C_3-Fullero-*tris*-methanodicarboxylic acid protects epithelial cells from radiation-induced anoikia by influencing cell adhesion ability

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Abstract Anoikia is a type of apoptotic cell death that occurs in cells that are substrate-restricted in their growth. Buckminsterfullerenes represent a new class of chemical compounds with wide potential pharmacological antioxidant activity. In this report we provide the first demonstration that a water-soluble fullerene derivative, C_3-fullero-*tris*-methanodicarboxylic acid, synthesized in our laboratories, is capable of inducing anoikia resistance in epithelial cells by a mechanism involving a 'trophic' effect on cell spreading-associated cytoskeletal components, i.e. on actin microfilaments.

Key words: Epithelial cell; Ultraviolet B; Anoikia; Fullerene; Cytoskeleton

1. Introduction

During recent years a large body of evidence has accumulated to suggest that oxidative stress may play a role as a common mediator of apoptosis [1]. It is conceivable that ultraviolet (UV)-induced reactions represent oxidative stress mediated by the formation of free radicals, reactive oxygen, lipid peroxidation, liberation of membrane phospholipids, and subsequent formation of prostaglandins by the cyclo-oxygenase pathway. In particular, UVB radiation generates superoxide anion radicals, intracellular hydrogen peroxide, and hydroxyl radicals [2]. We have previously demonstrated that UVB (1200 J/m^2) induces in A431 epithelial cells cytoskeleton-dependent surface blebbing and the type of apoptosis called anoikia [3,4]. This is an apoptotic process due to the loss of both homotypic and/or heterotypic cell interactions leading to an 'homeless' condition (anoikia) causing cell death [5]. Buckminsterfullerenes and their organofunctionalized derivatives have recently become a topic of interest in biochemistry and medicinal chemistry. In particular, two regioisomers of fullero-*tris*-methanodicarboxylic acid (F-*tris*-MDC) with C_3 and D_3 symmetry have been shown to be potent scavengers of hydroxyl radical (OH) and superoxide anion [6]. In the present study, we have investigated the possible role of C_3-fullero-*tris*-methanodicarboxylic acid (C_3-F-*tris*-MDC) as a defense against the harmful effects of UVB radiation. Recent investigations regarding their biological activity have revealed that fullerene derivatives exhibit several types of subcellular effects both in vitro and in vivo. Because of the hypothesized role of free radicals in UVB-induced cell damage, the aim of our study was to determine whether C_3-F-*tris*-MDC could be effective in protecting epithelial cells from radiation-induced injury, i.e. inhibiting (i) bleb formation, (ii) cell detachment and (iii) apoptosis.

2. Materials and methods

2.1. Cell cultures

A431 cells, a human epidermoid carcinoma cell line, were grown as monolayers in RPMI supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, UK), 1% non-essential amino acids, 5 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37°C in 5% CO_2/95% air. For light microscopy and scanning electron microscopy (SEM), controls and treated cells were seeded on 13 mm diameter glass coverslips in separate wells (50×10^3 cells/well).

2.2. UVB exposure

For UVB irradiation (1200 J/m^2) the dishes were placed without covers 10 cm under a Philips TL 20 W/12 lamp. In order to eliminate UV radiation not in the UVB range, a Kodak filter (type Kodacell TL-401) was used as described [3]. In these conditions the UVB radiant flux density to the cells was 2.2 W/m^2, as measured with an Osram Centra radiometer.

2.3. Synthesis of C_3-F-tris-MDC

C_3-F-*tris*-MDC (UPF) was prepared by a methodology which represents an improvement in terms of number of steps and overall yield with respect to previously reported procedures [6,7]. Accordingly, a solution of fullerene-C_{60} (1.0 g, 1.388 mmol) and dry diethyl bromomalonate (0.92 g, 3.87 mmol) in toluene was magnetically stirred for 15 h in an argon atmosphere and at room temperature in the presence of dry 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 0.59 g, 3.87 mmol). The reaction mixture was then evaporated and the brownish residue subjected to flash chromatography by means of the Biotage Flash 40 system (Biotage UK, Ltd., Flash 40M, KP-SIL cartridges) [8]. Elution with toluene-hexane (8:2) to toluene afforded a fraction (0.50 g) enriched in the tris adduct [*trans-3,trans-3,trans-3*-$C_{63}(CO_2Et)_6$] with D_3 symmetry. Following elution with toluene-acetonitrile (99.5:0.5) gave a fraction (0.49 g) enriched in the regioisomeric tris adduct (*e,e,e*-$C_{63}(CO_2Et)_6$] with C_3 symmetry which was further purified by flash chromatography using toluene-acetonitrile (99.9:0.1) thus obtaining pure C_3 adduct (0.250 g) in 15.5% yield. Alkaline hydrolysis of the C_3 adduct was performed with sodium hydride in toluene (30 min, room temperature). Methanol was added to the cooled (0°C) reaction mixture and stirring was continued for 45 min at room temperature. The red-orange precipitate thus formed was collected by centrifugation and washed with toluene and hexane. An equimolar amount of

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4 M sulfuric acid was then added to the residue dissolved in water and the resulting solution was concentrated in vacuo and re-dissolved in methanol. Filtration and evaporation of the solvent afforded pure C_3-F-*tris*-MDC (0.150 g) in 70% yield.

2.4. Cell treatments

In order to verify if C_3-F-*tris*-MDC was capable of protecting the cells from UVB damage, different concentrations were used (1, 10, 100, 300 and 500 μM). Since low concentrations (1, 10 μm) were ineffective (data not shown), only the results observed with the lowest dose able to exert a significant biological activity (100 μM final concentration) will be reported. D_3-F-*tris*-MDC was dissolved in RPMI and added to the cells following two different protocols: (1) incubation (24 h) of the cells in the presence of C_3-F-*tris*-MDC followed by washing in phosphate buffered saline (PBS) and exposure to UVB in PBS. Immediately after irradiation, PBS was replaced by complete medium; (2) cells were exposed to UVB in PBS with the compound. Immediately after irradiation the PBS with the compound was removed and the cells were incubated in medium without C_3-F-*tris*-MDC.

2.5. Evaluation of apoptosis

For apoptosis evaluation cells attached to the substrate were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature; detached cells were collected by centrifugation and re-suspended in PBS, seeded on polylysine-coated coverslips for 15 min and fixed with formaldehyde as above. After washing in PBS, cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min at room temperature. Cells were then incubated with Hoechst 33258 chromatin dye (Sigma) for 30 min at 37°C as previously described [9]. After washing, all samples were mounted with glycerol-PBS (2:1) and observed with a Nikon Microphot fluorescence microscope. Quantitative evaluation of apoptotic cells was performed by counting 500 cells at high magnification (550×) [10]. Student's *t*-test was used to evaluate statistical differences ($P < 0.01$ was considered significant).

2.6. Scanning electron microscopy (SEM)

Control and treated cells were washed in PBS and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.4) containing 3% (w/v) sucrose at room temperature for 20 min. Following post-fixation in osmium tetroxide for 30 min, cells were dehydrated though graded ethanol solutions, critical point-dried in CO_2 and gold-coated by sputtering with a Balzers Union SCD 040 apparatus. The samples were then examined with a Cambridge 360 scanning electron microscope.

2.7. Immunocytochemical analysis

For F-actin evaluation controls and treated A431 cells were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 30 min at room temperature. After washing in the same buffer, cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min at room temperature. Cells were stained with fluorescein-phalloidin (Sigma) at 37°C for 30 min. For the detection of focal adhesion kinase (FAK), control and treated cells were fixed in 70% ice-cold methanol, washed twice with cold PBS and stained with anti-FAK monoclonal antibodies (Upstate) at a final concentration of 0.1 mg/ml for 30 min at 4°C. After washing with cold PBS the cells were incubated with anti-rabbit IgG fluorescein-linked whole antibody (Sigma) at 4°C for 30 min. Finally, all the samples were mounted with glycerol-PBS (2:1) and observed with a Nikon Microphot fluorescence microscope.

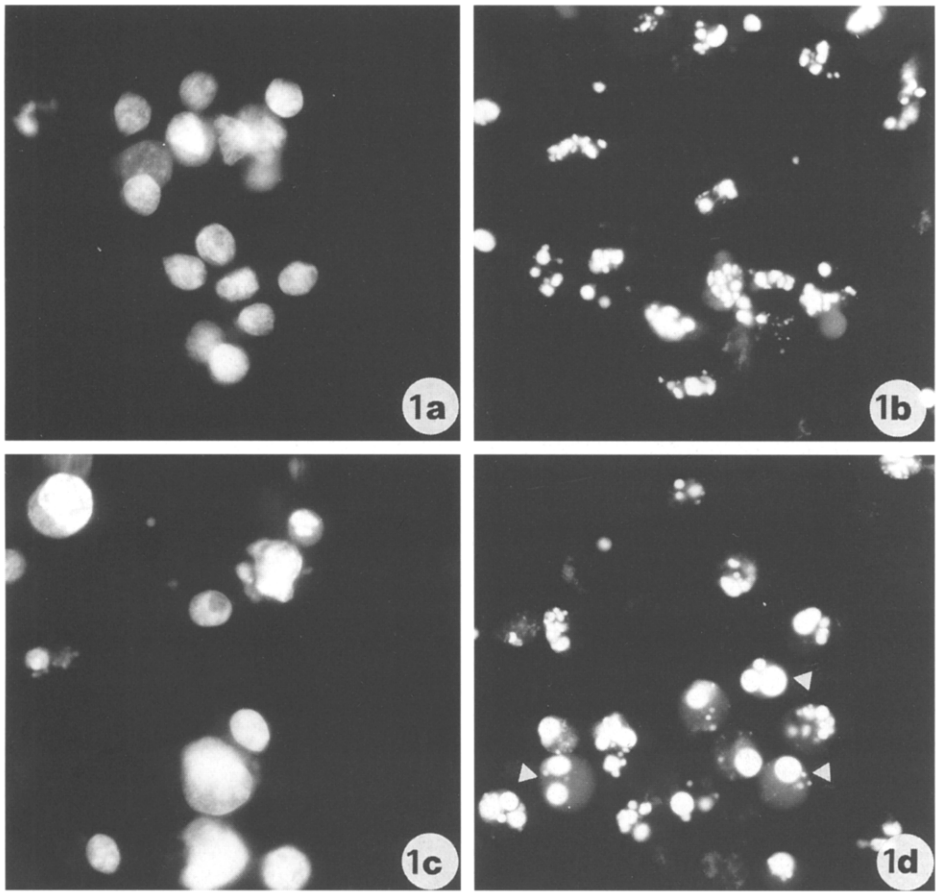

Fig. 1. Apoptotic evaluation. Morphological features of detached cells after nuclear staining with Hoechst dye. Normal nuclear appearance of control cells is shown in panel a. Exposure to UVB radiation resulted in the formation of chromatin clumps typical of apoptosis (b). The presence of C_3-F-*tris*-MDC during UVB exposure inhibited nuclear fragmentation (c). By contrast, pre-exposure to the drug was ineffective in protecting the cells from apoptosis (d). However, morphological features of nuclear fragmentation appeared to be affected: aggregated and condensed nuclear matrix, without clumping, was in fact often detected (arrows). Magnification, 900×.

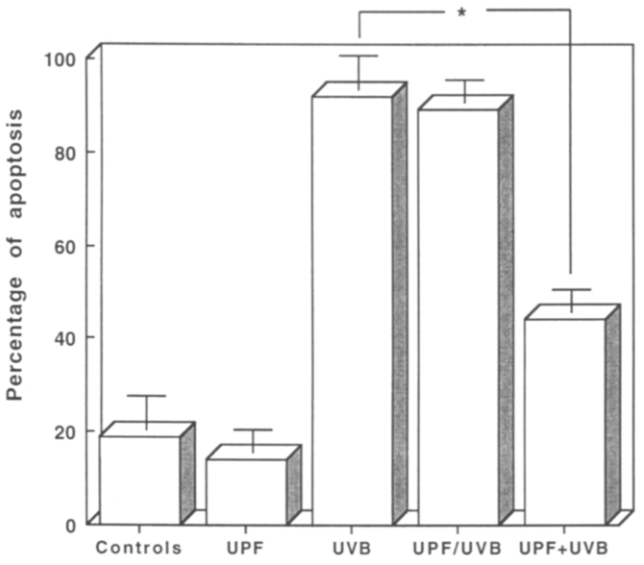

Fig. 2. Percentages of apoptosis in detached cells. A significant protection (*) was detected when the cells were exposed to UVB in the presence of C_3-F-*tris*-MDC (UPF+UVB). Pre-exposure to the drug (UPF/UVB) was ineffective.

3. Results

3.1. C_3-F-tris-MDC is capable of significantly protecting cells from UV radiation-induced detachment and apoptosis

In consideration of the lesions induced by UV radiation [3], specific studies were carried out in order to analyze injured and/or dying cells. While very few apoptotic cells were detected in control samples (1–2%), the percentage of apoptotic cells after radiation exposure was significantly higher ($42 \pm 3\%$). Treatment with C_3-F-*tris*-MDC during radiation exposure significantly hindered the apoptotic process ($19 \pm 3\%$ of apoptotic cells) while pre-treatment with the drug as stated in Section 2 was ineffective ($40 \pm 4\%$ apoptotic cells). However, interesting results came from the observation that apoptotic cells were found flowing freely in the supernatants while no sign of apoptosis was detected in adhering cells. In fact, in supernatants from control samples, very few apoptotic nuclei were detected (Fig. 1a). Several nuclei with fragmented chromatin were instead found after exposure to UVB alone (Fig. 1b). The presence of C_3-F-*tris*-MDC during UVB exposure appeared to significantly hinder the apoptotic process ($\Delta = -47\%$) and nuclear chromatin appears to be normally shaped (Fig. 1c). By contrast, exposure to C_3-F-*tris*-MDC before radiation did not protect the cells from apoptosis (Fig. 1d). These results on detached cells were also quantitatively evaluated as stated in Section 2 and the results ob-

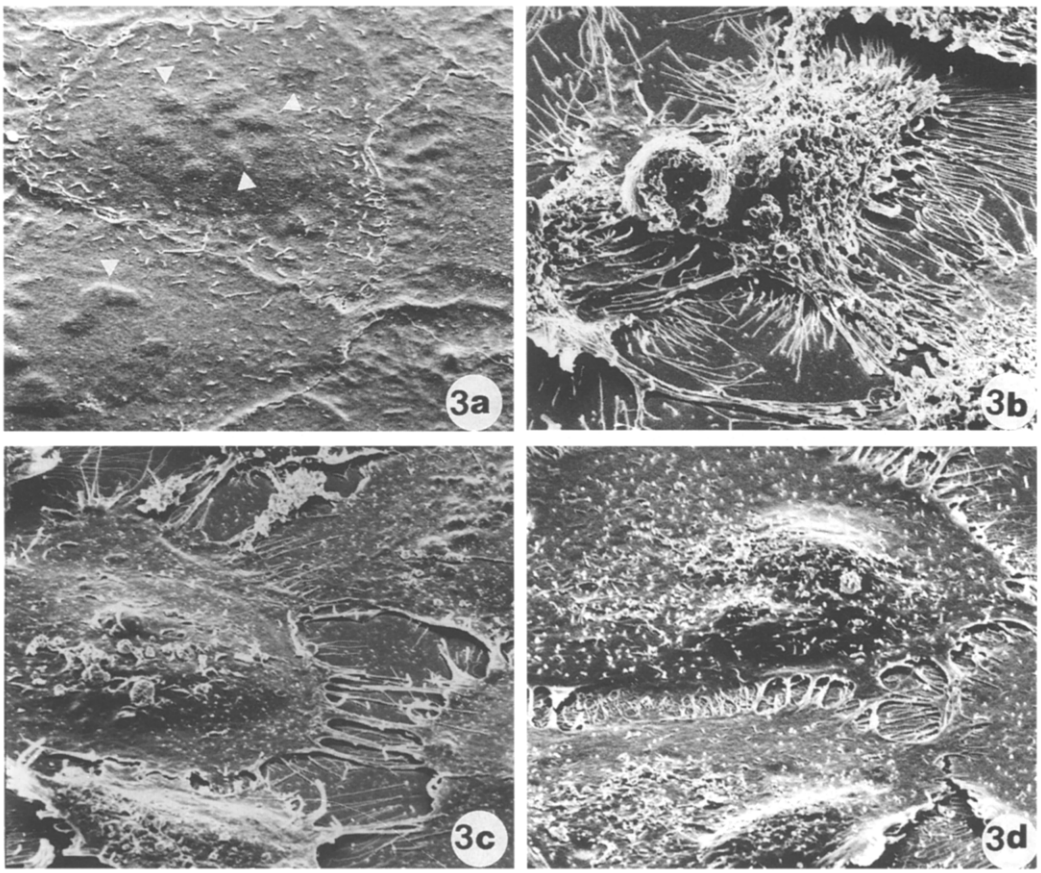

Fig. 3. SEM analyses showed that A431 epithelial cells appeared as extremely flat, polygonal cells well adhering to the substrate (a). Note small surface bulbs probably corresponding to underlying cell organelles (arrows). UVB radiation induced cell retraction and rounding with the formation of large surface blebs (b). Exposure to C_3-F-*tris*-MDC partially protects the cells from rounding, blebbing and from changes of cell-to-cell interaction. Pre-exposure to C_3-F-*tris*-MDC before radiation (c) was, however, less effective than the presence of the drug during UVB exposure (d). Magnification: 1125×.

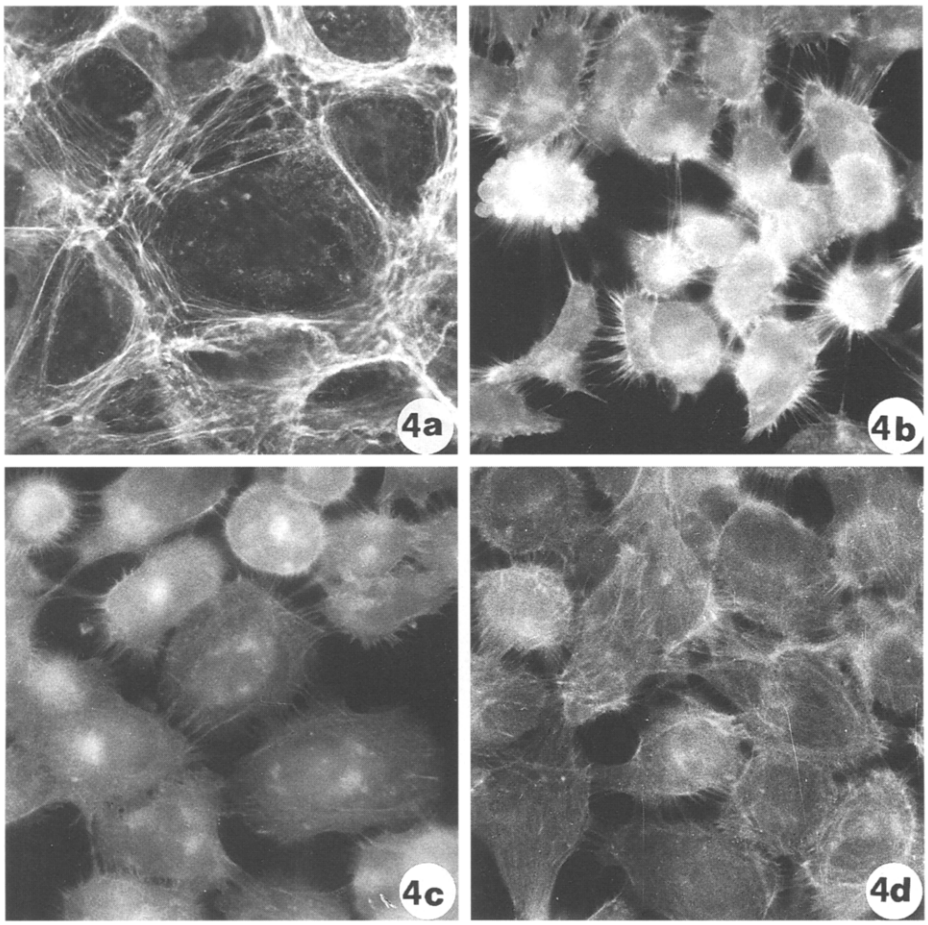

Fig. 4. F-actin microfilament organization as appeared in control cells (a) was completely subverted by radiation exposure (b). Pre-exposure to C_3-F-*tris*-MDC exerted a partial protective activity (c) while the presence of the drug during radiation was capable of powerfully protecting the cytoskeleton network from radiation-induced changes (d). Magnification: 900×.

tained are shown in Fig. 2. Accordingly, a significant positive correlation ($P < 0.01$) was found between cell detachment and apoptotic induction. In particular, exposure of A431 cells to UVB radiation induced cell detachment (60% detached cells after 24 h), while the presence of the fullerene derivative during UVB exposure reduced the percentage of detached cells (15%). Pre-exposure to C_3-F-*tris*-MDC, although not able to protect the cells from apoptosis (Fig. 2), induced an increased percentage of cells remaining firmly attached to the substrate after UVB exposure (30% detached cells).

3.2. *The protection from cell damage and death exerted by C_3-F-tris-MDC is accompanied by protection from the UVB-induced cell surface alterations*

In order to evaluate the activity of C_3-F-*tris*-MDC in hindering UVB-induced cell damage and apoptosis, surface features associated with both homotypic and heterotypic contacts were considered. A431 cells were then studied by SEM (Fig. 3). Control epidermoid cells appear to be perfectly flat, polygonal and with intimate cell-to-cell contacts (Fig. 3a). Cell rounding and the formation of many surface protrusions or blebs, a well-known marker of oxidative cellular damage and apoptosis, were found after UVB treatment (Fig. 3b). Exposure with C_3-F-*tris*-MDC seemed to prevent the UVB-induced cell surface damage. In these conditions, cell surface morphology as well as homotypic and heterotypic interactions appeared similar to those of control cells (Fig. 3c). Furthermore, the presence of the fullerene derivative during UVB exposure resulted in a very pronounced inhibition not only of cell detachment, but also of cell retraction and rounding (Fig. 3d).

3.3. *Immunocytochemical analyses*

In consideration of the importance of cytoskeletal elements in cell adhesion [4,11], a specific analysis was performed on F-actin microfilaments and on a cytoskeleton-related intracellular adhesion plaque-associated molecule, i.e. the FAK. Fig. 4a shows the normal appearance of F-actin filaments. A cortical boundary network of well-organized actin filaments was detected. The exposure to UVB radiation induced a remarkable alteration of actin filament organization (Fig. 4b). Pre-treatment with C_3-F-*tris*-MDC was ineffective in protecting the cytoskeleton network from radiation-induced damage. In fact, in A431 cells treated with this compound and subsequently exposed to UVB radiation, actin filaments appeared markedly altered (Fig. 4c). By contrast, the presence of C_3-F-*Tris*-MDC during UVB exposure seemed to be essential for the maintenance of actin filament distribution. In particular, radiation-induced cytoskeleton injury was prevented (Fig. 4d). Exposure to C_3-F-*tris*-MDC alone did not modify the actin network (not shown).

Cell adhesion also depends on the adhesion plaques, which are cytoskeleton-associated structures [12] also involved in cell

'homing' processes. We thus decided to evaluate the expression of FAK, an important molecule involved in cell attachment [13]. Fig. 5 shows that compared to control cells, in which the positivity appears scattered in the cell cytoplasm (Fig. 5a), radiation exposure resulted in a decreased positivity of FAK molecule which appears as small dot spots occasionally visible in the cytoplasm of retracted and roundish cells (Fig. 5c). C_3-F-*tris*-MDC induced a markedly increased expression and rearrangement of such molecule (Fig. 5b). The exposure to the fullerene derivative during radiation led to the partial maintenance of FAK molecule expression and arrangement in the cytoplasmic adhesion plaques (Fig. 5d).

4. Discussion

The present results demonstrate for the first time that C_3-F-*tris*-MDC, a water-soluble fullerene C_{60} derivative, can 'protect' cells from intracytoplasmic and/or membrane alterations induced by UVB which eventually lead to cell injury and, in a fraction of cells, to the apoptotic program for death. After UVB radiation, a specific series of events at the subcellular level resulting in cell rounding, surface blebbing, cell detachment from the substrate and, finally, cell death was described by various research groups including ours [3,14]. This could be ascribed to the specific role of reactive oxygen species in DNA damage as well as in metabolic changes associated with cell damage and apoptosis. The mechanism underlying this phenomenon could in fact be associated with an oxidative modification of membrane lipids and of cytoskeletal proteins, i.e. an impairment of cytoskeletal network plasticity and function. The capacity of C_3-F-*tris*-MDC to 'protect' A431 cells from UVB-mediated oxidative damage could thus be associ-

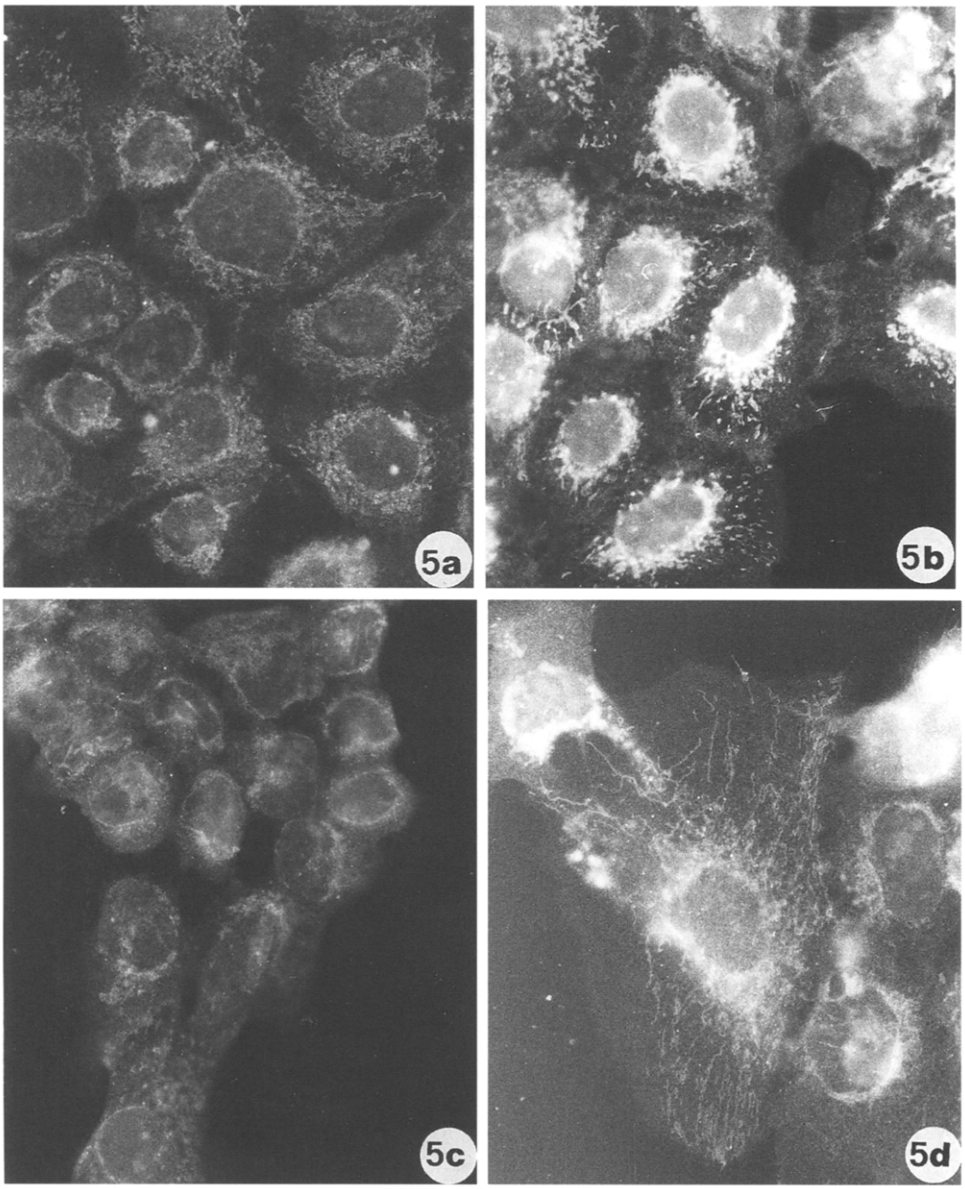

Fig. 5. Immunocytochemical analysis of FAK distribution indicated that normal distribution of this molecule, which appeared scattered in the cytoplasm of adhering cells (a), was markedly altered after radiation (c). The exposure to C_3-F-*tris*-MDC alone resulted in an increased positivity and rearrangement of this antigen (b). Cells exposed to radiation in the presence of the drug partially maintained the FAK molecule distribution (d). Note cell flattening in panel d and that all pictures are at the same magnification (900×).

ated with the ability of this drug to behave as an antioxidant compound specifically contributing to the maintenance of the cytoskeletal component network and to the integrity of focal adhesion plaques. This capability seemed to be mainly exerted only when the fullerene derivative was given during UVB exposure. This could be due to the ability of the drug to react with superoxide radical and with hydroxyl radical. Because the half-life of OH is extremely short (10^{-9} s) it may be difficult for compounds that are only capable of scavenging OH to achieve a sufficient concentration near the site of OH generation to out-compete endogenous targets of OH attack (lipids, proteins, DNA, and other macromolecules).

It may be that the ability of carboxyfullerenes to eliminate $O^{\bullet}$ before its conversion to $OH^{\bullet}$ is an important aspect of their biologically relevant antioxidant properties [6]. By contrast, cell pre-loading with the drug appears to be ineffective. This can indicate that, as suggested for other anti-oxidizing drugs [15], no alteration or improvement of cell anti-oxidizing machinery is induced by fullerenes but a direct activity might be hypothesized. In particular, the localization of the fullerene derivative into cell membranes could explain its role as a 'free radical sponge' [16,17] especially for superoxide anion and hydroxyl radicals generated by radiation. Our results indicate that C_3-F-*tris*-MDC, besides having a protective effect against some oxidative intracellular lesions, i.e. cell detachment and surface blebbing, is also capable, per se, of impairing apoptotic processes triggered by UVB radiation. Thus, C_3-F-*tris*-MDC could be considered an agent able to interfere with (or protect from) those alterations of cell homeostasis which are due to oxidative damage and lead to the type of cell death called anoikia, the homeless condition in which adhering cells lacking homotypic and heterotypic contacts are driven towards their removal from tissues.

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