Irradiation-induced Expression of \(O^6\)-Methylguanine-DNA Methyltransferase in Mammalian Cells

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ABSTRACT

\(O^6\)-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein which plays an important role in chemotherapy, mutagenesis, and carcinogenesis. The specific activity of MGMT in female rat liver can be induced by approximately 20-fold by treatment of the rats with \(\gamma\)-irradiation. Maximum response occurred 48 h after 15 Gy irradiation. MGMT levels in male rats were induced by only 3-fold. MGMT activity was also induced by irradiation of rat hepatoma H4IIIE cells with a 3-fold increase noted after treatment with 3 Gy. Northern analysis and nuclear run-on assays indicated that the induction of MGMT was regulated at the transcriptional level. The radiation-mediated increase in MGMT was blocked by H7, a protein kinase inhibitor, but not by H89, an inhibitor of protein kinase A. Hydroxyl radicals may play a role in the induction mechanism since dimethyl sulfoxide, a radical scavenger, blocked the radiation-mediated increase in MGMT. MGMT activity was also increased by treatment of the cells with \(H_2O_2\), in accordance with the involvement of activated oxygen species in the induction of MGMT. Finally, the addition of cycloheximide, an inhibitor of protein synthesis, prior to but not after irradiation, abolished the increase in MGMT activity.

INTRODUCTION

Alkylating agents, such as chloroethylnitrosourea and BCNU,\(^4\) interact with several of the bases of DNA and at several sites within these bases, resulting in the production of a variety of alkylated lesions in DNA. A major site of alkylation is the \(O^6\) position of guanine. In particular, \(O^6\)-alkylguanine in DNA eventually leads to the substitution of an A-T for the guanine and an opposing cytosine. The extent of DNA-DNA adducts rearranges to form a cross-link between this moiety in DNA. The result of this transfer is an inactive DNA-DNA interstrand cross-links appears to correlate to cytotoxicity that is induced by BCNU. Consequently, the intracellular level of MGMT, which is able to repair the \(O^6\)-methylguanine DNA adducts, plays an important role in countering the potential therapeutic efficacy of BCNU and analogous alkylating agents.

Exposure of the intact rat and of the rat hepatoma cell H4IIIE to ionizing radiation can significantly induce MGMT activity (6-8). Habraken and Laval (9) showed that the resistance of H4 cells to BCNU was increased when the cells were pretreated with ionizing radiation. The resistance to BCNU correlated well with the induction of MGMT as a result of irradiation. Based upon this observation, it might be predicted that resistance to BCNU could be significantly enhanced by treatment of tumors with this haloethylnitrosourea after the delivery of radiotherapy; hence such a schedule might be counterproductive.

A better understanding of the molecular mechanisms underlying the induction of MGMT after exposure to ionizing radiation may lead to a strategy for suppressing the induced resistance to BCNU. Consequently, we have studied the mechanism of the radiation-induced increase in MGMT in greater detail.

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\(^4\)The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MGMT, \(O^6\)-methylguanine-DNA methyltransferase; CHX, cycloheximide; DMSO, dimethyl sulfoxide; cDNA, complementary DNA.

MATERIALS AND METHODS

Cell Culture. H4IIIE cells were grown in minimal essential medium supplemented with 10% fetal calf serum and gentamycin (40 \(\mu\)g/ml) in a humidified 5% \(CO_2\)-95% air atmosphere. All media were obtained from Gibco. The doubling time for H4IIIE cells was 15 h.

Irradiation Treatment. Male and female Sprague-Dawley rats (200-250 g) were exposed to whole-body irradiation with a \(^{60}\)Co Picker V9 unit. The dose rate of \(\gamma\)-irradiation was 80 rad/min, and the total dose was controlled by altering the time of exposure. Exponentially growing H4IIIE cells were irradiated with \(\gamma\)-rays in a similar manner.

Preparation of Liver and H4IIIE Cell Extracts. At various times after irradiation, the rats were killed, and the livers were removed and homogenized at 4°C in 3 volumes of TEDGP buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, 5% glycerol) containing 0.5 M NaCl. After centrifugation for 70 min at 15,000 x g at 4°C, the supernatant fraction was removed and assayed for MGMT activity.

The H4IIIE cells were trypsinized at various times after irradiation, washed twice with phosphate-buffered saline, collected by centrifugation, and resuspended in TEDGP/0.5 M NaCl. The cells were lysed by homogenization with pestle B of a Dounce homogenizer. After centrifugation for 70 min at 15,000 x g, the supernatant fraction was removed and assayed for MGMT activity.

Assay of MGMT Activity. The methylated DNA substrate was prepared by reacting \(^3\)Hmethyl-A'-nitrosourea (0.5 or 17.9 Ci/mmol, obtained from New England Nuclear or Amersham Corp., respectively) with calf thymus DNA as described by Karren et al. (10). The final DNA substrate contained about 80% of its radioactivity in \(O^6\)-methylguanine. The MGMT activity was assayed by a modification of the method of Myrnes et al. (11). Tissue extracts were incubated for 30 min at 37°C with \([\text{methyl-}\text{H}]\text{DNA}\) (5 or 0.1 pmol \(O^6\)-methylguanine, with a specific activity of 0.5 or 17.9 Ci/mmol, respectively) and 200 \(\mu\)g bovine serum albumin in a final volume of 1 ml. The reaction was stopped by adding 250 \(\mu\)l 50% trichloroacetic acid, and the mixture was heated to 80°C for 30 min. The protein precipitate was collected by centrifugation, and the pellet was washed twice with 1 ml 5% trichloroacetic acid. The pellet was redissolved with 200 \(\mu\)l 0.1 N NaOH,
neutralized with 200 µl 0.4 M Tris-Cl (pH 8), and counted in Aquasol. The protein concentration in the supernatant fraction was determined by the Bio-Rad protein assay procedure with bovine serum albumin as the reference standard.

Northern Blot Analysis. Polyadenylated RNA was isolated as described (12) 12 h after irradiation. The RNA, 10 µg, was separated by denaturing agarose/formaldehyde gel electrophoresis, transferred to a nylon membrane (Schleicher and Schuell), and hybridized with a 32P-labeled human MGMT cDNA probe. The latter probe was produced exactly as described in Wu et al. (13). The source of the probe was a MGMT cDNA which was incorporated into a pET-plasmid system, pET-MGMT (13). After washing the membrane and autoradiography, the intensity of the positive signals was normalized to the extent of ethidium bromide fluorescence.

Nuclear Run-on Assay. Nuclei were prepared from irradiated and unirradiated H4IIE cells by the NP-40 lysis technique (14), frozen in liquid nitrogen, and stored until analyzed. The frozen nuclei (from 1 x 10⁸ cells) were thawed at room temperature and 200 µl of a 2x reaction buffer (10 mM Tris-Cl, pH 8, 5 mM MgCl₂, 0.3 M KCl) containing ATP, GTP, and CTP was added along with 10 µl of [α-32P]UTP (760 Ci/mmol, 10 mCi/ml). The reaction mixture was incubated for 1 h at 30°C, the nuclei were pelleted at 15,000 x g for a few seconds, and the supernatant fraction was aspirated to remove most of the unincorporated labeled nucleotide.

Nuclear RNA was extracted as described by Chomczynski and Sacchi (15), precipitated twice with sodium acetate-ethanol, resuspended in water, and used in the hybridization experiments. For the latter, 20 µg of pET-MGMT were blotted onto a nylon membrane and hybridized with the labeled nuclear RNA.

RESULTS

Hepatic MGMT from female rats was induced by 20-fold after whole-body γ-irradiation (Fig. 1). Maximum MGMT activity was observed in the liver 48 h after exposure of the rats to 15 Gy of γ-irradiation (Fig. 2). The effects of irradiation were pursued at the RNA level by northern blot analysis of rat liver polyadenylated RNA using a 32P-labeled human MGMT cDNA as probe. The size of the MGMT mRNA was approximately 1 kilobase, which was in accordance with that reported by Potter et al. (16). The hybridization signal for MGMT mRNA increased in a dose-dependent manner as indicated in Fig. 3. The steady-state level of MGMT mRNA in the livers of female rats after treatment with 7.5 and 15 Gy γ-irradiation was increased by 3- and 12-fold, respectively. The kinetics of irradiation-induced expression of liver MGMT mRNA was studied; these data are shown in Fig. 4. Maximum MGMT mRNA levels were observed at 12 h after irradiation, at which time a 15-fold increase over control was noted. The extent of induction of MGMT protein corresponded well to the increase in MGMT mRNA.

During the course of these studies, we noted a striking gender effect of irradiation upon the rat liver MGMT levels, although the basal activity was similar in the livers from the male and the female. Male rats responded far less to irradiation than did female rats. After 15 Gy irradiation, only a 2- to 3-fold induction of MGMT activity was observed in the livers from male rats (Fig. 5), in contrast to the 17- to 20-fold elevation in female rat liver. Northern blot analysis indicated that a similar refractoriness of male rat liver to irradiation was apparent in the steady-state level of MGMT mRNA (Fig. 3).

The gender effect of irradiation on MGMT levels was examined in nonhepatic tissues (Table 1). The extent of induction in spleen, kidney, and brain was similar for the male and female. Lung MGMT activity as published previously (8) was reduced by irradiation of the rats; the reduction was similar for the male and female.

The MGMT activity in rat H4IIE hepatoma cells also responded to γ-irradiation. A 3-fold induction of MGMT activity was observed after treating the cells with 3 Gy of γ-irradiation as shown in Fig. 6. The induction kinetics in H4IIE cells (Fig. 7) was similar to the results from the in vivo study. Maximum steady-state levels of MGMT mRNA were seen at 12 h after irradiation of the H4IIE cells, at which time an approximately 2-fold increase over control was observed. In order to establish an effect of irradiation upon transcription in the H4IIE cells, nuclear run-on experiments were performed. A 2.3-fold increase in the steady-state mRNA level was observed at 12 h after irradiation (results not shown). These results are in accordance with the regulation of MGMT induction at the transcriptional level, with posttranscriptional events playing only a minor role.

The need for protein synthesis in the radiation-mediated effect on MGMT was examined by the use of the inhibitor CHX (Fig. 8). H4IIE cells were treated with CHX 30 min before or 6 h after γ-irradiation. The MGMT mRNA was not induced when CHX was added to the medium prior to the radiation treatment. On the other hand, the addition of CHX 6 h after irradiation had no effect on the induced expression of MGMT mRNA.

We hypothesized that the radiation-induced expression of MGMT activity in the H4IIE cells might be influenced by hydroxyl radical-mediated damage to DNA. Accordingly, the
effects of a hydroxyl radical scavenger, DMSO, were studied on radiation-induced MGMT (Fig. 9). Although DMSO had no effect upon control MGMT activity, it strikingly eliminated the radiation-induced response. Further studies on H4IIE MGMT were conducted after treatment of the cells with different concentrations of H2O2 in order to generate reactive oxygen species (Fig. 10). A 2-fold induction in MGMT activity was observed after exposing these cells to 100 μM H2O2. These studies suggested that reactive oxygen species could induce MGMT activity.

A possible involvement of the protein kinases in the radiation response was studied through the use of several inhibitors. H7, a protein kinase inhibitor (17), at 12.5 and 25 μM, was also able to completely abolish the induction of MGMT activity after irradiation of the H4IIE cells (Table 2). H7 inhibits both protein kinase C and protein kinase A. In order to ascertain any role of protein kinase A in the induction mechanism, H89, a specific inhibitor of this enzyme, was added to the irradiated cells. The induction of MGMT activity in the H4IIE cells was not changed by H89 (Table 2), thus suggesting that the abolition of the radiation response by H7 might be caused by an effect on protein kinase C.

DISCUSSION

The induction of rat hepatic MGMT by whole-body γ-irradiation as first reported by Margison et al. (6) has been confirmed. However, the induced level of hepatic MGMT obtained in our experiments, i.e., 20-fold, exceeded that observed by the Margison group (6), i.e., 5-fold. Different dose rates of radiation and a different age of rats have been used, but no significant change in the induction response was observed. However, gender exhibited a marked effect, with male rats showing a diminished response to irradiation. The mechanism underlying this response is not clear, although estrogen may be involved through interaction of its receptor with a cis-regulatory element in the MGMT gene. The latter element has not yet been demonstrated in the MGMT gene. It is interesting that the normal MGMT activity in the livers of male and female rats is almost identical. The latter implies that a simple interaction of the estrogen receptor and the estrogen-responsive element cannot be responsible for the observed effects on MGMT activity.

A similar response to γ-irradiation was noted with the rat

Fig. 3. Northern blot analysis of hepatic MGMT mRNA in female and male rats after ionizing radiation. Rats were pretreated with 0, 7.5, and 15 Gy of γ-irradiation, the livers were removed 48 h later, and polyadenylated RNA was isolated and examined by northern analysis as described in "Materials and Methods." A, Northern blot hybridization; B, densitometric analysis of the intensities. The intensity is given in arbitrary units.

Fig. 4. Kinetics of induction of hepatic MGMT mRNA in irradiated rats. Female rats were exposed to 15 Gy γ-irradiation, polyadenylated RNA was isolated and electrophoresed on 1.2% agarose gels as described in "Materials and Methods," and Northern blot hybridization was performed with 32P-labelled human MGMT cDNA as the probe. A, Northern blot analysis of MGMT mRNA. Size markers (in kilobases) are listed on the right margin. The MGMT mRNA had a mobility of 1 kilobase. Lane 1, control RNA; Lane 2, 6 h after irradiation; Lane 3, 12 h; Lane 4, 24 h; Lane 5, 36 h. In B, the autoradiograph of A was subjected to laser densitometry, and the data were plotted as area (arbitrary units of density) as a function of time after irradiation.
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Fig. 5. Effect of γ-irradiation on hepatic MGMT activity in the female and male rat. Rats were pretreated with 15 Gy of γ-irradiation and killed 48 h later. The results are representative of two experiments.

Table 1 Effect of irradiation upon MGMT specific activity
All rats were treated with 15 Gy of γ-irradiation and sacrificed 48 h later. The liver results are averages ± SE for six determinations; the other values represent averages from two experiments.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control (pmol/mg protein)</th>
<th>Induced (pmol/mg protein)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.083 ± 0.015</td>
<td>1.46 ± 0.24</td>
<td>17.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.120</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.040</td>
<td>0.12</td>
<td>3.0</td>
</tr>
<tr>
<td>Brain</td>
<td>0.016</td>
<td>0.068</td>
<td>4.3</td>
</tr>
<tr>
<td>Lung</td>
<td>0.151</td>
<td>0.065</td>
<td>-2.3</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.092 ± 0.009</td>
<td>0.34 ± 0.04</td>
<td>3.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.116</td>
<td>0.23</td>
<td>2.0</td>
</tr>
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<td>Kidney</td>
<td>0.051</td>
<td>0.13</td>
<td>2.5</td>
</tr>
<tr>
<td>Brain</td>
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<td>5.0</td>
</tr>
<tr>
<td>Lung</td>
<td>0.147</td>
<td>0.115</td>
<td>-1.3</td>
</tr>
</tbody>
</table>

Fornace et al. (18) have suggested that the induction of MGMT may be regulated at the posttranslational level, since no change was noted in the MGMT mRNA level. In our hands, Northern blot analyses of female rat liver and H4IIE cell polyadenylated RNA after irradiation showed a dose-dependent increase in MGMT mRNA, which correlated with the increase in the specific activity of MGMT. These results tended to rule out a major effect of radiation upon some posttranslational mechanism. The reasons for the difference in our results and those of Fornace et al. (18) might be related to their selection of an inappropriate probe for normalizing the mRNA levels, i.e., actin, and an inappropriate time schedule. We as well as Woloschak et al. (19) have noted an increase in actin mRNA after treatment of cells with ionizing radiation. Therefore, the use of actin mRNA levels as a normalizing procedure would seem inappropriate. We used the ethidium bromide fluorescence of the mRNA as the basis for normalization. Second, in our hands, maximum MGMT mRNA levels were seen at 12 h.

H4IIE hepatoma cells, resulting in a 3-fold induction of MGMT activity after treatment with 3 Gy. Above that level, cytotoxicity was apparent. No further increase in MGMT was seen altering the growth conditions, e.g., different serum concentrations. The ability of this tumor cell line to respond to irradiation may have been significantly dampened by the neoplastic transformation process. Furthermore, the induction may be regulated by some plasma or hormonal factors that were not included in the cell culture medium. Finally, the H4IIE cells were isolated from a male rat and therefore might be expected to exhibit a reduced response to γ-irradiation, as is apparent in the intact male rat.

Fig. 6. Effect of γ-irradiation on the MGMT activity in rat hepatoma H4IIE cells. The cells were irradiated with various doses and analyzed 48 h later for MGMT activity. These results are representative of three experiments.

Fig. 7. Time course of induction of MGMT mRNA in H4IIE cells after γ-irradiation. The H4IIE cells were exposed to 3 Gy γ-irradiation and the mRNA fraction was isolated at various times as described in Fig. 4. A. Northern analysis; B, densitometric analysis.

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Fig. 8. Effect of CHX on the level of MGMT mRNA after irradiation of H4IIE cells. The H4IIE cells were treated with CHX, 5 μg/ml, 30 min prior to (−0.5 h) or 6 h after (6 h) the irradiation (3 Gy). The CHX treatment lasted 5 min. Polyadenylated RNA was isolated as described in the text and analyzed as discussed in Fig. 4.

Fig. 9. Effect of DMSO on the induction of MGMT activity after exposure to 3 Gy γ-radiation. One ml DMSO was added to the culture medium 5 min before irradiation of the H4IIE cells. The medium was immediately changed, and the cells were grown for 48 h after the irradiation. MGMT activity was determined as described in the text.

Fig. 10. Effect of hydrogen peroxide on the MGMT activity in rat hepatoma H4IIE cells. The H4IIE cells were treated at 4°C for 30 min with different concentrations of H₂O₂ as indicated in the figure. The peroxide was removed by washing the cells twice in fresh culture medium. MGMT activity was determined 48 h after the treatment.

Table 2 Effect of protein kinase inhibitors upon irradiation-induced MGMT activity in H4IIE cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>MGMT (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.28</td>
</tr>
<tr>
<td>+ 12.5 μM H7</td>
<td>1.13</td>
</tr>
<tr>
<td>+ 25 μM H7</td>
<td>1.41</td>
</tr>
<tr>
<td>Irradiation</td>
<td>2.18</td>
</tr>
<tr>
<td>+ 12.5 μM H7</td>
<td>1.41</td>
</tr>
<tr>
<td>+ 25 μM H7</td>
<td>1.48</td>
</tr>
<tr>
<td>Control</td>
<td>1.28</td>
</tr>
<tr>
<td>+ 20 μM H89</td>
<td>0.91</td>
</tr>
<tr>
<td>Irradiation</td>
<td>2.09</td>
</tr>
<tr>
<td>+ 20 μM H89</td>
<td>2.23</td>
</tr>
</tbody>
</table>

after irradiation. Fornice and colleagues (18) isolated the mRNA from their cells at 4 and 8 h after irradiation. An additional difference in their work is the lack of information about the inducibility of MGMT activity in their cell lines; they did not determine MGMT activity after their treatments. If MGMT activity is not induced in their cell lines by ionizing irradiation, a change in MGMT mRNA would be detected. These three differences may be responsible for the differences in the results. As reported in the present paper, we have used the nuclear run-off methodology to confirm regulation occurring at the transcriptional level. A similar observation has been published by Fritz et al. (20). Finally, it is germane to mention that no significant increase in the MGMT mRNA was noted in male rats, even though an elevation in MGMT (Fig. 5) was apparent.

Studies on the mechanism of the irradiation response have been initiated. Ionizing radiation can generate hydroxyl radicals that can cause single-strand and double-strand breaks in the DNA. The hydroxyl radicals can be neutralized by radical scavengers such as DMSO. The addition of DMSO to the cell culture medium abolished the irradiation response to MGMT in the H4IIE cells, suggesting that the hydroxyl radicals may play a role in the induction response. It has been reported by Von Hove and Kennedy (21) that the irradiation of cells in an anaerobic atmosphere, which would significantly reduce oxygen radical formation, dramatically reduced the induction of MGMT. This report strongly supports our hypothesis. We have examined the effects of reactive oxygen species on H4IIE MGMT by treating the cells with different concentrations of hydrogen peroxide. MGMT activity was induced by 2-fold after exposure of the cells to 100 μM hydrogen peroxide.

Exposure of mammalian cells to DNA-damaging agents elicits a specific response known as the genetic stress response (22), which is analogous to the SOS effect in bacteria. Ionizing radiation and hydrogen peroxide share many similar characteristics, and both can induce a genetic stress response. Early work suggested that a group of genes induced by irradiation and hydrogen peroxide may be activated by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (22), thus implicating a protein kinase C-dependent pathway. Whether the induction of MGMT is regulated by a similar pathway has been examined by treating the H4IIE cells with H7, a purported protein kinase C inhibitor (17). The induction of MGMT was abolished by 10 μM H7. However, H7 is not a specific protein kinase C inhibitor in that protein kinase A is also affected by this agent (17). In order to confirm that the induction of MGMT is regulated by a protein kinase C-dependent pathway, H89, a specific protein...
kinase A inhibitor (17), was applied to the cells before γ-irradiation. No significant effect on the induction of MGMT was observed, suggesting that protein kinase C may have been responsible for the induction response of MGMT.

While ionizing radiation and hydrogen peroxide act rapidly, the induction of MGMT occurs with rather slow kinetics. CHX has been used to see if protein synthesis is required for the increased expression of MGMT mRNA. H4IIE cells were treated with CHX before and 6 h after γ-irradiation. The MGMT mRNA was not induced when CHX was included in the medium before the irradiation treatment. On the other hand, the inclusion of CHX 6 h after irradiation had no effect on the induction, suggesting that the synthesis of certain important factor(s) may be required at an early stage in the induction process.

Ionizing irradiation induces MGMT activity, even though this protein does not play any obvious role in the repair of the resultant damage to DNA. Furthermore, ionizing radiation does not produce any O'-methylguanine lesions in DNA (21). Finally, MGMT does not protect the cells from further DNA damage and mutagenesis. The possibility that MGMT may play a protective role in other ways cannot be excluded. Ionizing radiation does induce the expression of two transcription factors, c-jun and EGR1, in HeLa cells and normal human fibroblasts immediately after treatment (23). A similar induction of c-jun after irradiation has been observed in rat liver and H4IIE cells in our laboratory (data not shown). If the MGMT gene has an AP-1 regulatory element, an elevation in the expression of c-jun could lead to an induction of MGMT expression. It is clear that an interplay among hydroxyl radicals, protein kinase C activity, c-jun, and MGMT gene expression must be studied in greater detail.

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