------------------------|
| Uninvolved skin |                 |                                            |                                   |                                                                   |
| Epidermis       | 5               | 0                                          | 895                               | 0                                                                 |
| Dermis          | 8               | 3                                          | 1 372                             | 0.2                                                               |
| Involved skin   |                 |                                            |                                   |                                                                   |
| Epidermis       | 8               | 5                                          | 1 452                             | 0.9                                                               |
| Dermis          | 8               | 4                                          | 1 682                             | 0.8                                                               |
| Total           |                 | 12                                         | 5 401                             | 0.6                                                               |



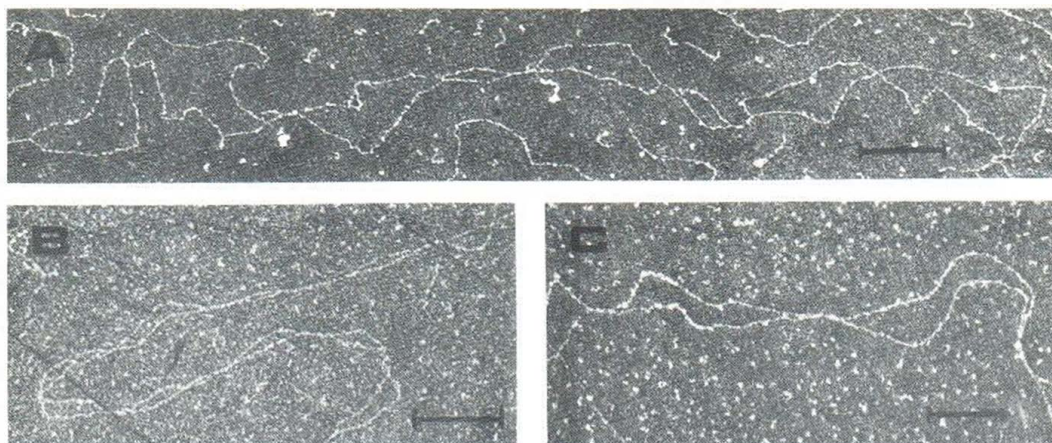


Fig. 2. The figure shows 3 examples of molecules with differing intramolecular percentages of crosslinking. Fig. 2 A is a molecule, 6.8% crosslinked. The total magnification is  $\times 32\,000$ . In Fig. 2 B, the percentage of crosslinking

is 31.8% and the total magnification is  $\times 36\,400$ . In Fig. 2 C, the percentage of crosslinking is 20.0%, and the total magnification is  $\times 30\,300$ . The bar on all 3 figures has the length of 1 kilobasepairs, equalling  $0.3\ \mu\text{m}$ .

system no DNA crosslinks could be detected in the absence of 8-MOP in the medium and neither could crosslinks be induced by 8-MOP alone without irradiation. In these *in vitro* experiments, however, the concentration of 8-MOP was higher than that used in our clinical experiments.

Our present results have recently been confirmed by Pathak *et al.* (16). Using both an electron-microscopic technique and biochemical methods for detection of interstrand DNA crosslinking they were unable to detect 8-MOP–DNA crosslinks in epidermis of guinea pigs receiving oral 8-MOP when given in therapeutic dose range ( $0.6\text{--}1.2\ \text{mg/kg}$  8-MOP and UVA  $12\text{--}16\ \text{J/cm}^2$ ). Crosslinks could be detected only when the drug dose exceeded  $6.0\ \text{mg/kg}$  and UVA  $4\ \text{J/cm}^2$ . Topical 8-MOP concentrations of  $20\ \mu\text{g/cm}^2$  revealed significant crosslinks.

The functional significance of induction of a single or a few crosslinks in epidermal DNA molecules is largely unknown. Previous *in vitro* experiments indicate that transcription (4) and replication (3) are inhibited and under *in vivo* conditions crosslinks inhibited epidermal DNA synthesis (18). However, even if the formation of interstrand DNA crosslinks in skin can be considered to be an indicator of the damage to the genetic material we were unable to demonstrate this as a consequence of PUVA treatment using 8-MOP.

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Table VI. Interstrand DNA crosslinks in epidermal and dermal tissue in patients with psoriasis on topical PUVA treatment

|                 | No. of biopsies | No. of biopsies with crosslinked molecules | No. of non-cross-linked molecules | No. of crosslinked molecules/No. of non-crosslinked molecules (%) |
|-----------------|-----------------|--------------------------------------------|-----------------------------------|-------------------------------------------------------------------|
| Uninvolved skin |                 |                                            |                                   |                                                                   |
| Epidermis       | 4               | 1                                          | 1 158                             | 1.8                                                               |
| Dermis          | 4               | 2                                          | 771                               | 1.6                                                               |
| Involved skin   |                 |                                            |                                   |                                                                   |
| Epidermis       | 4               | 4                                          | 710                               | 1.8                                                               |
| Dermis          | 7               | 3                                          | 1 463                             | 1.4                                                               |
| Total           |                 | 10                                         | 4 102                             | 1.6                                                               |

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