Modulation of γ-Irradiation Induced ROS Responses by Engineered Fullerenes in Human Epidermal Keratinocytes

Jun Gao*, Hsing Lin Wang*, Andrew P. Shreve and Rashi Iyer* [1]
*Bioscience Division, Los Alamos National Laboratory
TA 43, MS888, Los Alamos, New Mexico, 87544, rashi@lanl.gov
[1] Corresponding author

ABSTRACT

High-dose ionizing irradiations can cause acute tissue and organ damage, especially severe cutaneous lesions. These cutaneous injuries often result in further complications and incur high mortality. Therefore, it is critical to develop effective therapeutic approaches for acute high-dose ionizing irradiation injury. Intracellular reactive oxygen species (ROS) have been implicated in mediating irradiation-induced cell damage. In the present study, we investigated the protective effect of engineered fullerene nanoparticles on γ irradiation-induced intracellular ROS levels in human epidermal keratinocytes (HEK). Cells were exposed to water-soluble fullerene derivatives at concentrations of 25, 50, and 100 μg/ml. Exposure of HEK cells to high-dose gamma (γ) irradiation (10 and 17 Gy) resulted in a significant increase in cellular ROS levels. The observed γ-irradiation induced ROS response was notably attenuated by modified-fullerenes, CD-C_{60}, tris-C_{60} and hexa-C_{60}. Our results point to the potential use of fullerene derivatives as novel therapeutic agents for protection against high-dose irradiation generated ROS-induced cellular damage.

Keywords: fullerene, reactive oxygen species, keratinocyte, irradiation exposure and nanotechology.

1 INTRODUCTION

Response and injury of the cutaneous system are the main determinants of mortality on exposure to high-dose ionizing irradiation [1]. Ionizing irradiation hazards are present universally, such as ultraviolet radiation, radiation therapy, accidental or intentional radiation, including terrorism and nuclear power workers [2, 3]. Exposure to moderate (1~10 Gy) to high doses (10~20 Gy) of ionizing irradiation has the potential to cause life threatening cutaneous injuries. Furthermore, cutaneous insults are frequently combined with mechanical, chemical, and thermal cutaneous trauma. Currently, there are few, if any mitigation strategies available to treat irradiation-induced cutaneous injuries [4, 5]. There is an urgent need for identification of biological targets and the development of new radioprotective agents to protect or treat irradiation-induced cutaneous injury.

Skin epidermis is a first line of defense against pathogens and other environmental insults, including ionizing irradiation. Keratinocytes are the major cell type in human epidermis (>90%) and are the primary responders to cutaneous insults. Studies have shown that reactive oxygen species (ROS) act as mediators of irradiation-induced cell damage [6]. Though ROS is necessary for important cellular functions, excessive production of ROS can result in severe and acute cellular damage. Thus, decreasing intracellular ROS levels may play a dominate role in radio-protection.

Fullerenes are carbon based nanoparticles with a well defined structure and geometry. The best known of these molecules is the Carbon-60 Buckminster-fullerene. Fullerenes can be modified to become water-soluble, suggesting they potential use as diagnostic or therapeutic agents in medicine [7]. The type and placement of extra-structural substituents widely impacts the chemical and physical properties of the functionalized fullerene. Typically the more substituents added the higher the redox potential. Recent studies with C_{60}-based water-soluble derivatives have suggested a free radical modulation function for these engineered nanoparticles [manuscript submitted and 8]. Moreover, Dugan et al. have demonstrated that the anti-apoptotic activity of functionalized fullerenes is due to their ability to act as ROS 'sponges' [9, 10] . Therefore, in the current study, we investigated the ability of three water-soluble fullerene derivatives, CD-C_{60}, tris-C_{60} and hexa-C_{60} (Figure 1), to modulate irradiation-induced ROS generation in HEK cells.

2 MATERIALS AND METHODS

2.1 Preparation of fullerene derivatives

Water-soluble carboxyfullerenes adducts substituted with a total of 12 or 6 carboxyl groups, and γ-cyclodextrin-C_{60} fullerene molecules (Figure 1) were synthesized for this study [11]. The carboxyl groups were affixed as hexa- and tris-substituted malonic acid adducts, and for convenience we refer to the 12-carboxylate species as hexa-C_{60} and the 6-carboxylate species as the tris-C_{60}. We also prepared a γ-cyclodextrin-C_{60} fullerene complex (referred to as CD-C_{60}), which renders the otherwise insoluble C_{60} into a water-soluble fullerene. Purity of products was verified by C^{13} NMR.

2.2 Cell culture

Human epidermal keratinocyte (HEK) cells were grown in defined keratinocyte serum free medium (SFM) supplemented with 1ml Defined Keratinocyte-SFM growth

supplement (Invitrogen, Carlsbad, CA) in 37 ˚C incubator in a humidified atmosphere and 5% CO_2.

2.3 Irradiation exposure and fullerene derivatives treatment

HEK cells were trypsinized and incubated in flat-bottom 96-well (2×10^4 cells/well) cell culture plates. Cells were allowed to recover for 24 hrs, and then exposed to γ-ionizing irradiation at doses of 10 and 17 Gy using an MK-1-68A Cs-137 sealed source irradiator at 385 cGy/min (J. L. Shepard and Associates, Glendale, CA). Inhibition studies were performed using caged-C_{60} and carboxyfullerenes at concentrations of 25 µg/ml, 50 µg/ml and 100 µg/ml in warm DPBS. To determine their role in modulating irradiation-induced ROS generation, fullerene derivatives were added either 2 hr before irradiation exposure (pre-treatment), 0 hrs (co-treatment) or 1 hr after irradiation exposure (post-treatment).

2.4 Reactive oxygen species (ROS) analysis

The redox-sensitive fluorescent dye 2', 7'-dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes, Invitrogen, Eugene, OR) was used to measure the production of intracellular ROS. HEK cells were loaded with DCF-DA dye (5 µM) 1 hr before the irradiation exposure. After irradiation HEK cells were incubated for 30 min. As a positive control, cells were also exposed to 100 µM H_2O_2. The fluorescence of ROS converted DCF was monitored by fluorescence plate reader (excitation/emission 490nm/535 nm). A media control cell culture sample was used for each exposure condition.

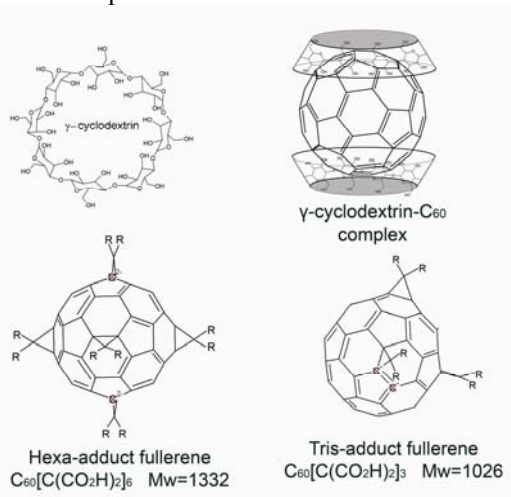

Figure 1. Schematic structures of fullerene derivatives. γ-cyclodextrin (top left) was used to synthesize γ-cyclodextrin caged C_{60} complex (top right) (CD-C_{60}). CD-C_{60}, fullerene hexa-carboxylic acid derivative (Hexa-C_{60}, R=-COOH) and fullerene tris-carboxylic acid derivative (Tris-C_{60}, R=-COOH) were employed for ROS production evaluation.

3 RESULTS

3. 1. ROS response of HEK cells on concurrent treatment with γ-irradiation and fullerene derivatives

We examined the ability of fullerene derivatives to inhibit the induction of intracellular ROS by γ-irradiation. The intracellular fluorescence intensity of cells loaded with DCF-DA is indicative of the amount of oxidative stress occurring. As expected, there was a significant increase in cellular ROS levels in cells exposed to 10 and 17 Gy of γ-irradiation compared to unexposed cells (Figure 2). Conversely, in γ-irradiated cells co-treated with fullerene derivatives CD-C_{60} a significant inhibition of irradiation-induced ROS levels was observed at all the doses. Though a similar response was observed at the lower doses of 25 and 50 µg/ml of tris-C_{60} at both 10 and 17 Gy, no decrease was seen at the higher dose of 100 µg/ml in cells exposed to 17 Gy. While hexa-C_{60} did prevent ROS generation in the 10 Gy exposed cells, no significant effect was observed in the cells treated with the doses of 25 and 100 µg/ml hexa-C_{60} at 17 Gy. When unirradiated cells were treated with the different fullerene derivatives, a small but statistically significant decrease in basal levels of intracellular ROS was observed with CD-C_{60} (at all doses), tris-C_{60} (25 and 50 µg/ml) and hexa-C_{60} (50 and 100 µg/ml) suggesting an ROS-scavenging role for these nanoparticles.

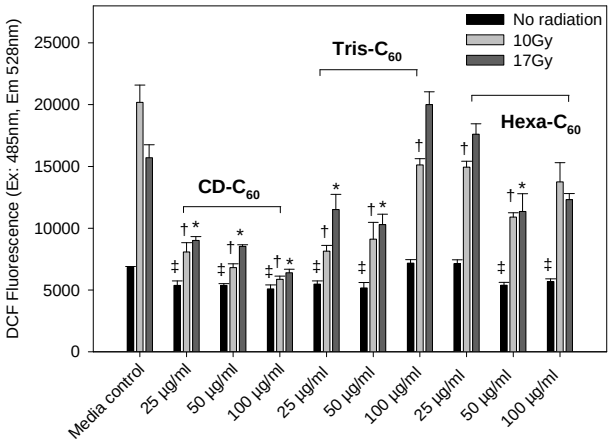

Figure 2. Intracellular ROS levels in irradiated keratinocytes co-treated with fullerene derivatives. $p<0.05$, statistically different from media control without irradiation exposure (‡), with 10 Gy (†) and 17 Gy irradiation exposure (*).

3.2 Investigation of ROS response in γ-irradiated cells on pre-treatment with fullerene derivatives

Results from the co-treatment experiments suggest either a free radical scavenging or inhibitory function for fullerenes in irradiation-induced ROS responses. To determine this cells were pre-treated for 2 hrs with fullerene

derivatives at 25, 50 and 100 μg/ml prior to exposing the cells to either 10 or 17 Gy γ-irradiation. Unlike the co-treatment results pre-treatment with CD-C_{60} did not cause a decrease in the ROS levels in the 10 Gy exposed cells, but afforded a slight reduction in ROS levels in the 17 Gy exposed cells. On the other hand, a considerable decrease in the intracellular ROS levels was observed in a dose dependent manner in the tris-C_{60} pre-treated cells at both γ-exposure doses (Figure 3). However, in the hexa-C_{60} pre-treated HEK cells, no decrease in irradiation-induced ROS was noted. In contrast, a synergistic increase in ROS levels was observed in irradiated cells co-treated with hexa-C_{60}.

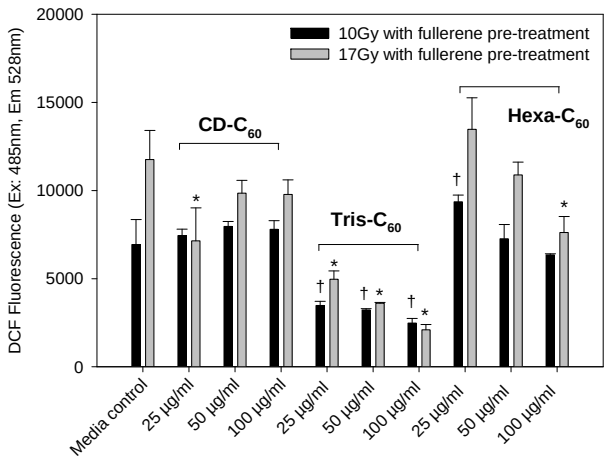

Figure 3. Effect of fullerene pre-treatment on ROS levels in γ-irradiated keratinocytes. $p<0.05$, statistically different from media control with 10 Gy irradiation exposure (†) and with 17 Gy irradiation exposure (*).

3.3 Post-treatment of cells with fullerene derivatives was less effective in protecting against γ-irradiation mediated ROS response.

To elucidate the mechanism by which fullerenes regulate irradiation-induced ROS responses, HEK cells were post-treated with fullerene derivatives 1 hr after γ-irradiation exposure. Although a slight decrease in cellular ROS level was observed in the CD-C_{60} and tris-C_{60} treated cells, there were no noteworthy changes in the γ-irradiation induced cellular ROS response on post-treatment with either of the fullerene derivatives (Figure 4). On the contrary, low dose of hexa-C_{60} slightly enhanced the ROS response in the 17 Gy irradiated cells.

Collectively, our results suggest that concurrent and pre-treatment of cells with tris-C_{60} can potentially inhibit γ-irradiation induced ROS generation. However, treatment of cells with either CD-C_{60}, hexa or tris-C_{60} after irradiation exposure did not afford any protection against γ-exposure.

3.4 CD-C_{60} Elevates γ-irradiation Induced ROS Production in a Cell-free System

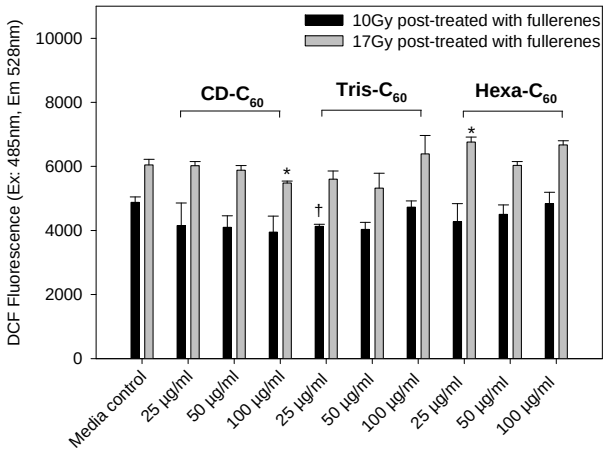

Figure 4. Effect of post-treatment of cells with fullerenes following γ-irradiation of HEK cells. $p<0.05$, statistically different from media control with 10 Gy irradiation exposure (†) and with 17 Gy irradiation exposure (*).

To determine possible effects of γ-irradiation on the fullerene derivatives we exposed CD-C_{60}, tris and hexa- C_{60} with 10 and 17 Gy in a cell-free system in media alone. A dose-dependent ROS increase was observed in 10 Gy and 17 Gy γ-irradiated media, however, compared to the intracellular ROS levels this increase was minimal (Figure 2). Moreover, unlike the cellular experiments, a prooxidant response was observed with CD-C_{60} in media. Interestingly, both tris-C_{60} and hexa-C_{60} carboxyfullerenes did not significantly alter ROS production after irradiation exposure. These results indicate that γ-irradiation and CD-C_{60} interactions can potentially alter redox status of CD-C_{60}.

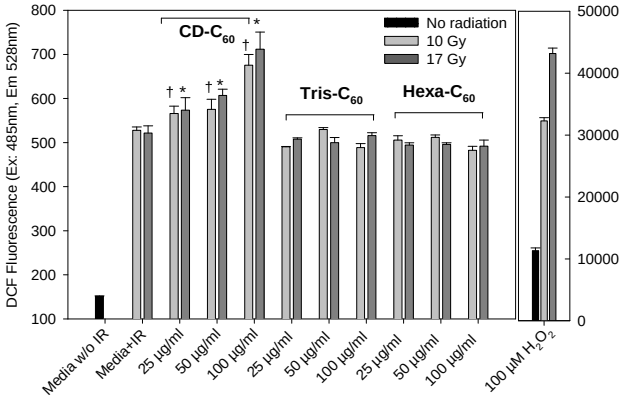

Figure 5. Fullerene derivatives alter ROS levels after γ-irradiation exposure in cell-free system. Fullerene derivatives in DPBS were co-treated with 10 and 17 Gy γ-irradiation to determine the background ROS response. $p<0.05$, statistically different from media control with 10 Gy irradiation exposure (†) and with 17 Gy irradiation exposure (*).

4 DISCUSSION

The reported findings of the current study suggest a protective role for engineered fullerenes in γ irradiation-induced cellular damage. The ability of fullerenes to modulate intracellular ROS levels is a well-recognized but poorly understood phenomenon [12, 13]. Our results clearly indicate that CD-C_{60}, tris and hexa-C_{60} can regulate the oxidative status of cells, even following acute exposure to ROS-inducing agents such as γ irradiation. In the current study, co-treatment of irradiated cells with CD-C_{60}, tris-C_{60} and hexa-C_{60} significantly reduced the ROS levels. This result indicates that these fullerenes may either scavenge the ROS generated by the γ-rays or potentially inhibit the irradiation-induced free radical production. In the fullerene pre-treated cells there was a notable, consistent and dose-dependent decrease in the tris-C_{60} treated cells. However, CD-C_{60} did not reduce cellular ROS concentrations, whereas, low dose of hexa-C_{60} induced an ROS response. Interestingly, post-treatment with fullerenes did not have a notable effect on cellular ROS levels. Given these results we can speculate that fullerenes, specifically, CD-C_{60} and tris-C_{60} do not function as ROS scavengers but rather may inhibit γ-irradiation induced ROS-generation. The observed difference in the radioprotective ability of the tested fullerenes in pre-treated and post-treated cells may be due to the ability of the functionalized fullerene to inhibit the generation of reactive oxygen species rather than scavenge free radicals. An alternative explanation for the lack of 'ROS protection' in the post-treatment samples may be that in the absence of fullerenes the levels of intracellular ROS generated on γ exposure may be well beyond or saturate the 'scavenging' capacity of these nanoparticles. However, results from the cell-free systems indicate otherwise, wherein CD-C_{60} causes an increase in ROS levels, and no significant change is noted in the tris and hexa-C_{60} irradiated samples, precluding a 'ROS sponge' function for these fullerenes. In addition, a role for cellular components involved in maintaining oxidative status in the cell cannot be totally overlooked. Thus, these radioprotective fullerenes could potentially modulate pathways or proteins involved in pro- or anti-oxidant responses.

While the mechanisms that underlie what appears to be a fullerene-mediated radioprotective response to γ-irradiation obviously requires further investigation, it is nevertheless tempting to speculate about how some potential mechanisms may contribute to the effect. Conceivably, there may be three possible mechanisms by which CD-C_{60} and tris-C_{60} reduce irradiation induced intracellular ROS levels, 1) function as free radical scavengers, 2) inhibit ROS generation or 3) induce antioxidant responses. In addition, the different responses observed might be a function of the surface modification of the core fullerene structure. Properties and effects can dramatically change when structure and substituents of exohedral fullerenes are altered [14]. Thus, the differential responses observed with the three distinctly functionalized fullerenes maybe a function of their chemical modifications impacting not only their reduction potential but also their uptake by cells and targeted tissue. In conclusion, our study demonstrated that, tris-C_{60} more so than CD-C_{60} and hexa-C_{60} significantly suppress γ-irradiation induced ROS generation if administered either before or during exposure to irradiation. Further investigation is ongoing to determine the mechanism(s) underlying this response.

Acknowledgments: supported by LANL (LDRD-DR)

REFERENCES

[1] T. J. Cerveny, T.J.M.V., R.W. Young, Acute radiation syndrome in humans in Medical Consequences of Nuclear Warfare. 1989.

[2] Little, J.B., Environmental hazards. Ionizing radiation. N Engl J Med, 275(17): 929-37. 1966.

[3] Barnett, D.J., et al., Understanding radiologic and nuclear terrorism as public health threats: preparedness and response perspectives. J Nucl Med, 47(10): 1653-61. 2006.

[4] Wagner, R.H., M.A. Boles, and R.E. Henkin, Treatment of radiation exposure and contamination. Radiographics, 14(2): 387-96. 1994.

[5] Waselenko, J.K., et al., Medical management of the acute radiation syndrome: recommendations of the Strategic National Stockpile Radiation Working Group. Ann Intern Med, 140(12): 1037-51. 2004.

[6] Isoir, M., et al., Human keratinocyte radiosensitivity is linked to redox modulation. J Dermatol Sci, 2006. 41(1): 55-65.

[7] Bosi, S., et al., Fullerene derivatives: an attractive tool for biological applications. Eur J Med Chem, 38(11-12): 913-23. 2003.

[8] Chen, Y.W., et al., Fullerene derivatives protect against oxidative stress in RAW 264.7 cells and ischemia-reperfused lungs. Am J Physiol Regul Integr Comp Physiol, 287(1): R21-6. 2004.

[9] Dugan, L.L., et al., Buckminsterfullerenol free radical scavengers reduce excitotoxic and apoptotic death of cultured cortical neurons. Neurobiol Dis, 3(2): 129-35. 1996.

[10] Dugan, L.L., et al., Carboxyfullerenes as neuro-protective agents. Proc Natl Acad Sci U S A, 94(17): 9434-9. 1997.

[11] Lamparth, I.a.H., A., Water-soluble malonic acid derivatives of C_{60} with a defined 3-D structure. J. Chem. Soc., 1727-8. 1994

[12] Markovic, Z., et al., The mechanism of cell-damaging reactive oxygen generation by colloidal fullerenes. Biomaterials, 28(36): 5437-48. 2007.

[13] Kamat, J.P., et al., Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications. Toxicology, 155(1-3): 55-61. 2000.

[14] Chellat, F.; Merhi, Y.; Moreau, A.; Yahia, L. Biomaterials, 26, (35), 7260-75. 2005.