Urinary thymine dimers and 8-oxo-2′-deoxyguanosine in psoriasis

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Abstract Psoralen in conjunction with UVA (PUVA) is perhaps the most effective treatment for psoriasis. It is, however, a risk factor for skin cancer in these patients and there is a need to develop non-invasive assays reflective of treatment-induced DNA damage. We report here the assessment of two important lesions, thymine dimer (T⟨⟩T) and 8-oxo-2′-deoxyguanosine (8-OHdG), in the urine of psoriasis patients. It was found that, once corrected for urine concentration, the psoriatic group had significantly higher ($P < 0.0001$) urinary levels of thymine dimers compared to the control group. No significant differences in urinary 8-OHdG levels were noted between the psoriatic, atopic dermatitis and control groups. Therefore biomonitoring of therapy from the very start with this simple and non-invasive assay could perhaps be an effective measure of the risk involved with the treatment allowing optimization for minimal-risk therapy.

Key words: PUVA; 8-Oxo-2′-deoxyguanosine; Thymine dimer; Psoriasis; Antibody; Urine

1. Introduction

The major features of psoriasis are abnormal differentiation and hyperproliferation of keratinocytes, along with infiltration of inflammatory cells into the skin [1]. About 2% of the world's population is affected with psoriasis and it is believed that immunological factors, abnormal epidermal growth and abnormal keratinization are of pathogenic relevance [2]. A variety of treatments are available for psoriasis [3], traditionally aimed at reducing the abnormal epidermal proliferation. However these treatments are genotoxic and must be considered potentially carcinogenic [4].

The discovery in 1974 of a new photobiologic principle, light and drug, paved the way for a new and effective treatment of psoriasis. Psoralens, an important class of compounds widely distributed in nature [5,6], are used extensively as therapeutic agents for the treatment of skin diseases like psoriasis. This approach, which combines psoralen with UVA radiation (PUVA), whilst effective for treating psoriasis has long term side effects including an increased risk of cutaneous malignancy related, in part, to the cumulative dose of UVA received during PUVA therapy [7,8]. It has been suggested that psoralens owe their photosensitizing and photochemotherapeutic effectiveness to their ability to intercalate into DNA and form psoralen-DNA photoadducts upon subsequent exposure to UVA light [9]. Although PUVA therapy can achieve 80–90% clearance of plaques in 8–12 weeks [10], this treatment is done on a long term basis to prevent recurrence, or to treat new lesions. Such chronic treatment poses a threat through the development of skin cancer [11]. It is therefore essential to develop procedures to increase the safety of the treatment by predicting and hence minimizing carcinogenic effects.

Progress has been made in understanding the molecular events of non-melanoma skin cancer, through associating characteristic UVR-induced mutations at dipyrimidine sites with DNA photolesions such as cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts [12], which are inducible at clinically relevant doses of UVB and UVA [13]. A major biomarker of oxidative stress is the DNA damage product 8-hydroxy-2′-deoxyguanosine [14]. Since reactive oxygen species (ROS) are assumed to be involved in the pathogenesis of chronic inflammatory skin diseases, such as atopic dermatitis, it is possible that increased ROS generation may be one of the contributing factors to tissue damage in psoriasis [15]. Indeed, it has recently been shown that patients with atopic dermatitis have significantly higher levels of urinary 8-OHdG [16]. A role for 8-OHdG in carcinogenesis has been postulated [17] and its involvement in skin damage is based on evidence that UV-derived hydroxyl radicals and singlet oxygen induce 8-OHdG in vitro [18]. Furthermore, high levels of 8-OHdG appear in normal human epidermis after a single dose of UV radiation suggesting a significant role in UV-induced carcinogenesis [19].

Damaged DNA products are eliminated by a variety of repair enzymes and may be detected as nucleoside derivatives [20]. The level of these products depends on the equilibrium between the rates of damage and repair, reflected by the amount of lesion excreted into urine. In the present study we examined the urine of psoriasis patients post-PUVA treatment for two markers of genotoxic insult: 8-OHdG, an acknowledged marker of oxidative stress and thymine dimer (T⟨⟩T), a unique marker of UV exposure. The measurement of 8-OHdG was by a competitive ELISA using a specific monoclonal antibody obtained commercially, whereas T⟨⟩T measurement utilized a polyclonal antiserum raised against UVC-irradiated poly(dT), characterization of which indicated specific recognition of T⟨⟩T. We predict that the development of such non-invasive assays to measure oxidative and non-

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Abbreviations: UVC-poly(dT), UVC-irradiated polythymidylic acid; PUVA, psoralen+ultraviolet A; T⟨⟩T, thymine dimer; 8-OHdG, 8-oxo-2′-deoxyguanosine; NER, nucleotide excision repair; UVR, ultraviolet radiation

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oxidative DNA damage may represent a means by which an individual's ability to process DNA lesions could be determined and a therapy-related risk assessment performed.

2. Materials and methods

2.1. UVC irradiation of polythymidylic acid and synthetic oligonucleotides

Polythymidylic acid was purchased from Sigma Chemical Company (Poole, UK) and the synthetic oligonucleotides were prepared by the Protein and Nucleic Acid Laboratory, Department of Biochemistry, University of Leicester, UK. All irradiations were performed on ice, using a 254 nm UVC source (Knight Optical Technologies) and given a total exposure of 21 J/cm^2. Proof that T⟨⟩T are successfully induced by UVC irradiation was provided by GC-MS analysis [21].

2.2. Production and purification of polyclonal antibodies

UVC-irradiated poly(dT) was complexed with methylated bovine serum albumin (BSA) as described earlier [22]. Each rabbit was immunized according to Cooke et al. [23]. The resulting antiserum was purified for IgG class antibodies with a Protein A Sepharose CL-4B column and the specificity evaluated by a competitive binding assay.

2.3. Enzyme-linked immunosorbant assay

The detection of antibodies to UVC-poly(dT) by direct ELISA and competitive ELISA to define specificity was performed in a manner similar to that described by Cooke et al. [23], with the knowledge that damaged and native DNA bind equally well to ELISA plates [24]. In both instances, UVC-DNA was used as the solid phase antigen, bound to 96-well ELISA plates (Life Technologies Ltd., Paisley, Scotland) and the primary antiserum used diluted 1:5000 in milk/PBS. Detection of the primary antiserum was by peroxidase-labelled goat anti-rabbit IgG (DAKO Ltd., High Wycombe, UK) diluted to 1:2000 (in milk/PBS; 50 μl/well). Absorbances were read at 492 nm using an Anthos 2001 plate reader (Anthos Labtec Instruments, UK).

2.4. Detection of T⟨⟩T in urine

The ability of antiserum to detect T⟨⟩T in urine was demonstrated by 'spiking' urine samples with known concentrations of T⟨⟩T-containing oligomers prior to analysis by competitive ELISA.

2.5. Urine samples

Urine samples were obtained from a total of 33 patients (21 female, 12 male; median age 36 years, age range 14–85 years), who were classified with mild to moderate psoriasis (mean Psoriasis Assessment Severity Index [PASI] score 8.5; range 1.2 to 17.4). All were on topical therapy comprising tar and emollients of vitamin D_3 analogues. A control group of 16 subjects was used (10 males and 6 females; median age 30.7 years, age range 17–43 years). Seven individuals with atopic dermatitis were also examined as a reference dermatitis group (three male, four female; median age 38 years, age range 13–74 years), three of which were assessed as severe or post-flare. The urinary creatinine values of all the groups were examined (Department of Chemical Pathology, Leicester Royal Infirmary, Leicester, UK) as impaired renal function may affect levels of any lesion excreted into the urine. Collected urine samples were stored, without any additives, at −80°C in 20 ml plastic Universal tubes, until analysis. Following thawing and centrifugation (300×*g* for 10 min), the supernatants were applied to the competitive ELISA plate according to the protocol described earlier.

2.6. Urinary 8-OHdG measurements

Samples of urine were analyzed by competitive ELISA, without prepurification, using '8-OHdG Check' (JaICA, Fukuroi City, Japan) [25].

2.7. Urinary thymine dimer measurements

Samples of urine were analyzed by competitive ELISA, without prepurification, using the anti-thymine dimer antibody described above.

Statistical analysis was performed using Graph Pad Prism, version 2.01.

3. Results

The immunogen UVC-poly(dT)/BSA conjugate induced a strong immunogenic response in rabbits, such that at a 1:5000 dilution of the antibody produced an ELISA absorbance at 492 nm of 1.0 whilst the binding of pre-immune serum was negligible. Isotyping revealed the majority of the response to be of the IgG class. The antigenic specificity of the antibody was determined by a series of competitive ELISA experiments. The concentration of competitor giving 50% inhibition (IC_{50}) is an index of the antigenicity of the compound and the lower the value the better the competitor.

UVC-poly(dT) was a very effective inhibitor of antibody binding with the IC_{50} less than 0.07 μg/ml whilst unirradiated poly(dT) inhibited to a much lesser extent with an IC_{50} of 13.85 (Table 1). UVC-irradiated DNA, made single-stranded (ss) was a better inhibitor than double-stranded (ds) UVC-DNA, whereas no significant inhibition was observed with native DNA irrespective of whether single- or double-stranded (data not shown). Further identification of epitopes recognized by the antibody was performed with a series of thymine-containing oligonucleotides. Initially the effect of length of thymidylate oligonucleotides was investigated, with the effectiveness of inhibition increasing with chain length (Table 1). Furthermore poly(A12), poly(C12) and poly(G12) whether UVC-irradiated or not, consistently failed to inhibit in the competition ELISA (Table 1), directly implicating thymine dimerization in the epitope formation.

In order to confirm that adjacent thymine residues are necessary for binding we used a septanucleotide containing alternating A and T residues which was found to be an ineffective inhibitor when UVC-irradiated. Another oligomer of alternating A-A and T-T units, when UVC-irradiated, also inhibited poorly implying that dimerization of thymine residues alone was not sufficient to form the preferentially recognized antigen

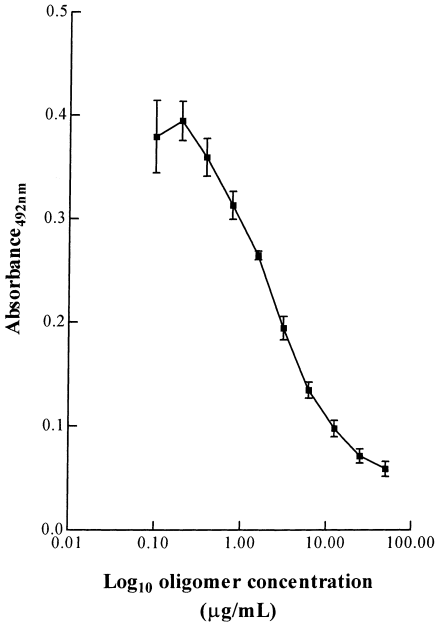

Fig. 1. Antibody recognition of thymine dimer-containing oligomers in urine. Urine samples were spiked with increasing concentrations of oligomer prior to competitive ELISA.

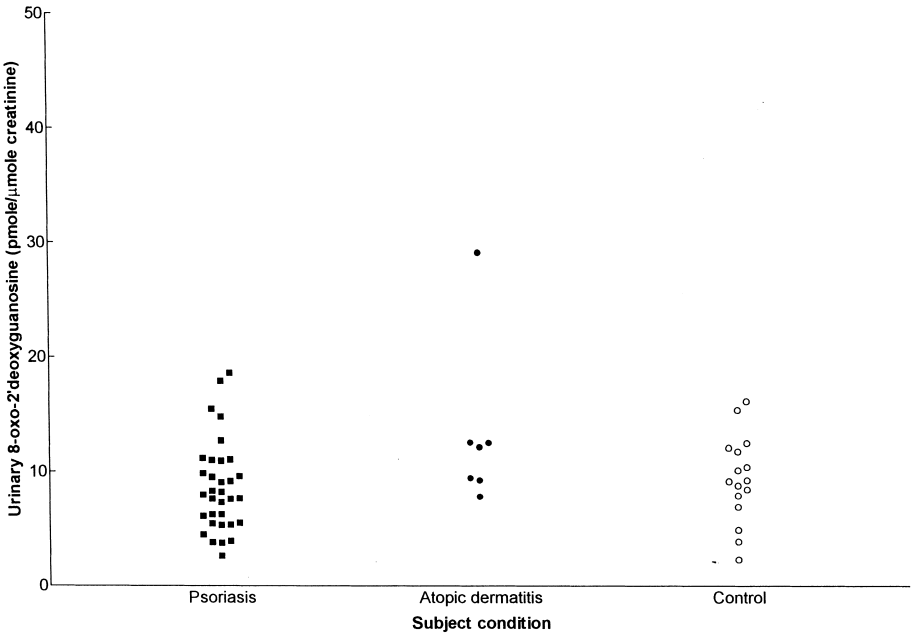

Fig. 2. Detection of 8-OHdG in the urine samples of patients with psoriasis, using a monoclonal antibody-based competitive ELISA. (Only $n = 29$ psoriatic patients are represented as four had urinary levels of 8-OHdG below the limit of detection.) Also represented is the inflammatory disease control, atopic dermatitis patients and healthy control subjects.

for the majority of antibodies present in the serum. However, when a UVC-irradiated oligonucleotide with three thymine residues in sequence flanked by adenine residues was used, a strong inhibitory response was seen. This strong recognition was not improved appreciably by four thymine residues in a sequence flanked by adenine residues (Table 1). Putative urinary constituents which might interfere with the assay were also investigated and shown not to inhibit antibody binding (Table 2). The ability of the antiserum to detect thymine dimers in urine was further demonstrated by the linearity of the competitive ELISA response in spiking experiments (Fig. 1).

Assessment of urinary creatinine concentration indicated the psoriatic and atopic dermatitis patients to be free from renal disease and hence this would not affect urinary 8-OHdG or T⟨⟩T levels. Creatinine levels were successfully applied to correct for urine concentration. No significant differences in urinary 8-OHdG levels were noted between the control (9.78, S.D. = 3.80 pmol/μmol creatinine), atopic dermatitis (12.29, S.D. = 7.72 pmol/μmol creatinine) and psoriasis (9.36,

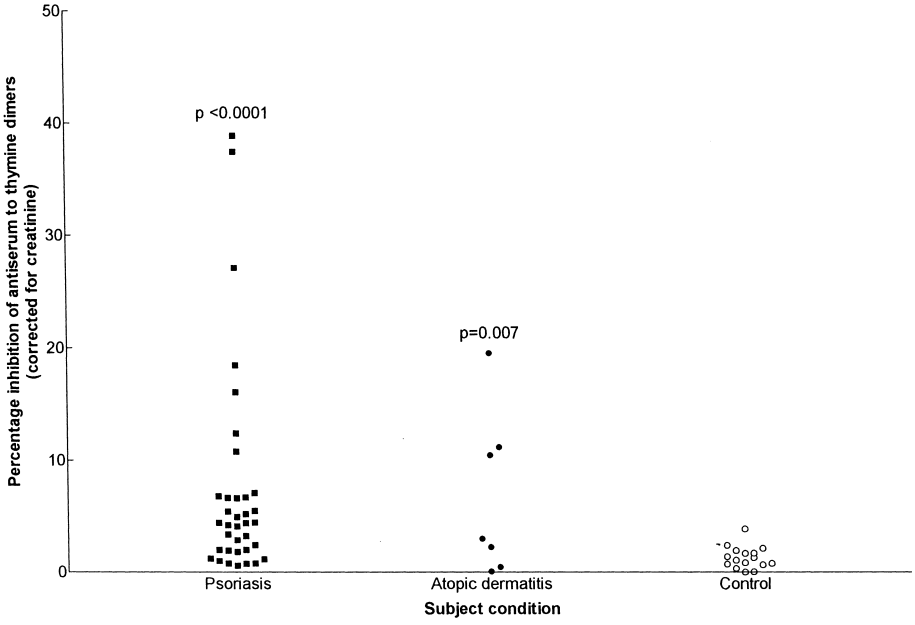

Fig. 3. Detection of urinary thymine dimers in psoriasis patients using a polyclonal antiserum-based competitive ELISA. Also represented is the inflammatory disease control, atopic dermatitis patients and healthy control subjects.

Table 1
Index of inhibition by a variety of oligonucleotide modified/native DNA and protein competitors, as determined by competitive ELISA with the anti-thymine dimer antiserum

Characterization competitor		IC_{50} (μg/ml)
UVC-poly(dT)		< 0.07
poly(dT)		13.86
T3	UVC-irradiated	19.39
T6	UVC-irradiated	0.74
T9	UVC-irradiated	0.11
TATATAT	UVC-irradiated	> 50.00
TAATTA	UVC-irradiated	17.11
AATTTAA	UVC-irradiated	4.64
AATTTTAA	UVC-irradiated	3.98
poly(G12)	Irradiated/unirradiated	> 100.00
poly(C12)	Irradiated/unirradiated	> 100.00
poly(A12)	Irradiated/unirradiated	> 100.00
Keyhole limpet haemocyanin		> 250

S.D. = 7.80 pmol/μmol creatinine) groups (Fig. 2). No correlation was seen between PASI scores and urinary 8-OHdG (data not shown). Urinary levels of thymine dimer were found to be significantly raised in both the psoriasis ($P < 0.0001$) and atopic dermatitis ($P = 0.007$) patients compared to the control group (Fig. 3).

4. Discussion

PUVA is a highly effective treatment for psoriasis, although prolonged use is associated with an increased risk of developing non-melanoma skin cancer, in particular squamous cell carcinoma [26]. Several studies have shown that psoriasis patients who receive extensive PUVA therapy have a 10-fold increase in the incidence of squamous cell carcinoma over that in the general population [27]. There could be several explanations for the increased incidence of skin cancer in PUVA-treated patients. First, PUVA is mutagenic and carcinogenic and may itself induce skin cancer [28]. Second, because PUVA treatment is immunosuppressive [29], it may permit the growth of skin cancers induced by other carcinogenic agents. Third, because many psoriasis patients undergo treatment with UVB (290–320 nm) in addition to PUVA [30] or because the light sources sometimes used in PUVA therapy contain small but significant wavelengths in the UVB region these might be the inducing carcinogen.

Since UV and PUVA induce different types of lesion in DNA, it has been hypothesized that mutations induced by these agents may also be different [31]. While UV induces primarily cyclobutane-type pyrimidine dimers and pyrimidine (6-4)-pyrimidone photoproducts [32], PUVA induces monofunctional adducts and DNA cross-links [33]. The main repair mechanism responsible for the elimination of both types of DNA lesions is probably nucleotide excision repair (NER) which removes bulky and helix-distorting lesions from DNA [34], which are subsequently excreted in the urine. Psoralen is also known to generate ROS, presumably singlet oxygen, through type II photodynamic action [35] and it has recently been hypothesized that PUVA-induced oxidative DNA damage may occur in vivo [36]. Urinary 8-OHdG, derived from the nucleotide pool or DNA, via an endonuclease or NER is an accepted marker of oxidative stress [25]. Additionally, cell turnover, due to apoptosis or necrosis may also contribute to urinary levels of any lesion but are still reflective of lesion levels induced by treatment [37].

Table 2
Index of inhibition by a variety of putative urinary constituents, as determined by competitive ELISA with the anti-thymine dimer antiserum

Putative urinary constituent	IC_{50} (μg/ml)
Thymine	> 250
Guanine	> 250
Cytosine	> 250
Adenine	> 250
Xanthine	> 250
Albumin	> 250
Uric acid	> 6000
Urea	> 6000
Creatinine	> 6000

Our work represents the first report of urinary 8-OHdG measurements in psoriasis and the results suggest that there is no chronic oxidative stress associated with mild to moderate forms of this disease. However, oxidative stress may be induced during PUVA treatment, with the assay still applicable to biomonitoring. Contrary to a recent report [16] we did not detect elevated levels of 8-OHdG in atopic dermatitis patients although this may be due to the limited number of subjects studied.

We have developed a unique assay utilizing a polyclonal antibody specific for T⟨⟩T for the detection of this lesion in the urine of psoriasis patients. When compared with controls and once corrected for creatinine and hence urine concentration, the psoriatic group was found to contain significantly higher urinary levels of the lesion, thus suggesting a relationship between its presence and the treatment. Three patients within the atopic dermatitis group possessed elevated urinary T⟨⟩T, raising the levels for that group to one of significance. Examination of these three patients revealed them to be either particularly severe, or post-flare. UV exposure is routinely used for treatment of such cases, which would be expected to generate elevated levels of this lesion. These findings warrant further study of this patient group.

Taken together, these data implicate the UV given to psoriasis patients as part of PUVA therapy as the source of thymine dimers in urine. Therefore a constant monitoring of the treatment for thymine dimers and perhaps 8-OHdG in the urine may provide a good measure of the risk involved and aid intervention to provide a minimal-risk therapy.

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References

[1] Duvic, M., Asano, A.T., Hager, C., Mays, S., Morhein, Acklatek, Carter (1998) J. Am. Acad. Dermatol. 39, S129–S133.
[2] Van de Kerkhof, P.C.M. (1998) Pharm. Weekbl. 133, 1481–1491.
[3] Feldman, S.R. and Clark, A.R. (1998) Med. Clin. North Am. 82, 1135–1144.
[4] Moller, P., Knudsen, L.E., Frentz, G., Dybdahl, M. and Wallin, H. (1998) Mutat. Res. 407, 25–34.
[5] Pathak, M.A., Danieles, F. and Fitzpatrick, T.B. (1961) J. Invest. Dermatol. 39, 225–239.
[6] Beier, R.C. and Oertli, E.H. (1983) Phytochemistry 22, 2595–2597.
[7] Chuang, T.-Y., Heinrich, L.A. and Schultz, M.D. (1992) J. Am. Acad. Dermatol. 26, 173–177.
[8] Lever, L.R. and Farr, P.M. (1994) Br. J. Dermatol. 131, 215–219.
[9] Stern, R.S., Morison, W.L., Thibodeau, L.A., Kleinerman, R.A., Parrish, J.A., Geer, D.E. and Fitzpatrick, T.B. (1979) Arch. Dermatol. 115, 1320–1324.
[10] Weinstein, G.D. and White, G.M. (1993) J. Am. Acad. Dermatol. 28, 454–459.
[11] Olsen, J.H., Moller, H. and Frentz, G. (1992) J. Am. Acad. Dermatol. 27, 716–722.
[12] Ziegler, A., Jonason, A.S., Lefell, D.J., Simon, J.A., Sharma, H.W., Kimmelmann, J., Remington, L., Jacks, T. and Brash, D.E. (1994) Nature 372, 773–776.
[13] Young, A.R., Potten, C.S., Nikaido, O., Parsons, P.G., Boenders, J., Ramsden, J.M. and Chadwick, C.A. (1998) J. Invest. Dermatol. 111, 936–940.
[14] Halliwell, B. (1993) in: DNA and Free Radicals (Halliwell, B. and Aruoma, O.I., Eds.), pp. 67–79, Ellis Horwood, New York.
[15] Nagata, M., Sedgwick, J.B., Bates, M.E., Kita, H. and Busse, W.W. (1995) J. Immunol. 155, 2194–2202.
[16] Tsuboi, H., Kouda, K., Takeuchi, H., Takigawa, M., Masamoto, Y., Takeuchi, M. and Ochi, H. (1998) Br. J. Dermatol. 138, 1033–1035.
[17] Toyokuni, S., Tanaka, T., Hattori, Y., Nishiyama, Y., Yoshida, A., Uchida, K., Hiai, H., Ochi, H. and Osawa, T. (1997) Lab. Invest. 76, 365–374.
[18] Kasai, H., Yamaizumi, Z., Berger, M. and Cadet, J. (1992) J. Am. Chem. Soc. 114, 9692–9694.
[19] Ahmed, N.U., Ueda, M., Nikaido, O., Osawa, T. and Ichihashi, M. (1999) Br. J. Dermatol. 140, 226–231.
[20] Simic, M.G. (1992) Mutat. Res. 267, 277–290.
[21] Podmore, I.D., Cooke, M.S., Herbert, K.E. and Lunec, J. (1996) Photochem. Photobiol. 64, 310–315.
[22] Plescia, O.J., Braun, W. and Palczuk, N.C. (1964) Proc. Natl. Acad. Sci. USA 52, 279–285.
[23] Cooke, M.S., Mistry, N., Wood, C., Herbert, K.E. and Lunec, J. (1997) Free Rad. Biol. Med. 22, 151–157.
[24] Blount, S., Griffiths, H.R., Emery, P. and Lunec, J. (1990) Clin. Exp. Immunol. 81, 384–389.
[25] Cooke, M.S., Evans, M.D., Podmore, I.D., Herbert, K.E., Mistry, N., Mistry, P., Hickenbotham, P.T., Hussieni, A., Griffiths, H.R. and Lunec, J. (1998) FEBS Lett. 363, 363–367.
[26] Forman, A.B., Roenigk Jr., H.H., Caro, W.A. and Magid, M.L. (1989) Arch. Dermatol. 125, 515–519.
[27] Stern, R.S. (1991) Blood Cells 18, 91–99.
[28] Coven, T.R., Walters, I.B., Cardinale, I. and Krueger, J.G. (1999) Photodermatol. Photoimmunol. Photomed. 15, 22–27.
[29] Morison, W.L. and Kripke, M.L. (1984) Cell. Immunol. 85, 270–277.
[30] Lindelof, B. and Siguregeirsson, B. (1993) Br. J. Dermatol. 129, 39–43.
[31] Nataraj, A.J., Wolf, P., Cerroni, L. and Ananthaswamy, H.N. (1997) J. Invest. Dermatol. 109, 238–243.
[32] Rosenstein, B.S. and Mitchell, D.L. (1987) Photochem. Photobiol. 45, 775–780.
[33] Gasparro, F., Chan, G., Edelson, R.L. and Yale, J. (1985) J. Biol. Med. 58, 519–534.
[34] Sancar, A. and Tang, M.-S. (1993) Photochem. Photobiol. 57, 905–921.
[35] Pathak, M.A. and Joshi, P.C. (1984) Biochim. Biophys. Acta 798, 115–126.
[36] Liu, Z., Lu, Y., Lebwohl, M. and Wei, H. (1999) Free Rad. Biol. Med. 27, 127–133.
[37] Lindhal, T. (1993) Nature 362, 709–715.