Inhibition of γ-Radiation Induced DNA Damage in Plasmid pBR322 by TMG, a Water-soluble Derivative of Vitamin E

REMA RAJAGOPALAN¹, KHALIDA WANI², NAGARAJ G. HUILGOL², TSUTOMU V. KAGIYA³, and CHERUPALLY K. KRISHNAN NAIR¹*  

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Alpha-tocopherol monoglucoside (TMG), a water-soluble derivative of α-tocopherol, has been examined for its ability to protect DNA against radiation-induced strand breaks. Gamma radiation, up to a dose of 6 Gy (dose rate, 0.7 Gy/ minute), induced a dose-dependent increase in single strand breaks (SSBs) in plasmid pBR322 DNA. TMG inhibited the formation of γ-radiation induced DNA single strand breaks (SSBs) in a concentration-dependent manner; 500 μM of TMG protected the single strand breaks completely. It also protected thymine glycol formation induced by γ-radiation in a dose-dependent manner, based on an estimation of thymine glycol by HPLC.

INTRODUCTION

The most important target in a living cell damaged by ionizing radiation is genomic DNA. The types of damage suffered by DNA are strand breaks of the single and double-strand types, base damage, sugar damage and cross-links of the intra and inter-strand types. Several of these types of damage are also produced due to oxidative stress induced by free radicals. Many natural and synthetic compounds, such as curcumin, vitamins A, C and E, melatonin and chlorophyllin, having an anti-oxidative property, could mitigate deleterious effects of ionizing radiation.

Compounds with antioxidant properties have been shown to prevent the deleterious effects of ionizing radiation in living systems and bio-molecules. The radioprotective ability of these compounds have been attributed to their ability to scavenge free radicals. The water-soluble derivative of vitamin E-2-(α-D-glucopyranosyl)-methyl-2,5,7,8-tetra methyl chroman-6-ol exhibited a higher antioxidant property than that of ascorbic acid in vitro. TMG has been shown to have a radioprotective effect in mammalian systems and yeast. It was reported that the administration of TMG to mice, five minutes prior to whole-body X-irradiation by an oral route or immediately after by an ip route, protected them from the lethal effects of radiation. The administration of TMG by ip to mice following irradiation prevented gamma radiation-induced chromosomal aberrations in bone-marrow cells. In this report we present data on the protection of DNA against radiation-induced DNA damage by TMG, a glucoside derivative of α-tocopherol (vitamin E).

Plasmid DNA constitutes a useful model system for investigating interactions between topologically constrained DNA and radiation fields and other envi-

¹Corresponding author: Phone: 91-22-5593869, Fax: 91-22-5560750/91-22-5505151, E-mail: cknair@magnum.barc.ernet.in

¹ Radiation Biology Division, Bhabha Atomic Research Centre, Mumbai 400 085,
² Department of Radiation Oncology, Nanavati hospital & MRC, Vile Parle, India
³ Kinki Research Foundation, Kyoto, Japan.
ronmental stresses. Plasmids have served as useful model systems for numerous radiation studies in addition to their role as vectors.\textsuperscript{16–18)}

**MATERIALS AND METHODS**

**Chemicals**

TMG was obtained from CCI Corporation, Gifu, Japan by Prof. V.T. Kagiya.

Trolox was obtained from Sigma Chemical Company. Molecular biology-grade agarose, ethidium bromide and thymine were purchased from Sigma Chemical Company, USA. Plasmid pBR322 DNA was prepared from an Escherichia coli culture and purified by CsCl\textsuperscript{2}-density gradient centrifugation\textsuperscript{19)}. It was ensured that these preparations contained more than 90\% of form I (native super coiled form) and the rest form II (open circular form).

**Exposure to gamma-radiation and estimation of DNA Damage**

Plasmid pBR322 DNA (1 to 1.5 \(\mu\)g) suspended in 50 \(\mu\)l of 50 mM sodium phosphate buffer (pH 7.4) was exposed to various doses of \(\gamma\)-radiation at a dose rate of 0.7 Gy/min at 0\degree C using a \(^{60}\)Co Theraton Junior Teletherapy unit. (Atomic energy Canada Ltd, Ottawa, Canada). The supercoiled and open circular forms of DNA were separated by agarose gel electrophoresis using 0.8\% agarose gels in 90 mM Tris borate and 2 mM EDTA buffer (pH 8.3)\textsuperscript{20)}. DNA bands after staining with ethidium bromide, were quantitated by scanning negative prints with a Shimadzu CS-9000 densitometer. Radiation-induced DNA Damage was estimated as an increase in the open circular form of DNA. All irradiation mixtures of TMG and DNA were prepared in a buffer just prior to irradiation in required concentrations. The DNA damage was calculated according to a method of T. Ashikaga et al.\textsuperscript{21)}.

The DNA strand breaking index (SBI) is defined as follows:

\[
\text{SBI} = \frac{\text{Open circular DNA}}{\text{Total DNA}} \times 100
\]

\[
\text{Inhibition \%} = 1 - \frac{\text{SBI in the presence of sample - control SBI}}{\text{SBI in the absence of sample - control SBI}} \times 100
\]

**Estimation of thymine glycol by HPLC**

Thymine glycol formation in the presence of 10 mM sodium nitrite was studied using 5 mM thymine at high doses of (200–800 Gy) \(\gamma\)-radiation. Thymine glycol was separated by isocratic elution using 5\% methanol in water on an ODS C18 column using a HPLC system from Applied Biosystems\textsuperscript{22)}. An irradiated thymine sample was eluted at a flow rate of 1 ml / min, and the peaks were detected at 260 nm. Thymine glycol was eluted at a retention time of 2.59 minutes, whereas thymine was eluted at a retention time of 3.60 minutes from a reverse-phase column.

**RESULTS**

Figure 1 depicts a dose-response curve showing the formation of SSBs following the exposure of plasmid pBR322 DNA to \(\gamma\)-rays. It is evident from the figure that there is a linear decrease in single strand breaks per DNA molecule up to a dose of 6 Gy, above which the curve reaches a plateau. A radiation dose of 6 Gy was selected for further experiments to study the effect of TMG. The reverse of a photonegative of the agarose gel electrophoresis of pBR322 DNA irradiated at 6 Gy in both the presence and absence of different concentrations of TMG is given in Fig. 2. As compared to the control (lane 1), the exposure of plasmid DNA to a \(\gamma\)-radiation dose of 6 Gy (lane 2) increased the relative intensity of the band corresponding to form II. The addition of TMG prior to irradiation in increasing concentrations reduced the intensity of the form-II bands, corresponding to the open circular form of form II. Similar radioprotection of DNA damage has been observed in plasmid DNA exposed to higher doses of \(\gamma\)-radiation. The TMG required for radioprotection was also high (1mM)\textsuperscript{23)}. From Fig. 3, it is evident that TMG reduced radiation-induced single strand breaks in plasmid DNA in a dose-dependent manner. Even at a concentration of 50 \(\mu\)M of TMG, significant protection (33.5\%) was observed. At 500
µM of TMG it caused about a 90% reduction in the radiation-induced SSBs. The radiation protection by trolox, a vitamin E anologue, is given in figure 3. It was observed that trolox protected DNA less efficiently than TMG. At a 50 µM concentration TMG afforded 33.5% protection to DNA against 25% by trolox. The extent of radiation protection of DNA by these chemicals increased along with an increase in the concentration. However, it can be seen that the presence of 100 µM TMG resulted in 81.05% protection, while trolox, even at 300 µM, protected DNA only to the extent of 37% (Fig. 3).

The inhibition of thymine glycol formation due to 400 Gy γ-radiation in the presence of TMG is shown in Fig. 4. TMG at 500 µM concentration inhibited γ-radiation induced thymine glycol formation due to γ-radiation by 13%. At 1 mM concentration TMG brought down the yield of thymine glycol to 80% (20% inhibition) and 2.0 mM TMG reduced the formation of thymine glycol to 75% (25% inhibition).
DISCUSSION

Several Chemicals and ionizing-radiation produce mutagenic and carcinogenic effects through the generation of reactive oxygen species. Various compounds that are capable of scavenging free radicals have been tried for protecting DNA against environmental genotoxins\(^\text{10,24–27}\). Vitamin E present in living cells has been shown to be an inhibitor of free radical-mediated damage\(^\text{28}\). It acts as a free-radical scavenger-antioxidant by effectively removing oxidative radicals\(^\text{28,29}\).

Unlike vitamin E, TMG is highly soluble in water (100g/100ml). TMG has been reported to be an effective radioprotector, as is evident from survival studies using mice\(^\text{12–14}\), and also in yeast\(^\text{15}\). *Saccharomyces cerevisiae* D7 yeast cells showed advanced radioresistance due to the presence of 10 mM TMG. Trolox, a structural analogue of alpha-tocopherol, is also a free-radical scavenger and protector against DNA.

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**Fig. 3.** Protection of γ-radiation induced DNA damage in plasmid pBR322 by TMG and trolox (see Materials and Methods).

**Fig. 4.** Inhibition of thymine glycol formation due to the irradiation of thymine by TMG.
damage\textsuperscript{10}. From our results, the protective effect of TMG has been observed to be higher than that of trolox. In TMG the carboxylic acid group of trolox is replaced by a methyl glycosidic unit at C-2. The presence of the sugar moiety could presumably be responsible for its higher radioprotective ability.

The \textit{in vitro} system in which DNA damage was elicited by free radicals generated during radiolysis gives direct evidence for the protection of radiation damage by TMG. In our previous report, pulse radiolysis studies provided direct evidence of free-radical scavenging by TMG\textsuperscript{23,30}. The radiation-induced damage to cell is mainly a consequence of damage to the DNA, and thymine is a highly radiosensitive base in DNA. The radiation-induced hydroxylation of thymine as a base constituent to form thymine glycol has been used as a chemical measure to assess the effect of radiomodifiers\textsuperscript{22}. The formation of thymine glycol can be taken as a measure of DNA damage; further, it has been found that it increases with increasing dose of radiation exposure\textsuperscript{5,22}. Our studies show that TMG effectively inhibits thymine glycol formation. Among several sulphhydryl compounds, such as cysteine and other similar compounds tested, amifostine appear to be the best radioprotector, and is being used during radiation therapy\textsuperscript{26}. Many compounds extracted from plants have also been shown to be radioprotectors\textsuperscript{6,7,10}. The ability of the natural polyamines putrescine and spermine to radioprotect DNA and cells has been reported\textsuperscript{24,31}. The mutagenic and carcinogenic effects of radiation through the generation of free radicals have been well-studied. Compounds which are capable of scavenging free radicals are of considerable interest for protecting against radiation and other genotoxins\textsuperscript{32}. Melatonin has been reported to be a good radioprotector because of efficient free radical scavenging and antioxidant properties\textsuperscript{33}. This hormonal product of the pineal gland was shown to protect against radiation-induced genetic damage in humans\textsuperscript{34}. The radioprotective effects of sodium tungstate against hematopoietic injury resulting from exposure to $^{60}$Co $\gamma$-rays in Wistar rats has also been demonstrated\textsuperscript{35}. Recent studies have tested, the use of electron-spin resonance for probing the redox reaction of nitroxyl for the \textit{in vivo} screening of the radioprotective activity of cystamine in mice\textsuperscript{36}. Nontoxic compounds which can protect DNA against ionizing radiation have considerable potential as radioprotectors of normal tissue during the radiotherapy of various tumors. They may also be useful for the prevention of diseases like cancer as well as other degenerative diseases arising from gene mutation. TMG does not induce DNA damage by itself, but inhibits $\gamma$-radiation induced single-strand breaks (SSBs) in a concentration-dependent manner; 500 $\mu$M of TMG was shown to protect single-strand breaks almost completely. In our previous report, pulse radiolysis studies of TMG involving electron oxidants, like Br$_2^-$ and N$_3^-$, indicated the formation of phenoxy radicals upon reaction with electron oxidants\textsuperscript{23,30}. These results suggest that TMG manifests radiation protection to biomolecules owing to its ability to scavenge free radicals. This is the first report that shows direct evidence of the radioprotection of DNA by TMG. This compound was shown to effectively protect plasmid DNA against ionizing radiation in an \textit{in vitro} system independent of all cellular defense mechanisms, including DNA repair. A demonstration of the radioprotective effect of TMG in the plasmid pBR322 system and its ability to scavenge free radicals perhaps suggests that it may be useful as an efficient radioprotector for normal tissues in radiation therapy.

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