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Original Contribution

MODULATION OF EXOGENOUS AND ENDOGENOUS LEVELS OF THIOREDOXIN IN HUMAN SKIN FIBROBLASTS PREVENTS DNA DAMAGING EFFECT OF ULTRAVIOLET A RADIATION

CHRISTINE DIDIER,* JEAN-PIERRE POUGET,[†] JEAN CADET,[†] ALAIN FAVIER,* JEAN-CLAUDE BÉANI,* and
MARIE-JEANNE RICHARD*

*LBSO/LCR7 CEA n°8—Université Joseph Fourier, Grenoble, France; and [†]"Laboratoire Lésions des Acides Nucléiques",
Département de Recherche Fondamentale sur la Matière Condensée, Service de Chimie Inorganique et Biologie and UMR 5046
CNRS, CEA/Grenoble, Grenoble, France

Abstract—Thioredoxin (Trx) plays important biological roles both intra- and extracellularly via thiol redox control. We have previously demonstrated that Trx exhibited protective effects against UVA cytotoxicity in human skin fibroblasts. As an extension of the latter investigation, the present work is aimed at assessing ability of Trx to maintain genomic integrity in human skin fibroblasts upon exposure to UVA radiation. Indeed, UVA (320–380 nm) is mutagenic and induces genomic damage to skin cells. The alkaline comet assay was used in association with DNA repair enzyme including formamido pyrimidine glycosylase (Fpg) and endonuclease III (endo III) to estimate the amount of modified bases together with the level of strand breaks and alkali-labile sites. The HPLC-EC assay was applied to assess 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) levels and to permit the calibration of comet assay as previously described. We reported that overexpression of human Trx (transient transfection) as well as exogenous human recombinant Trx added to the culture medium, decreased the level of DNA damage in UVA irradiated cells. Interestingly, transfection appeared to prevent UVA-induced 8-oxodGuo (3.06 au per Joules.cm^{-2} compared to 4.94 au per Joules.cm^{-2} for nontransfected cells). Moreover, Trx accumulates into nuclei in transfected cells. This finding supports the notion that Trx is important for the maintenance of the integrity of genetic information. This work demonstrated that under conditions of UVA oxidative stress, Trx prevented the UVA-induced DNA damage. © 2001 Elsevier Science Inc.

Keywords—Thioredoxin, DNA damage, Ultraviolet radiation, Comet assay, Free radicals

INTRODUCTION

The UV radiation component of sunlight is the most important environmental factor involved in the pathogenesis of skin cancers [1]. UVA radiation (320–400 nm) is able to create mutations [2], some of which may lead to malignant transformation [3]. UVA radiation may be absorbed by intracellular chromophores like riboflavin or nicotinamide coenzyme, ultimately resulting in the generation of reactive oxygen (ROS) [4] and nitrogen species (NOS) including nitric oxide (NO•) [5]. Indeed, excited photosensitizers can react with DNA by electron abstraction (type I) or via the production of singlet oxygen (type II). Type I reaction can give rise to superoxide anion, $O_2^{\bullet-}$ as the end-product of the reaction of oxygen with the radical anion of the photosensitizer. The rather unreactive $O_2^{\bullet-}$ species can be converted into the highly damaging hydroxyl radical •OH via a Fenton reaction. It may be added that singlet oxygen $^1O_2$ (type II reaction) is involved in the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) [6]. Therefore, the relative proportion of strand breaks versus 8-oxodGuo can be used as an indicator of the contribution of both type I and type II effects. The measurement of 8-oxodGuo is important because this lesion, which is known to be mutagenic, is commonly used as a marker of oxidative stress [7,8].

Thioredoxin (Trx) is a small ubiquitous multifunctional protein [9]. It contains a redox-active disulfide bridge in its

Address correspondence to: Marie-Jeanne Richard, Ph.D., LBSO, Laboratoire de Biochimie C, C. H. U. Albert Michallon, 38043 Grenoble Cedex 03, France; Tel: +33 (0) 4 76 76 54 84; Fax: +33 (0) 4 76 76 56 64; E-Mail: Marie-Jeanne.Richard@ujf-grenoble.fr.

oxidized form, which is reduced by the NADPH-dependent FAD-containing thioredoxin reductase (TR) enzyme. There is emerging evidence that the Trx system is as important as the glutathione system in the cellular redox regulation against oxidative stress. Human Trx was originally cloned by Wollman et al. [10] and identified as adult T cell leukemia-derived factor (ADF), which was characterized as a growth factor secreted by human T lymphotropic virus-I-transformed leukemic cell lines [11]. More recently, it was shown that intracellular Trx plays an important role in the regulation of protein-nucleic acid interactions through the redox regulation of its cysteine residues [12]. Trx that is secreted from cells [13] exhibits a cytokine-like extracellular activity to promote cell growth [14]. Mammalian Trx has been shown to be a stress-inducible protein [15]. Recently we demonstrated that Trx was induced by UVA and was able to protect cells against UVA (submitted for publication). Furthermore, Trx is a scavenger of ROS [16,17] and NOS [18], whereas recombinant Trx (rhTrx) exerts a protective activity against cytotoxicity, in which the generation of ROS seems to be involved [19,20]. These data suggest that Trx plays a number of important biological roles in both intra- and extracellular compartments.

Based on these considerations, we studied the protection provided by Trx in UVA-irradiated human skin fibroblasts. Attempts were made to determine whether exogenous and endogenous Trx by itself can modulate ROS-induced DNA damage. The measurement of single and double strand breaks (SSB, DSB) together with alkali-labile sites (ALS) was performed using the single cell gel electrophoresis method named *comet assay*. When associated with DNA repair enzymes such as *E. coli* formamidopyrimidine DNA *N*-glycosylase (Fpg) or *E. coli* endonuclease III (endo III), the comet assay provides insights into the level of modified purines and pyrimidines, respectively [21,22]. Modified bases are thus converted into strand breaks that can be further assessed by the single cell gel electrophoresis method. 8-OxoGua, together with 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde) were detected using the Fpg protein [23,24]. Endo III was used in a similar way to assess the level of degradation products of pyrimidine bases, which may include among others, 5-hydroxy-5-methyldantoin and thymine glycols [22,25].

MATERIALS AND METHODS

Materials

RPMI-1640 and fetal calf serum (FCS) were purchased from ATGC (ATGC Biotechnologie, Noisy-le-Grand, France). L-glutamine and fungizone were obtained from Boehringer-Mannheim (Mannheim, Germa-

ny), penicillin and streptomycin from Polylabo (Paul Block, Strasbourg, France) and kanamycin and Puck's saline from Gibco (Grand Island, NY, USA). Phosphate-buffered saline (PBS) that contains calcium and magnesium, was purchased from Eurobio (Les Ullis, France). Agarose and low melting-point agarose were obtained from Promega (Madison, WI, USA). Slides and cover glasses were from Touzart et Matignon (Vitry-sur-Seine, France) and Marienfeld (Bad Mergentheim, Germany), respectively. The *E. coli* Fpg and Endo III were kindly provided by Dr. Serge Boiteux (CEA, Fontenay-aux-Roses, France). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

Cultures of normal human skin fibroblasts were established from excess tissues of breast plastic surgery specimens and were further propagated in RPMI-1640 supplemented with 10% FCS, 300 μg.ml^{-1} L-glutamine, 0.5 μg.ml^{-1} fungizone, 0.17 μg.ml^{-1} penicillin and streptomycin and 54 μg.ml^{-1} kanamycin. Cells with population showing doubling levels between 7 and 14 were seeded in 9 cm^2 NUNC-petri dishes from Gibco and grown for 5 d to near confluency. The culture medium was replaced by fresh medium 48 h before the experiment.

Transfection experiments

Transfections of fibroblasts were performed by Fu-GENE 6 reagent (Roche, Meylan, France) according to the manufacturer protocols. This was achieved using the eukaryotic expression vector pCMV-ADF encoding human Trx, which was a gift from P. A. Baeuerle. Originally vector without cDNA for human Trx was used as control. Briefly, cells were seeded in 35 mm petri dishes, and transfected at 60% confluence with 5 μg plasmid. Then, 48 h later, cells were harvested for the comet assay.

Purification of recombinant human thioredoxin (rh Trx)

Thioredoxin was expressed as a fusion protein that contains 6 histidine residues attached to their C-terminus (pQE60 vector from Qiagen, Courtaboeuf, France). Expression was induced in logarithmic cultures of *E. coli* M15 by the addition of 2 mM isopropyl β-D-thiogalactopyranoside. After 3 h bacteria were centrifuged and lysed by sonication under nondenaturing conditions. Thioredoxin was purified by using a nickel-chelated affinity resin (Qiagen). The purified protein fraction was reduced

by addition of a 5-fold molar excess dithiothreitol, and subsequent incubation for 30 min at 37°C. Then, it was dialyzed against 25 mM Tris/1 mM EDTA, pH 7.5 in order to remove the excess DTT [26]. The rh Trx showed a dithiol-dependent reducing activity using the insulin reduction assay [27]. Rh Trx was added to the cell culture medium 3 h before UVA irradiation.

UVA-irradiation

The UVA source was a high-pressure Tecimex apparatus (Verre & Quartz-Dixwell, Bondy, France). The maximum emission of the lamp was centred at 372 nm. The dose rate was determined to be 42 mW.cm^{-2} using a radiometer (Verre & Quartz-Dixwell). Temperature was controlled (22°C). Control cells were similarly treated but left in the dark instead to be irradiated. Cells were irradiated in PBS as previously described [28].

Alkaline single-cell gel electrophoresis

The procedure previously described [29] was slightly modified as follows. Typically, 150 μl of 1% normal melting agarose in PBS buffer, were dropped on frosted microscope slides (Touzart et Matignon, Les Ullis, France), covered with a coverslip, and kept at room temperature until their subsequent use. Human skin fibroblasts, pCMV-ADF transfected-cells or rh Trx-treated cells were trypsinized, and the cell pellet was suspended in PBS. About 20,000 cells were mixed with 75 μl of 1.2% low melting point agarose (FMC Bioproducts, Rockland, ME, USA) in PBS buffer, held at 37°C. Slides were kept at 4°C a few minutes to allow the gel to solidify. Embedded cells were immediately irradiated using low UVA fluences (2 J/cm^2). Then, all slides were immersed overnight in cell lysis buffer (1% triton X-100, 10% DMSO, 2.5 mM NaCl, 100 mM Na$_2$EDTA, 100 mM Tris, 10% sodium lauroyl sarcosinate, pH 10) in the dark at 4°C, in order to prevent nonspecific DNA damage and repair process to occur. Next, the slides were placed in a horizontal electrophoresis unit containing freshly prepared electrophoresis buffer (1 mM Na$_2$EDTA, 300 mM NaOH). The DNA was allowed to unwind for 40 min before electrophoresis was performed at 25 V, 300 mA for 45 min. Afterwards, the slides were gently immersed in neutralization buffer (0.4 M Tris-HCl, pH 7.4) for 5 min, and this step was repeated three times. Finally, 50 μl of 0.5 mg/ml ethidium bromide was pipetted on the slides to stain the DNA. The slides were placed in a humidified air-tight container to prevent drying of the gel, until the analysis was performed.

Two *E. coli* DNA-glycosylases, namely Fpg and endo III, were used separately to convert some of the UVA-radiation-induced base modifications into single strand breaks. For this purpose, the experiments were performed after the cell lysis and before the electrophoresis step. The slides were washed three times for 10 min with 0.1 M KCl, 0.5 mM Na$_2$EDTA, 40 mM HEPES, 0.2 mg/ml bovine serum albumin, adjusted to pH 8 with 4 M KOH. Then, 100 μl of either 1.5 μg/ml of Fpg or 1.5 μg/ml of endo III were laid on the slides, prior to incubation at 37°C for 45 min in a humidified atmosphere.

Slide analysis

The slides were examined using an epifluorescence microscope, Zeiss Axioskop 20 (Carl Zeiss, Microscope Division, Oberkochen, Germany), equipped with a mercury lamp HBO (50 W, 516–560 nm, Zeiss), and filters 5 and 15 (Zeiss) at 20× magnification. Fifty randomly selected comets on each slide were scored with a pulmix TM 765 camera (Kinetic Imaging, Liverpool, UK) and linked to a Komet 3.0 image analysis system (Kinetic Imaging). The software allows measurement of the fluorescence intensity of the head and tail regions, together with the determination of their length. The quantification of DNA damage was performed using the tail moment (TM), the product of the tail distance (i.e., the distance between the center position of the head and the center of gravity of the tail) and the percentage of DNA in the tail (relative to the total amount of DNA in the entire comet [head + tail]). For each condition, the averaged tail moment was determined using three different slides prepared as described previously.

HPLC-EC measurements

The chaotropic method reported by Helbock et al. [30] was used for the DNA extraction. The procedure described by Pouget et al. [22] was applied for the measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo). Typically, the HPLC system consisted of a Merck-Hitachi HPLC pump, model 6200, connected to a SIL 9A automatic injector (Shimadzu, Kyoto, Japan). The isocratic mobile phase constituted of 50 mM KH$_2$PO$_4$ that contained 8% methanol. The flow-rate was 1 ml.min^{-1}. Separation of the nucleosides was performed using a C18 reversed-phase Uptisphere (5 μm, 4.6 mm × 250 mm) column from Interchim (Montluçon, France) maintained at 30°C. The retention times of 8-oxodGuo and 2′-deoxyguanosine (dGuo) were 18.6 and 13 min, respectively. A Coulochem II, model 5200 A, electrochemical detector (ESA, Chemlsford, MA, USA) was used for the detection of 8-oxodGuo. The oxidation potentials of the electrodes of the analytical cell (model
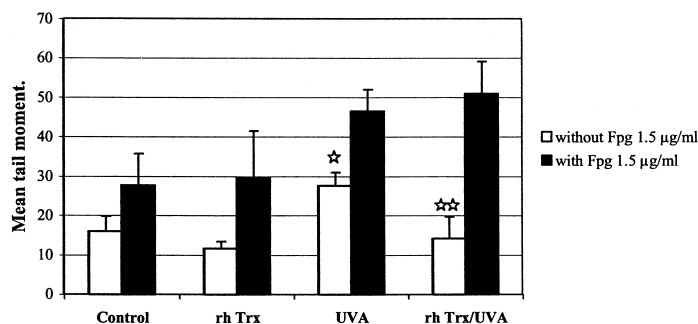
Fig. 1. Mean tail moment in human skin fibroblasts or rh Trx-treated cells exposed to UVA radiation (2 J/cm^2): with or without Fpg. Yield of damage is represented by mean tail moment of 50 cells per slide ($n = 3$). ☆$p < .01$ irradiated cells versus control cells and ☆☆$p < .01$ rh Trx-treated and irradiated cells versus irradiated cells.

5011, ESA), which were connected to the column, was set at 200 mV and 450 mV for E1 and E2, respectively. In addition, the potential of the guard cell placed prior to the inlet of the injector was set at 450 mV. Elution of unmodified nucleosides was monitored using an UV detector (model 2151, LKB Bromma) set at 280 nm. For each sample, the amount of DNA injected onto the column was estimated using the UV signal of dGuo after appropriate calibration.

Immunohistochemical cell staining

Fibroblasts (2×10^4) were seeded on 4 well plastic Lab-Tek chamber slides (Nunc, Naperville, IL, USA), fixed by 4% (w/v) paraformaldehyde in PBS for 10 min and then placed for 10 min in ice-cold methanol. Then, they were washed three times with PBS pH 7.4, and blocked for nonspecific antibody binding by incubating for 30 min in a milk buffer (2% dried milk powder, 0.1% tween 20 in PBS pH 7.4). Cells were washed and then incubated with 1 μg/ml of goat anti-human Trx (IMCO Corporation Ltd, Stockholm, Sweden) in PBS pH 7.4 containing 0.1% tween 20, 1% BSA, and 0.1% sodium azide. After washing with PBS, pH 8.6 and saturation for 5 min with the milk buffer, the cells were submitted to 2.3 μg/ml of biotinylated donkey F(ab')2 fragment anti-goat IgG (Jackson Immunoresearch Laboratories, West-grove, PA, USA) in PBS that contained 0.1% tween 20, 1% BSA, and 0.1% thymerosal. After two washes with PBS, pH 8.6, the cells were incubated with the avidin-biotin complex (StreptABC Complex/HRP, Dako, Glostrup, Denmark) for 1 h. Then, they were washed three times in 50 mM Tris-HCl buffer (pH 7.6), and incubated with 3,3'-diaminobenzidine (Sigma FAST DAB peroxidase substrate tablets) for 3 min according to the manufacturer. After washing with tap water, the cells were counterstained with Harris haematoxylin and the slides were mounted with Aquamount. Blanks were ei-

ther 1 μg/ml nonimmune goat immunoglobulin, or 1% (w/v) BSA in PBS. Cells in the present experimental conditions did not exhibit any endogenous peroxidase activity.

Flow cytometry analysis of thioredoxin expression

The cell suspension adjusted to 10^6 cells was fixed with 3% paraformaldehyde in PBS for 10 min at room temperature. Then, the cells were washed with PBS containing 1 w/v bovine serum albumin (BSA), and incubated for 10 min with goat antihuman Trx antibody at 1/100 in PBS containing 1% (w/v) BSA and 0.6% (w/v) saponin. After wash in PBS-BSA, cells were incubated for 10 min with fluorescein isothiocyanate (FITC) rabbit anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) conjugate, diluted 1/30 in PBS-BSA. In each case, controls were performed by incubating cells with irrelevant antibody. Analysis was carried out using a FACS Scan Flow cytometer (Becton-Dickinson, Le Pont de Claix, France). Fluorescence was recorded using the FL1-H filter (530 nm).

Statistical analysis

Each experiment was repeated three times. One-way analysis of variance (ANOVA) and a Newman-Keuls test were performed to determine whether differences in tail moment values were statistically significant among the various experimental conditions.

RESULTS

Effect of added exogenous rh thioredoxin

The comet assay was used to determine the ability of reduced rh Trx to prevent DNA damage (Fig. 1, open bars). The low applied UVA dose (2 J/cm^2) did not
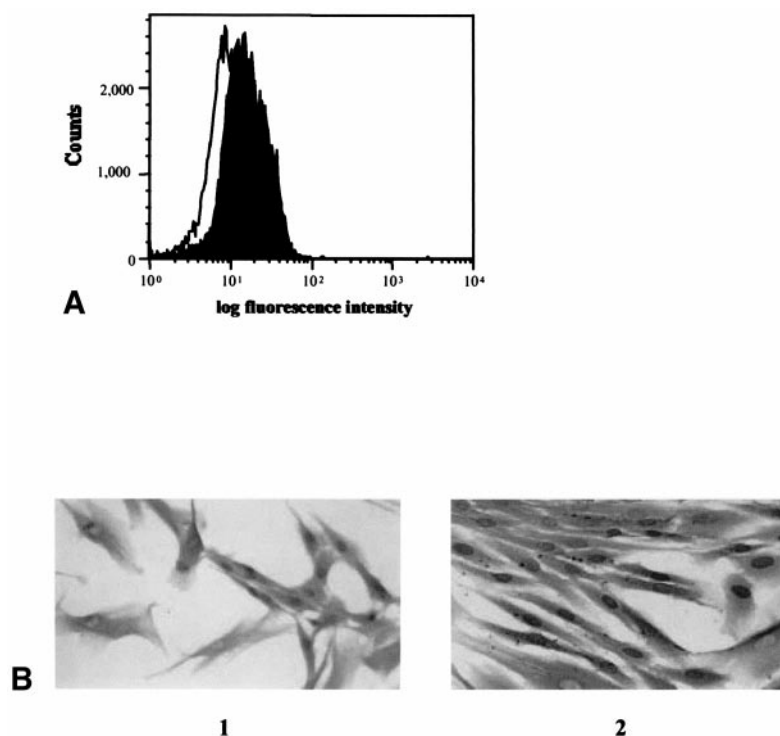
Fig. 2. Expression and localization of Trx protein in transient transfected human skin fibroblasts. (A) Cytofluorometric analysis of Trx expression. Trx contents in control and transient transfected cells (5 μg—48 h) were analyzed after staining of permeabilized cells. Increasing fluorescence intensity is plotted on the horizontal axis in log fluorescence intensity units versus cell numbers on the vertical axis. Plain line open curves represent control fibroblasts and dark shaded curves represent transient transfected cells. (B) Analysis of Trx localization in fixed/permeabilized fibroblasts by immunohistochemical staining. Cells were labeled with polyclonal antibody directed against human Trx and a biotinylated secondary antibody. (1) Control fibroblasts express low diffuse Trx staining. (2) pCMV-ADF-transfected fibroblasts (5 μg—48 h) shown higher Trx staining in the nuclear compartment. Cells were counterstained with Harris haematoxylin solution.

induce significant cytotoxicity [31] but generated direct strand breaks and/or alkali-labile sites (TM = 27.63 ± 3.35, $p < .01$). Interestingly, exogenous rh Trx (1 μM) added to the culture medium 3 h before the irradiation reduced significantly the tail moment of irradiated cells (TM = 14.17 ± 5.59, $p < .01$). The TM reached values identical to those measured in control cells (TM = 15.99 ± 3.82) demonstrating that rh Trx exerts a protective effect in UVA-irradiated human skin fibroblasts, whereas no modification of the TM was observed under basal conditions (TM = 11.67 ± 1.86).

In order to gain insights into the mechanism of prevention of DNA damage, we applied the comet assay with two DNA-glycosylases, Fpg and endo III involved in the removal of modified purine and pyrimidine bases, respectively. Results are represented in Fig. 1, dark bars. The mean tail moment determined in control cells treated with Fpg (1.5 μg/ml), was 2-fold higher (TM = 27.71 ± 8.05) than in absence of glycosylase. Pouget et al. reported a similar (×1.7) mean tail moment increase using 10 μg/ml of Fpg [22]. The tail moment was not significantly modified in rh Trx-treated cells. The tail moment measured using the modified comet assay strongly in-

creased (TM = 46.52 ± 5.59) in UVA-irradiated cells confirming that 8-oxodGuo is generated by UVA even at low doses. Exogenous Trx did not modify the tail moment of irradiated cells (TM = 50.98 ± 8.16). Rh-Trx did not appear to play a major role in preventing UVA-induced 8-oxodGuo. The endo III-sensitive sites were found to be quite low with respect to the lesions recognized by Fpg (data not shown).

Effect of thioredoxin overexpression

The application of suitable antioxidant can improve the intracellular antioxidant status and thus help to reduce the DNA damaging effects of UVA. We tested the effect of Trx by transfecting cells with pCMV-ADF. Under these conditions, we have measured the level of Trx by flow cytometry in basal conditions and 48 h after transient transfection. Control cells (mean fluorescence 51.2%) expressed Trx. Transient transfection induced a significant increase of fluorescence (mean 83.7%) compared to sham transfected cells (Fig. 2A). The negative control was performed using an irrelevant isotypic matched antibody (mean fluorescence 3.4% ± 0.7).
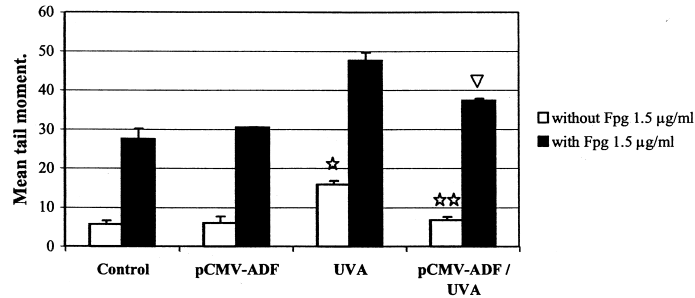
Fig. 3. Mean tail moment in human skin fibroblasts or pCMV-ADF-transfected cells exposed to UVA radiation (2 J/cm^2): with or without Fpg. Yield of damage is represented by mean tail moment of 50 cells per slide ($n = 3$). ☆$p < .01$ irradiated cells versus control cells; ☆☆$p = .001$ transfected and irradiated cells versus irradiated cells. $^\nabla p = 0.007$ transfected and irradiated cells versus irradiated cells in the presence of Fpg.

These results indicated that transient transfection induced about 30% increase of intracellular Trx expression by fibroblasts. We have also demonstrated that Trx translocated to the nucleus where it accumulates (Fig. 2B).

As shown in Fig. 3, the tail moment measured in control cells was low (TM = 5.67 ± 0.97), showing that the applied experimental procedure was well tolerated by the cells. These values were comparable with those obtained using the same experimental electrophoretic conditions on HaCaT cell line (TM = 6.45 ± 0.78) [28] and THP-1 monocytes cell line (10.1 μm) [32]. In cells overexpressing Trx (5 μg of expression vector pCMV-ADF), the mean tail moment (TM = 6.02 ± 1.70) was similar to those determined in control cells or sham transfected cells (vector alone, TM = 6.11 ± 1.02). The comparison of the values TM measured in UVA irradiated control cells and UVA-irradiated Trx-transfected cells indicates that the Trx overexpression strongly reduced the number of DNA strand breaks or alkali-labile sites (TM = 6.76 ± 0.90, $p < .01$). The transfection with vector alone have no significant effect (TM = 16.11 ± 0.98, $p < .01$).

In transfected fibroblasts, the mean tail moment

(TM = 27.60 ± 2.66) determined using the modified comet assay was 5-fold higher than for the standard comet assay. The tail moment strongly increased (TM = 47.72 ± 1.98) in UVA-irradiated cells. The combined treatment involving UVA irradiation (2 J/cm^2) and transient transfection (5 μg pCMV-ADF) significantly reduced the number of DNA strand breaks or alkali-labile sites (TM = 37.35 ± 0.59, $p = .007$). These results show that overexpression of Trx prevents oxidative injury to DNA. The mean tail moment determined in these conditions for sham transfected cells varied to TM = 28.90 ± 1.08 to TM = 46.80 ± 2.13 upon UVA radiation.

When the slides were treated with endo III, the mean tail moment (TM = 10.2 ± 1.65) in control cells was comparable to that of transfected cells (TM = 9.94 ± 2.72) (Fig. 4). Upon UVA radiation, no detectable increase in the number of endo III-sensitive sites was observed (TM = 16.97 ± 2.63). The value of TM is very similar to that determined on the monocytic cell line THP-1 in a previous work (19.9 μm with background levels of 10.6 μm) [32]. The low level of endo III-sensitive sites suggests that few modified pyrimidines are
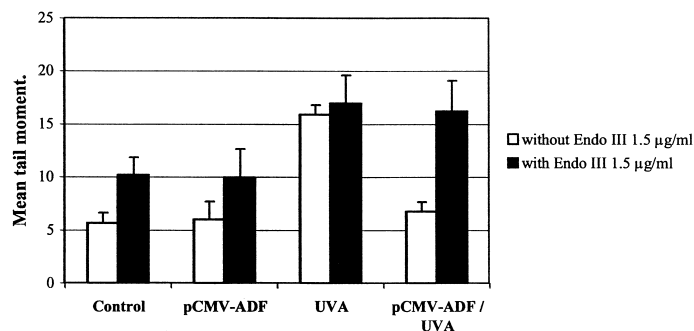

Fig. 4. Mean tail moment in human skin fibroblasts or pCMV-ADF-transfected cells exposed to UVA radiation (2J/cm^2): with or without endo III. Yield of damage is represented by mean tail moment of 50 cells per slide ($n = 3$).
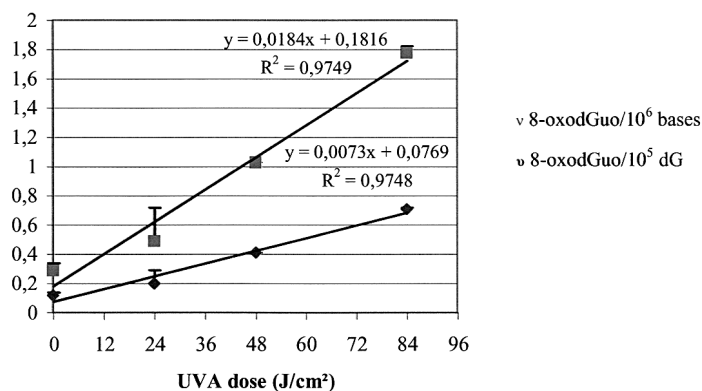
Fig. 5. Measurement of 8-oxodGuo by HPLC-EC in human skin fibroblasts exposed to UVA irradiation. Values represent the mean ± SD of three independent experiments.

involved in the damaging effects of UVA. In addition, the similar level of endo III-sensitive sites in irradiated and transfected fibroblasts (TM = 16.22 ± 2.87) indicates that Trx overexpression has no effect on the formation of oxidized pyrimidine bases.

Calibration

For calibration purpose [22], the HPLC-EC method was used to determine the level of 8-oxodGuo formed upon exposure of the cells to UVA radiation. The measurement of 8-oxodGuo in cellular DNA was achieved by HPLC-EC after enzymatic digestion of DNA to nucleosides. The level of 8-oxodGuo plotted as a function of the dose is shown in Fig. 5. No significant increase in the formation of 8-oxodGuo in the DNA of irradiated cells was observed for doses ranging from 0 to 24 J/cm^2. This could be accounted for by a high artifactual background of 8-oxodGuo as the result of spurious oxidation of guanine residues during the DNA extraction process [30]. This explains why doses over 25 J/cm^2 have to be delivered in order to obtain levels of 8-oxodGuo significantly higher than the background. The yield of UVA-induced 8-oxodGuo was determined to be about $0.0184/10^6$ bases/J/cm^2 within the range of doses comprised between 24 and 84 J/cm^2. This value is 2-fold higher than the yield of $0.0098/10^6$ base/J/cm^2 previously measured in THP1-myc [32].

It was first considered that UVA radiation-induced induction of 8-oxodGuo in human skin fibroblasts is linear within the dose range explored. Thus, the rate of formation of 8-oxodGuo measured for doses higher than 24 J/cm^2 was extrapolated to the low-dose region used for the comet assay (2 J/cm^2). The values in arbitrary units (au) of the tail moment were thus expressed as a function of 8-oxodGuo. It was also supposed that the Fpg-sensitive sites mostly consist of 8-oxodGuo resi-

dues, and that the excision of the modified base is quantitative. According to these assumptions, the formation of additional strand breaks formed per $J.cm^{-2}$ (about 4.94 au per J/cm^2) in the presence of Fpg could be correlated with the yield of 8-oxodGuo ($0.0184/10^6$ bases per joule). Consequently it was found that a value of one 8-oxodGuo/10^6 bases corresponds to 268.75 au of the tail moment. These results were compared with those obtained from pCMV-ADF-treated cells. In the latter cells, the formation of additional strand breaks in the presence of Fpg was 3.06 au per J/cm^2. This formation of additional strand breaks formed per joule could be correlated with the yield of 8-oxodGuo ($0.0114/10^6$ bases per joules/cm^2). It could be concluded that the 8-oxodGuo level induced by UVA irradiation is lower in transfected cells compared to control cells. Consistent with the scavenging capacity of ROS, we demonstrated the efficacy of Trx to prevent the induction of 8-oxodGuo by UVA irradiation.

DISCUSSION

The formation of oxidative DNA damage in cells is expected to depend on the rate of production of free radicals and other oxidants. Considering the central role played by DNA in cell viability, alterations of this macromolecule may be at the origin of the cytotoxic, mutagenic, and carcinogenetic effects of solar radiation including UVA radiation known to generate reactive oxygen species. Due to the long-term effects of unrepaired DNA lesions, it is important to better delineate the possible effects of cellular antioxidant status. Although the interaction of antioxidants and oxidative DNA damage is complex, it is reasonable to assume that antioxidants, like ascorbate, SH-containing compounds (glutathione, Trx) play a role in neutralizing free radicals and oxidants that cause DNA damage. Considering that

highly damaging hydroxyl radicals react rapidly with nearly all biological compounds and thus do not diffuse very far from their site of generation, a place of choice is assumed by intranuclear antioxidant. We demonstrated here that Trx accumulates into the nucleus following transient transfection and protects against the formation of oxidative DNA damage.

Trx was originally studied for its ability to act as a cofactor of ribonucleotide reductase, an enzyme essential for DNA synthesis [33]. It was shown that cells defective in Trx exhibit an increased chromosomal breakage, a characteristic of Fanconi anemia cells [17]. Human Trx was subsequently found to modulate the DNA binding of some transcription factors that regulate cell proliferation and DNA repair. This modulation was shown to be related to the redox properties of the protein. In order to shed light on the putative role of thioredoxin against the formation of oxidative DNA damage, cells were exposed to UVA radiation.

The standard comet assay, which allows the detection of the single and double strand breaks together with alkali-labile sites, was applied to cultured human skin fibroblasts. The assay has been previously demonstrated to detect damage following exposure to physiologically relevant range of UVA (0.5 to 2.0 J/cm^2) [29]. Using HPLC methods, induction of 8-oxodGuo was only observed when cells were exposed to over-physiological doses. More, the level of 8-oxodGuo in cellular DNA reported in the literature varies by greater than three orders of magnitude depending on the method used for its determination [23,34]. Basically, the problem is related at least partly to the fact that DNA extraction and subsequent work-up can result in artifactual oxidation of DNA bases mediated by intrinsic oxidants. The measurement of 8-oxodGuo in cellular DNA by the comet assay in association with the use of purified DNA repair enzymes, allows the determination of levels of damage that are 10-fold lower than by HPLC-EC [23,34]. The results were in accordance with those reported previously on γ-irradiated THP-1 [22]. The former assay constitutes an interesting approach for the measurement of UVA radiation-induced DNA damage in human skin fibroblasts. As suggested previously, we showed that UVA was able to induce the formation of 8-oxodGuo in human skin cells at physiological doses. Considering the high specificity of singlet oxygen to predominantly produce 8-oxodGuo [35], the modified comet assay allowed to rule out the contribution of Trx.

In the present study, the modified comet assay was used for evaluating the intracellular antioxidant effect of thioredoxin against the extent of oxidative DNA damage in human skin fibroblasts. We focused on the measurement of 8-oxodGuo level in control, Trx-treated cells and Trx-transfected cells. The results demonstrated that both

rh-Trx added in the medium and overexpression of Trx following transient transfection, strongly reduced the number of DNA strand breaks or alkali-labile sites in UVA-irradiated cells. However, only the Trx overexpression appeared to play a major role in preventing the UVA-induced formation of 8-oxodGuo (3.06 au per Joules/cm^2 compared to 4.94 au per Joules/cm^2 for nontransfected cells). We previously found that Trx translocated and accumulated into the nucleus following UVA irradiation (C. Didier et al., submitted for publication) [36]. Here, we observed that Trx accumulates into the nucleus in transfected cells. We previously reported the protective potential of thiols against UVA-induced oxidative damage to DNA [29]. The reducing environment created by Trx could explain the relative maintenance of the integrity of the genetic information [37]; indeed, endogenous Trx is kept in its redox active form by TR. Under these conditions, it will be interesting to evaluate the efficacy of selenium, an essential trace element for thioredoxin reductase (TR) activity [38]. The involvement of TR in biological functions such as cell growth and protection against oxidative stress has, to date, centred on its role as a reductant for Trx.

Similar results have been observed on human MCF-7 breast cancer cells stably transfected with human Trx cDNA both in terms of nuclear localization and genoprotection. As determined by immunofluorescent staining and confocal microscopy, the Trx-transfected cells showed increased levels of Trx. Trx-like fluorescent staining was principally observed in the nucleus. The comet assay was used to determine the TM in both stably transfected cells and vector-alone-transfected cells following UVA irradiation. The mean tail moment determined in stably transfected cells compared to vector-transfected cells was, respectively, TM = 11.49 ± 0.4 versus TM = 26.82 ± 1.45 for a dose of 20 J/cm^2 and TM = 19.69 ± 1.05 versus TM = 40.36 ± 0.75 after exposure to a dose of 60 J/cm^2.

Considering now exogenous rh Trx, which did not protect the cells against 8-oxodGuo generation, we postulate that only a small amount of this protein penetrates into the cells. Indeed, no specific receptor for Trx has been identified hitherto, nor has any active uptake of Trx over the plasma membrane been described [39,40]. The mechanism by which extracellular Trx acts is largely unknown, but Trx may promote the uptake of cystine into cells and upregulate the intracellular levels of glutathione [41]. Since cystine is necessary for glutathione synthesis, the genoprotection of exogenous Trx may be partly dependent on intracellular glutathione. An alternative hypothesis is that exogenous Trx may cooperate with related membrane-bound molecules such as TR [42], a hydroperoxide reductase by itself [43], and may

remove peroxides, or with thioredoxin peroxidase [44], that catalyzes the reduction of H_2O_2.

Assuming that oxidative DNA damage contributes to cancer, it should be possible to prevent the occurrence of the latter pathology by maintaining sufficiently high levels of intracellular thioredoxin. Unfortunately, it is difficult to consider such a treatment in photoprotection. The limiting factors for using such compounds in human are the degree of toxicity of the compounds and their ability to be absorbed. Experimentally, it will be interesting to investigate whether Trx derivatives can show similar photoprotection potential in human skin against the deleterious effects of UVA radiation.

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ABBREVIATIONS

ADF—Adult T cell leukemia-derived factor

ALS—alkali-labile site

BSA—bovine serum albumin

dGuo—2′-deoxyguanosine

DSB—double strand break

Endo III—*Escherichia coli* endonuclease III

FapyAde—4,6-diamino-5-formamidopyrimidine

FapyGua—2,6-diamino-4-hydroxy-5-formamidopyrimidine

FCS—fetal calf serum

Fpg—*Escherichia coli* formamidopyrimidine DNA *N*-glycosylase

H_2O_2—hydrogen peroxide

$O_2^{\bullet-}$—superoxide anion

$^1O_2$—singlet oxygen

8-oxodGuo—8-oxo-7,8-dihydro-2′-deoxyguanosine

PBS—phosphate-buffered saline

rh Trx—recombinant human thioredoxin

ROS—reactive oxygen species

SSB—single strand break

TM—tail moment

TR—thioredoxin reductase

Trx—thioredoxin

UVA—ultraviolet A radiation (320–400 nm)