Sensitization of Human Carcinoma Cells to Alkylating Agents by Small Interfering RNA Suppression of 3-Alkyladenine-DNA Glycosylase

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Abstract

One of the major cytotoxic lesions generated by alkylating agents is DNA 3-alkyladenine, which can be excised by 3-alkyladenine DNA glycosylase (AAG). Inhibition of AAG may therefore result in increased cellular sensitivity to chemotherapeutic alkylating agents. To investigate this possibility, we have examined the role of AAG in protecting human tumor cells against such agents. Plasmids that express low AAG protein levels were sensitized to alkylating agents. Two HeLa cell lines with AAG protein levels reduced by at least 80% to 90% displayed a 5- to 10-fold increase in sensitivity to methyl methanesulfonate, N-methyl-N-nitrosourea, and the chemotherapeutic drugs temozolomide and 1,3-bis(2-chloroethyl)-1-nitrosourea. These cells showed no increase in sensitivity to UV light or ionizing radiation. After treatment with methyl methanesulfonate, AAG knockdown HeLa cells were delayed in S phase but accumulated in G2-M. Our data support the hypothesis that ablation of AAG activity in human tumor cells may provide a useful strategy to enhance the efficacy of current chemotherapeutic regimens that include alkylating agents. (Cancer Res 2005; 65(22): 10472-7)

Introduction

Alkylating agents are used in the treatment of several human cancers. They are mostly methylating or chloroethylylating agents such as temozolomide and dacarbazine or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Cytotoxicity of these anticancer drugs results from the alkylation of DNA and is strongly attenuated by the ability of cells to repair DNA lesions (for reviews, see refs. 1–3). The major cytotoxic lesions generated by methylating agents are DNA 3-methyladenine and DNA O6-methylguanine, whereas BCNU induces a more complex array of DNA damage including ethano bases and DNA inter- and intranudand cross-links (4, 5). Besides being cytotoxic, these agents are also mutagenic and the occurrence of secondary cancers has been linked to the use of high-dose chemotherapeutic regimens. To reduce the risk of secondary cancers by decreasing treatment doses, cells must be sensitized to the cytotoxic but not to the mutagenic effects of alkylating agents. One possible approach to achieving this is to inhibit the repair of lethal DNA lesions that are not highly mutagenic.

O6-Methylguanine DNA methyltransferase (MGMT) directly demethylates DNA O6-methylguanine. This lesion is highly mutagenic due to its ability to mispair with thymine (reviewed in ref. 6). Inhibition of MGMT activity may only increase the chemotherapeutic effects of alkylating agents but also enhance the risk of secondary tumors caused by accumulation of mutagenic O6-alkylated G residues (reviewed in ref. 7). Nevertheless, inhibitors of MGMT have been tested in clinical trials as adjuncts to chemotherapy (2, 8). 3-Alkyladenine-DNA glycosylase (AAG), also called 3-methyladenine DNA glycosylase, excises 3-alkyladenine from DNA and initiates the base excision repair pathway for alkylated G residues (reviewed in ref. 8). Inhibition of MGMT has been linked to the use of high-dose chemotherapeutic agents; thus, unrepaired DNA 3-methyladenine is cytotoxic (12, 13).

Different Aag−/− murine cell lines display a range of methyl methanesulfonate (MMS) sensitivities. Aag−/− embryonic stem cells are hypersensitive to simple alkylating agents such as MMS and also to the chemotherapeutic agents BCNU and mitomycin C (14, 15). They undergo S-phase arrest on exposure to MMS, consistent with 3-methyladenine being a block to DNA replication (14, 15). E. coli and S. cerevisiae mutants lacking AAG are very sensitive to killing but not mutagenesis by methylating agents; thus, unrepaired DNA 3-methyladenine is cytotoxic (12, 13).

To determine whether AAG may be a suitable target for inhibition during chemotherapy, rather than using murine cells, we have examined the role of AAG in the defense of two human
carcinoma cell lines against alkylation toxicity. The exploitation of small interfering RNAs (siRNA) to selectively inhibit gene expression has been used previously to knockdown DNA repair activities and to examine their biological importance (22). In this article, we have used vector-based RNA interference to stably knockdown AAG protein levels in cervical and ovarian carcinoma cell lines and determined whether these cells are sensitized to alkylation agents, including those used in chemotherapy.

Materials and Methods

3-Alkyladenine-DNA glycosylase antibodies and Western blot analysis. AAG cDNAs were obtained from the IMAGE consortium and sequenced. The AAG coding sequence of IMAGE clone 359782 was subcloned into the pET29a expression vector (Merck Biosciences, Novagen, Nottingham, United Kingdom). Expression constructs were transformed into BL21-CodonPlus (DE3) (Stratagene, La Jolla, CA) and overexpressed on 10 histidine-tagged AAG proteins (tagged at the amino terminus), which were purified by affinity chromatography on Ni²⁺-nitrotetraacetic acid agarose columns (Qiagen, Crawley, United Kingdom). Polyclonal antibodies were raised against the purified protein by immunization of rabbits. Proteins (10-16 μg) in whole-cell extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane [Whatman (Schleicher & Schuell), Brentford, United Kingdom] using standard protocols. AAG was detected using the rabbit antisera at 1:100 dilution. Tubulin was also monitored using antitubulin antibodies to check for equal loading of cell extracts. The immunoblot was developed using horseradish peroxidase–conjugated anti-rabbit immunoglobulins (Amersham Biosciences, Chalfont St. Giles, United Kingdom) and a chemiluminescent substrate (Pierce, Rockford, IL).

Generation of stable cell lines with low levels of 3-alkyladenine DNA glycosylase protein. Palindromic oligonucleotides (64-mer) that will code for hairpin RNAs targeted to nucleotides 424 to 442 (CCGAGGCATGTT- CATGAA9) or 670 to 688 (CAAGAGCTTTGACCAGAGG) of the AAG open reading frame (882 nucleotides) were cloned into pSUPER (23). The resulting plasmids were sequenced to confirm correct insertion of the oligonucleotides and are referred to as pSUPER-AAG-siRNA424 and pSUPER-AAG-siRNA670. HeLa Ohio and A2780-SCA5 (clone 12780 in ref. 24) cells were transfected with the pSUPER-AAG-siRNA constructs together with the pPUR vector (BD Biosciences Clontech, Oxford, United Kingdom) that carries a selectable puromycin resistance marker. The cells were serially diluted 48 hours posttransfection and cultured for 3 weeks in DMEM containing 2 μg/ml puromycin. Twelve independent clones from each transfection were expanded. These clones were screened for reduced levels of AAG protein by Western blotting and cell lines with low AAG expression were established. Stable puromycin-resistant derivatives of cells transfected with the empty pSUPER vector together with pPUR were also cloned and had unchanged AAG protein levels.

Cytotoxicity assays. MMS and BCNU (carmustine) were obtained from Sigma (Poole, United Kingdom). N-Methyl-N-nitrosourea (MNU) and temozolomide were gifts from Peter Swann (University College London, London, United Kingdom) and Julie Silvester (Cancer Research UK, London, United Kingdom), respectively. Solutions of 0.1 mol/L MNU in 10 mmol/L potassium acetate (pH 4.8) and 0.5 mol/L BCNU in cold absolute ethanol were freshly prepared for each experiment. Aliquots of a stock solution of 0.15 mol/L temozolomide in DMSO were stored at −80°C. Cells were seeded in duplicate or triplicate in 10-cm dishes at 1,000 to 3,000 per dish. At 16 to 20 hours after seeding, the cells were treated with an alkylating agent or exposed to UV at 254 nm or to γ-radiation. Cells were treated with MMS, temozolomide, or BCNU for 1 hour, washed, and cultured in fresh medium. MNU was left in the culture overnight, during which time the MNU decomposes. Cells were exposed to UV in PBS, washed, and cultured in fresh medium. To monitor sensitivity to γ-radiation, cells were washed, resuspended in 10 ml PBS, and exposed to γ-rays from a 185Cs /C-137 source. The γ-irradiated cells were plated in triplicate (1,000-3,000 per dish) and cultured in fresh medium. After treatments, cells were cultured for 10 to 14 days and colonies were stained with Giemsa.

Flow cytometry. Cells were exposed to 1 mmol/L MMS for 1 hour and washed and fresh culture medium was added. After incubation for 6 to 48 hours, cells were harvested, washed with PBS, and fixed in 70% ethanol. After washing twice with PBS, they were treated with 100 μg/ml RNase and DNA was stained with 40 μg/ml propidium iodide. At each time point, 20,000 cells were analyzed using a FACSCalibur analytic cytometer (BD Biosciences Clontech) and the CellQuest program.

Results

3-Alkyladenine-DNA glycosylase protein and antibodies. Three plasmids (IMAGE clones 359782, 1839537, and 1723180) encoding three human AAG cDNAs were obtained from the IMAGE consortium and sequenced. All three cDNA sequences were identical to a previously reported colon adenocarcinoma AAG cDNA (accession no. L10752; ref. 25) except for two nucleotides (GC instead of CG) at positions 570 and 571 of the coding sequence. These two AAG isoforms result in two amino acid differences (Gln-Leu instead of His-Val at amino acids 190 and 191) and have been previously observed (accession no. P29372). The predicted amino-terminal sequence of AAG encoded by the three IMAGE consortium cDNAs also agreed with the result of Vickers et al. (25). A liver cDNA encoding AAG with a different amino terminus may be a splice variant (25, 26). Plasmids were constructed that encoded either full-length AAG or this protein deleted for its amino-terminal 74 amino acids (AAGΔ74). Both proteins with 10 his tags were overexpressed in E. coli and purified by affinity chromatography. 10his/AAGΔ74 is more thermostable (27) and also more active in in vitro assays (data not shown) than the full-length protein. Polyclonal antibodies were raised in rabbits against recombinant 10his/AAGΔ74 protein. These antibodies recognized full-length recombinant AAG and native AAG in crude HeLa cell extracts on Western blots (Fig. 1). They detected two closely migrating protein bands in HeLa cell extracts (Fig. 1). The same two protein bands were also recognized by a different...
polyclonal AAG antiserum (a gift from T. O’Connor, Biology Division, Beckman Research Institute, Duarte, CA; data not shown), indicating that they are both forms of AAG. Furthermore, a doublet AAG protein band was observed also when overexpressed AAG was monitored in human cell extracts using a monoclonal antibody (28). The two protein bands might correspond to the products of two AAG mRNA splice variants that have been found in several cell lines and tissues (25, 29), might have resulted from an uncharacterized posttranslational modification of AAG, or, as proposed by Rinne et al. (28), might be due to proteolytic removal of a small number of amino acids from a terminus.

Knockdown of 3-alkyladenine DNA glycosylase in HeLa cells. Two derivatives of pSUPER were constructed that express siRNAs targeted to two different regions of AAG mRNA (nucleotides 424-442 or 670-688 of the 882-nucleotide coding sequence). The constructs, pSUPER-AAG-siRNA424 or pSUPER-AAG-siRNA670, were cotransfected with pPUR into HeLa cells and stable puromycin-resistant clones were selected. The level of AAG protein in these clones was monitored by Western blotting. After transfection with either construct, 2 of 12 clones had significantly reduced AAG protein levels. The stable cell lines referred to as HeLa/pSUPER-AAG-siRNA424 and HeLa/pSUPER-AAG-siRNA670 had AAG protein levels reduced by 80% and 90%, respectively (Fig. 1). Both bands recognized by the polyclonal antibodies were reduced, suggesting that they were both forms of the AAG protein. The AAG protein level was unchanged in stable puromycin-resistant clones of HeLa cells transfected with the empty pSUPER vector.

Increased sensitivity of HeLa cells with low 3-alkyladenine DNA glycosylase protein levels to methyl methanesulfonate but not to UV or γ-radiation. To determine whether AAG protein protects HeLa cells against the toxicity of a methylating agent, we examined the sensitivity of the two AAG knockdown HeLa cell lines to MMS. Cells were exposed to MMS and survival was estimated by clonogenic assays. Both AAG knockdown cell lines were 4- to 5-fold more sensitive to killing by MMS than the control (HeLa cells stably transfected with pPUR and the empty pSUPER vector; Fig. 2). The two knockdown cell lines express siRNAs targeted to different regions of the AAG mRNA. Hence, increased MMS sensitivity of both lines indicates that this phenotype is due to low AAG protein levels and not a result of nonspecific changes of a different activity. As a further control, sensitivities of the cells to 254-nm UV light and γ-radiation were examined. The two AAG knockdown lines and the control displayed similar sensitivities to UV light or γ-rays (Fig. 2). These observations support the conclusion that MMS sensitivity is due to the specific depletion of AAG and that other DNA repair pathways such as base excision repair of oxidative damage, nucleotide excision repair, and recombination remain intact.

Sensitivity of 3-alkyladenine DNA glycosylase knockdown HeLa cells to the S₉₄ methylation agents N-methyl-N-nitrosourea and temozolomide. The major toxic methylated DNA base generated by MMS, an S₉₂ methylation agent, is 3-methyladenine (10% of total alkylation) whereas O₆-methylguanine is a minor product (0.3% of total alkylation). In contrast, MNU, an S₉₁ agent,
generates both 3-methyladenine (9% of total alkylation) and O\textsuperscript{6}-methylguanine (6.3% of total alkylation) as major products (4). Sensitivity of the AAG knockdown cells to MNU was examined. Strikingly, sensitivity of these cell lines to MNU was 10-fold greater than the control (Fig. 3A). Temozolomide is used in chemotherapy and generates the same alkylation intermediate (the methylazanionium ion) as MNU and, consequently, the same array of methylation products (2). It was therefore of interest to verify whether AAG can protect HeLa cells against this drug. Sensitivity of both AAG knockdown cell lines to temozolomide was also increased 10-fold (Fig. 3B). These observations strongly indicate that AAG affords a high degree of resistance against both MNU and temozolomide in HeLa cells.

MGMT activity was undetectable in crude cell extracts of control HeLa cells and of the two AAG knockdown derivatives (data not shown). Differences in MGMT activity are therefore unlikely to explain the enhanced sensitivity of the AAG knockdown cells to MNU or temozolomide by comparison with the control cells. Loss of MGMT gene expression is frequently associated with epigenetic silencing (30), has been previously observed in HeLa sublines (31), and may account for the lack of MGMT activity in the HeLa Ohio cells employed here.

Sensitivity of 3-alkyladenine DNA glycosylase knockdown HeLa cells to 1,3-bis(2-chloroethyl)-1-nitrosourea. Chlornitrosoureas are used as anticancer drugs and induce multiple lesions, including larger and more complex adducts than those induced by methylating agents. BCNU generates chloroethyl and hydroxyethyl base derivatives, ethano bases, and DNA intra- and interstrand cross-links (4, 5). Aag\textsuperscript{−/−} murine stem cells are sensitive to cell killing by MMS, MNU, and BCNU (14, 15). To determine whether AAG protects HeLa cells against BCNU toxicity, sensitivity of the AAG knockdown cells was examined. Both AAG knockdown cell lines showed a 10-fold increased sensitivity to the cytotoxicity of BCNU compared with the control cell line (Fig. 3C).

Cell cycle progression after methyl methanesulfonate treatment. Asynchronous populations of the HeLa/pSUPER-AAG670 knockdown cell line and control HeLa/pSUPER cells were treated with 1 mmol/L MMS for 1 hour and then allowed to grow in the absence of alkylating agent. At this MMS dose, 80% of the control cells survive compared with only 0.2% of the AAG knockdown cells (Fig. 2). Progression of the cells through the cell cycle was monitored by flow cytometry at 6, 12, 24, 36, and 48 hours after MMS treatment. Six hours after treatment, control cells were delayed in S phase but progressed to return to the same asynchronous distribution as the untreated cells at 24 hours. The AAG knockdown cells were more markedly delayed in S phase and then accumulated in G2-M (Fig. 4). These cells remained in G2-M at 24, 36, and 48 hours after MMS treatment (Fig. 4 and data not shown).

Knockdown of 3-alkyladenine DNA glycosylase in A2780 cells. To determine whether AAG depletion sensitizes a different tumor cell line to alkylation, AAG knockdown derivatives of an ovarian carcinoma cell line, A2780, were isolated. A2780 cells have MGMT activity and are proficient in mismatch repair and p53 responses (24). AAG protein levels of two isolates were reduced by 80% to 90% (Fig. 5A). MMS sensitivity of the AAG knockdown A2780 cells was examined and found to be increased by at least 2-fold at doses >1 mmol/L (Fig. 5B).

Discussion

By expressing siRNAs targeted to AAG mRNA, we have established stable HeLa cell lines with very low levels of the AAG DNA repair enzyme. These cells have a 5- to 10-fold increased sensitivity to the simple methylating agents MMS, MNU, and temozolomide and also to the chloroethylyating agent BCNU (carmustine). They were not sensitive to UV light or γ-radiation. AAG-depleted A2780 ovarian carcinoma cells were also sensitized to MMS. The phenotype of HeLa cells is comparable with that of the murine Aag\textsuperscript{−/−} knockout embryonic stem cells, which are also sensitive to MMS, MNU, temozolomide, and BCNU (14, 15), but contrasts with those of murine Aag\textsuperscript{−/−} embryonic fibroblasts, neurons, and bone marrow progenitor cells, which are less sensitive or even resistant to alkylation (17–20). Hence, in murine stem cells and human HeLa cells, as well as in E. coli and S. cerevisiae (32, 33), 3-methyladenine DNA glycosylase is a major determinant of MMS resistance whereas in other murine cell types and also in S. pombe (34), other processes such as nucleotide excision repair, recombinational repair, or DNA polymerases, which can bypass DNA damage, may play a significant role (20, 21).
MMS, MNU, and temozolomide methylate DNA at several different sites (4) but they all induce substantial amounts of 3-methyladenine, which is the major substrate of AAG (35). Hence, defective repair of DNA 3-methyladenine in the AAG knockdown cell lines is consistent with their hypersensitivity to these three agents. Formation of the analogous lesion 3-hydroxyethyladenine by BCNU does not seem to have been investigated. BCNU induces a complex array of DNA damage including several chloroethylated and hydroxyethylated bases, four different tricyclic ethano bases, and two types of DNA cross-links (4, 5). Both the E. coli AlkA and S. cerevisiae MAGE 3-methyladenine DNA glycosylases excise N2-chloroethylguanine (the most abundant lesion) and N7-hydroxyethylguanine from DNA, and E. coli AlkA has also been reported to remove diguanosinyl ethane (from N7-diguanyl DNA cross-links), N2,3,6-ethano guanine, 1,6-ethano adenine, and 3,6-ethano cytosine (33, 36–39). Activity of human AAG on these DNA lesions has thus far been observed only for 1,N6-ethano adenine (40, 41). This lesion probably arises by reaction of the chloroethyl adduct of 1-chloroethylnucleoside with the N6-amino group to form the tricyclic ethano base (Fig. 6). 1,N6-Ethano adenine interferes with base pairing and blocks in vitro DNA synthesis by both DNA polymerases α and β (42). Hence, deficient repair of this modified base is consistent with the sensitivity of AAG knockdown HeLa cells to BCNU. In this case, resistance of Aag−/− murine embryonic fibroblasts to BCNU could result from replication past these lesions by polymerase γ (42). It may now be of interest to examine the ability of AAG to repair other BCNU-induced lesions, such as N3-chloroethynucleoside, N3-hydroxyethyladenine, N7-chloroethylguanine, N7-hydroxyethylguanine, and N7-diguanyl DNA intrastrand cross-links, which are formed by interaction of abundant N7-chloroethynucleoside lesions (Fig. 6). Inhibition of apurinic endonuclease or treatment with methoxyamine that binds to abasic sites also sensitizes cells to BCNU, providing further evidence that lesions induced by this drug are repaired by the base excision repair pathway (43, 44).

Knockdown of AAG protein sensitized HeLa cells to the chemotherapeutic methylating agents temozolomide and BCNU. This is the first indication that targeted inhibition of human AAG activity might sensitize tumor cells to cytotoxic drugs. Other anticancer drugs that generate the same DNA lesions as temozolomide and BCNU include the S91 methylating agents dacarbazine, procarbazine, and streptozotocin and chloronitrosoureas such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine). Hence, inhibition of AAG might increase the efficacy of all these agents. Inhibitors of MGMT used as adjuncts to temozolomide chemotherapy have reached clinical trials (2, 8). Although inhibition of MGMT may increase cell killing by methylating drugs, it will also be expected to increase the risk of mutagenesis and the occurrence of secondary tumors (7, 30). In contrast, inhibition of AAG could increase the cytotoxicity of alkylation with a lesser risk of carcinogenicity. Simple assays for DNA glycosylases are available; thus, they are practical targets for screening of potential inhibitors. We have initiated a search for small chemical inhibitors of AAG using a high-throughput screen,1 more potent inhibitors are being sought by further screening and modeling of these compounds into the active site of the AAG three-dimensional structure (45). The level of sensitization of the AAG-depleted HeLa cells to the alkylating agents tested was high. Doses that allowed ~80% survival of the control cell line killed the AAG-depleted cells to 0.1% to 0.2% survival. AAG-depleted A2780 ovarian carcinoma cells were also sensitized but not as dramatically as the HeLa cells. AAG expression has been observed to be high in cervical neoplasia (46). Future work must therefore determine whether extreme sensitization is a particular characteristic of HeLa cells, human papillomavirus–transformed tumor cells, or rapidly proliferating malignant carcinoma cells. Further cell lines derived from different tumor types as well as human tumor explants in

1 T. Hammonds, T. Duncan, T. Lindahl, B. Sedgwick, unpublished data.
immunosuppressed mice should be examined. Secondary tumors induced by \( S_1 \) chemotherapeutic methylating agents, such as temozolomide, and some primary tumors such as hereditary nonpolyposis colorectal cancer are often deficient in mismatch repair and resistant to alkylating agents (47, 48). A further relevant extension of this work is, therefore, to determine whether AAG depletion will sensitize mismatch repair-deficient tumor cells to alkylations. These questions may be answered more succinctly when potent low-molecular weight chemical inhibitors of AAG are available.

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