Chemical oxidation and DNA damage catalysed by inorganic sunscreen ingredients

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Abstract Titanium dioxide (TiO_2) has been noted (US Federal Register, 43FR38206, 25 August 1978) to be a safe physical sunscreen because it reflects and scatters UVB and UVA in sunlight. However, TiO_2 absorbs about 70% of incident UV, and in aqueous environments this leads to the generation of hydroxyl radicals which can initiate oxidations. Using chemical methods, we show that all sunscreen TiO_2 samples tested catalyse the photo-oxidation of a representative organic substrate (phenol). We also show that sunlight-illuminated TiO_2 catalyses DNA damage both in vitro and in human cells. These results may be relevant to the overall effects of sunscreens.

Key words: Sunscreen; Titanium dioxide; Zinc oxide; DNA damage; Hydroxyl radical

1. Introduction

Titanium dioxide in sunscreens is formulated as 'micronised' or 'ultrafine' (20–50 nm) particles (so-called microreflectors) because they scatter light according to Rayleigh's law, whereby the intensity of scattered light is inversely proportional to the fourth power of the wavelength [1]. Consequently, they scatter UVB (290–320 nm) and UVA (320–400 nm) more than the longer, visible wavelengths, preventing sunburn whilst remaining invisible on the skin. However, TiO_2 also absorbs UV light efficiently, catalysing the formation of superoxide and hydroxyl radicals which can initiate oxidations [2]. The crystalline forms of TiO_2, anatase and rutile, are semiconductors with band gap energies of about 3.23 and 3.06 eV respectively [1], corresponding to light of about 385 nm and 400 nm (1 eV corresponds to 8066 cm^{-1}). Light at or below these wavelengths contains enough energy to promote electrons from the valence band (vb) to the conduction band (cb), generating single electrons and positively charged spaces called holes (h^+). After formation, electrons and holes either recombine or migrate rapidly (ca. 10^{-11} s) to the particle surface, where they react with adsorbed species. In aqueous environments, electrons react with oxygen, and holes with hydroxyl ions or water, forming superoxide and hydroxyl radicals:

$$TiO_2 + h\nu \rightarrow TiO_2(e^-/h^+) \rightarrow e^-(cb) + h^+(vb)$$

$$e^-(cb) + O_2 \rightarrow O_2^{\bullet -} \rightarrow HO_2^{\bullet}$$

$$h^+(vb) + OH^- \rightarrow {}^{\bullet}OH$$

This has been studied extensively in connection with total oxidation of environmental pollutants [3], especially with anatase, the more active form [4]. Such photo-oxidations may explain the toxicity of illuminated TiO_2 [5,6]. As TiO_2 can enter human cells [7], it is imperative to examine its possible consequences in detail, including effects on DNA. Although an evaluation of the safety of TiO_2 [8] concluded that it is not mutagenic and hence cannot damage DNA, it did not report on the effects of sunlight or on the particular preparations of TiO_2 that are used in sunscreens. This aspect is important because sunscreen TiO_2 particles are often coated with compounds (e.g. alumina, silica, zirconia) that form hydrated oxides which can capture hydroxyl radicals and may reduce photosensitivity [1]. However, some TiO_2/Al_2O_3 and TiO_2/SiO_2 preparations exhibit enhanced activity [9]. Here, we use chemical methods to examine photo-oxidations catalysed by TiO_2 from various sunscreens available in Europe and North America. We also test the ability of both illuminated TiO_2 and zinc oxide (ZnO), a sunscreen semiconductor with a band gap of 3.3 eV [10], to attack DNA.

2. Materials and methods

2.1. Chemical oxidation by titanium dioxide preparations

TiO_2 samples were extracted from over-the-counter sunscreens by washing with organic solvents (methyl cyanide, acetone, chloroform), and their anatase and rutile contents were determined by X-ray diffraction methods. Anatase and rutile standards were a gift from Tioxide Group Services Ltd., Grimsby, UK. TiO_2 concentrations were assayed according to the method of Codell [11] using standards made from pure TiO_2 (Aldrich); the molar extinction coefficient for the complex was assayed as 827 M^{-1} cm^{-1} at 404 nm. The photo-oxidative degradation of phenol by illuminated TiO_2 was monitored using high pressure liquid chromatography [12] to measure its disappearance, employing isocratic procedures at ambient temperature on a Waters 501 liquid chromatograph equipped with a Waters 441 detector set at 214 nm and a HP 3396A recorder. The column was a Waters μBONDAPAK C-18 reverse phase and the mobile phase was a 50:50 mixture of methanol (BDH Omnisolv grade) and distilled/deionised water. Each sunscreen TiO_2 was illuminated at 0.05% by weight in 58 ml of phenol (200 μM in air-equilibrated aqueous media, pH 5.5; retention time of phenol in the HPLC chromatogram was 5 min) using a 1000-W Hg/Xe lamp and a 365 nm (± 10 nm) interference filter, giving a light flux between 310 and 400 nm of ca. 32 mW cm^{-2}. Appropriate aliquots (1 ml) of the irradiated dispersion were taken at various intervals and filtered through a 0.1 μm membrane to remove the TiO_2 prior to analysis.

2.2. Illumination of DNA in vitro

The solar simulator [13] consists of a 250-W ozone-free lamp, a WG 320 filter and a quartz lens, resulting in an estimated fluence between

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300 and 400 nm of 12 W m^{-2}. DNA was the plasmid pBluescript II SK^+ (Stratagene) prepared and analysed on agarose gels according to Maniatis et al. [14]. Relaxed standards were made by depurinating plasmid in 25 mM sodium acetate pH 4.8 at 70°C for 20 min followed by cleaving with exonuclease III at 37°C [17] in 50 mM Tris-HCl, 5 mM $CaCl_2$ (the Ca^{2+} inhibits exonuclease but not cleavage at apurinic sites), 0.2 mM DTT, pH 8; linear standards by cutting with *Eco*RI. The authentic TiO_2 standards (confirmed by X-ray diffraction to be 100% anatase or 100% rutile) were suspended in water at 2% w/v; ZnO (Aldrich, < 1 μm) at 0.4% w/v. 25 μl of each were added to 25 μl of plasmid (2–3 μg of DNA) in 100 mM sodium phosphate pH 7.4 and illuminated as droplets (50 μl) on siliconised microscope slides placed on a brass block embedded in ice. A sunscreen containing only TiO_2 (7% w/v) was vortexed with water and centrifuged. The white pellet was washed 3 times with a mixture of chloroform and methanol (1:1), then with methanol alone, and dried. The powder was suspended in water at 2%, but most quickly settled out, leaving a cloudy supernatant with a TiO_2 content assayed at 0.025% w/v. This was mixed with an equal volume of plasmid DNA in buffer and illuminated. Direct strand breaks were assayed from the conversion of supercoiled plasmid to the relaxed form.

2.3. Illumination of DNA in vivo (comet assays)

Human cells (MRC-5 fibroblasts) were illuminated on ice with or without sunscreen TiO_2 (0.0125% w/v). The lens was omitted, giving an intensity similar to that found under the stratum corneum [13]. Samples were taken at increasing times, kept on ice, and analysed at the same time. For analysis, cells were embedded in low-melting agarose, lysed with 1% Triton X-100, subjected to alkaline gel electrophoresis and stained with ethidium bromide [15], and classified according to the five main standard classes [16].

3. Results and discussion

Oxidation of organic materials by hydroxyl radicals from illuminated TiO_2 can be examined conveniently by following the oxidation of a test molecule such as phenol [12,17]. Table 1 compares the oxidative degradation of phenol by TiO_2 samples from 10 different sunscreens with oxidation catalysed by pure rutile and pure anatase. All TiO_2 samples oxidise phenol, but activity does not depend solely on crystal type. The most active sample, SN10, also contains ZnO. However, as we do not know the precise composition of the samples (particle size, surface area per unit weight, presence/absence of coatings) we cannot assess the relative importance of these factors.

Hydroxyl radicals inflict direct strand breaks on DNA, and to test for such damage we illuminated supercoiled plasmids with simulated sunlight and TiO_2. Fig. 1 shows that plasmids

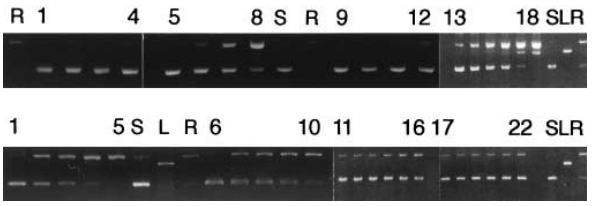

Fig. 1. Relaxation of plasmids caused by illuminated TiO_2 and ZnO and suppression by DMSO and mannitol. In both panels, S, L and R show the migration of supercoiled, linear and relaxed plasmid. Top panel: Plasmid relaxation found after illumination with sunlight alone for 0, 20, 40 and 60 min (lanes 1–4) and with 1% anatase (lanes 5–8) or 1% rutile (lanes 9–12) TiO_2 for the same times. Lanes 13–18: illumination with TiO_2 from sunscreen SN8 for 0, 5, 10, 20, 40 and 60 min. The results are typical of those found with various samples. Bottom panel: Illumination with 0.2% ZnO for 0, 10, 20, 40 and 60 min before (lanes 1–5) or after (lanes 6–10) adding DMSO; and with 0.0125% sunscreen TiO_2 for 0, 5, 10, 20, 40 and 60 min after adding 200 mM DMSO (lanes 11–16) or 340 mM mannitol (lanes 17–22).

are converted first to the relaxed form and ultimately to the linear form, demonstrating strand breakage. Sunlight alone has very little effect, while anatase is more active than rutile, consistent with photochemical comparisons (Table 1 and [4]). TiO_2 extracted from a sunscreen is also photo-active, and so is pure ZnO. The sunscreen illuminations contain much less TiO_2 than the anatase and rutile ones, suggesting that the sunscreen variety is especially active. Damage is suppressed by the quenchers [18] dimethylsulphoxide (DMSO) and mannitol, strongly suggesting that it is indeed caused by hydroxyl radicals.

Fig. 2 shows (top panel) that damage is very slightly suppressed by catalase, but also (bottom panel) that heat-inactivated catalase and bovine serum albumin have similar effects, suggesting that this limited quenching is due to the protein present rather than to catalase activity. Superoxide dismutase did not suppress the damage either (data not shown). It appears therefore that the strand breaks are not caused by superoxide ($O_2^{\bullet-}$), an active oxygen species formed by reaction between e^-(cb) and O_2 (Section 1), and do not depend upon the intermediate formation of hydrogen peroxide by reaction between 2 $^{\bullet}OH$ radicals. Rather, they appear to be due to direct attack by hydroxyl radicals, which is consistent with indications that hydroxyl radicals formed on TiO_2 remain

Table 1
Photodegradation of phenol by TiO_2 samples

Sample	Anatase/rutile ratio (%)	Phenol photodegradation (mmol h^{-1})	Relative rate
SN1[a]	50/50	0.008 ± 0.016	1.0
SN2	0/100	0.023 ± 0.008	2.8
SN3	0/100	0.043 ± 0.010	5.2
SN4	54/46	0.043 ± 0.007	5.2
SN5	0/100	0.086 ± 0.015	10.4
SN6	100/0	0.146 ± 0.014	17.6
SN7[a]	0/100	0.189 ± 0.008	22.7
SN8	100/0	0.44 ± 0.11	53.3
SN9	63/37	1.11 ± 0.03	134
SN10[b]	0/100	1.50 ± 0.04	180
Pure rutile	0/100	3.55 ± 0.12	427
Pure anatase	100/0	31.6 ± 0.8	3803

SN1–SN10 are over-the-counter sunscreens.
[a]Also contains $Al(OH)_3$.
[b]Also contains 1.95% ZnO.

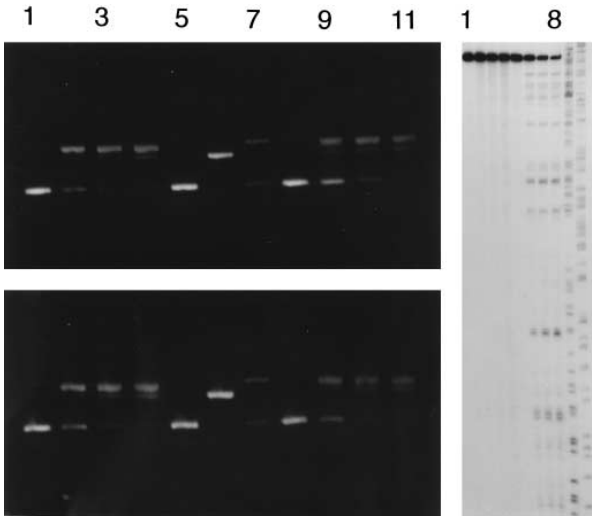

Fig. 2. Effect of catalase on damage inflicted by illuminated TiO_2 and location of lesions in DNA. Top panel: Plasmid DNA was illuminated (see Fig. 1) with sunscreen TiO_2 alone for 0, 20, 40 and 60 min (lanes 1–4) and for the same times (lanes 8–11) after adding 2.5 units/μl of catalase (0.1 mg/ml of protein). Lanes 5–7 show supercoiled, linear and relaxed plasmid. Bottom panel: Illumination with sunscreen TiO_2 as above after adding boiled catalase (lanes 1–4) or 0.1 mg/ml of bovine serum albumin (lanes 8–11). Right panel: A 426 bp fragment of double-stranded DNA labelled at one 5′-end was illuminated in 0.0125% sunscreen TiO_2 and samples were analysed on a sequencing gel. Lanes 1–4: illumination for 0, 20, 40 and 60 min. Lanes 5–8: illumination for the same times followed by treatment with *N,N′*-dimethylethylenediamine for 30 min at 90°C before analysis. This reagent displaces many damaged residues from DNA and then cleaves the sugar-phosphate chain, leaving homogeneous, phosphorylated termini with consistent mobility, thus clarifying the spectrum of lesions generated [19]. Lanes 9–10: G and A dideoxy sequencing standards.

on the surface of the particles [20]. By cleaving end-labelled DNA, other lesions were revealed (right panel), principally at some, but not all, guanine residues. Evidently, DNA damage is not confined to strand breaks.

Comet assays (Fig. 3) show that DNA in human cells is also damaged by illuminated TiO_2, consistent with endocytosis of TiO_2 [7]. Suppression by DMSO again implies that the damage is caused by hydroxyl radicals. These assays detect direct strand breaks and alkali-labile sites, and reveal the damage attributable to TiO_2.

Our results demonstrate that sunscreen TiO_2 and ZnO can catalyse oxidative damage to DNA in vitro and in cultured human fibroblasts. The fate of these materials applied to skin is uncertain. Autoradiographic studies using ^{65}ZnO suggest that it passes through rat [21] and rabbit [22] skin, probably through hair follicles, although the chemical form of the ^{65}Zn detected under the skin (and hence of the form that crosses the skin) is not clear. Some reports raise the possibility that ZnO [23] and pigmentary TiO_2 [24,25] pass though human skin, and a recent one suggests that micronised TiO_2 in sunscreens does too [26], although more systematic studies are clearly needed. It is important to characterise the fate and photochemical behaviour of sunscreens, which certainly prevent sunburn, because they are also intended to reduce skin cancers, which have increased rapidly recently [27,28]. While they can reduce the formation of cyclobutane dimers in DNA [29], which are induced by direct absorption of UVB, the ability of TiO_2 and at least one organic sunscreen to form reactive species such as hydroxyl radicals which can inflict other forms of damage [13,30] highlights the importance of investigating this aspect too.

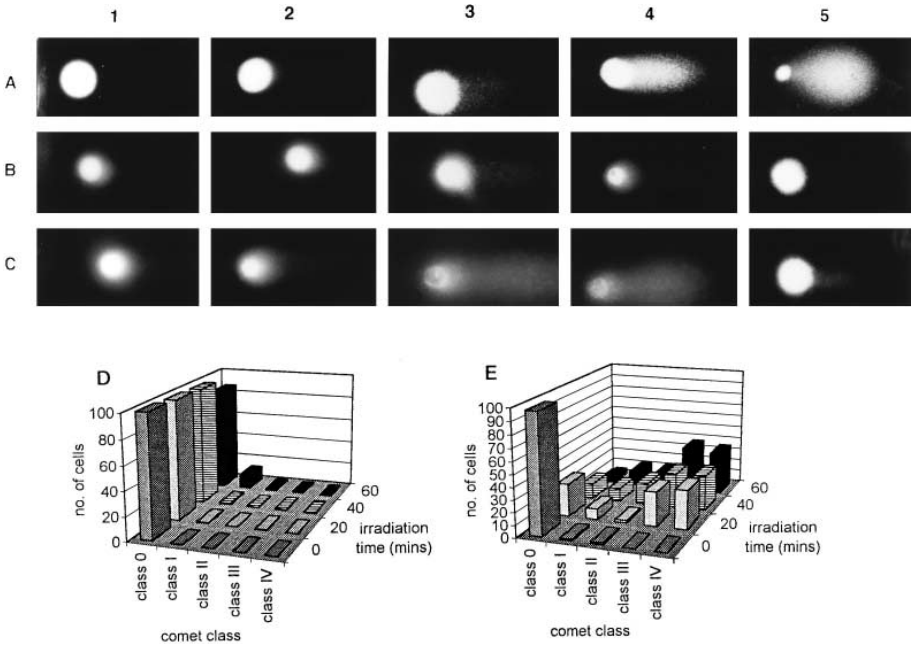

Fig. 3. Damage inflicted on human cells revealed by comet assays. Comet assays were performed as in Section 2. Row A: comets obtained using X-rays from a Gavitron RX30 source. The dose rate was 8.9 Gy min^{-1} and cells were exposed on ice for 0, 15, 30 and 60 s, giving comets falling into the five main standard classes [16] shown. 1, class 0; 2, class I; 3, class II; 4, class III; 5, class IV. Rows B and C: examples of comets obtained using simulated sunlight, MRC-5 fibroblasts and sunscreen TiO_2 (0.0125%). For each exposure, 100 cells were scored, and comets were classified by comparison with the standards (row A). Row B: no treatment (1); sunlight alone for 20, 40 and 60 min (2–4); and effect of TiO_2 in the dark for 60 min (5). Row C: sunlight with TiO_2 for 0, 20, 40 and 60 min (1–4); and for 60 min with TiO_2 and 200 mM DMSO (5). The charts summarise results from five independent experiments. D shows that sunlight alone inflicts few strand breaks and/or alkali-labile sites and E that inclusion of TiO_2 catalyses this damage.

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