DNA Damage Induced by a New 2-Chloroethyl Nitrosourea on Malignant Melanoma Cells

Denise Godeneche, Maryse Rapp, Alain Thierry, Françoise Laval, Jean-Claude Madelmont, Philippe Chollet, and Annie Veyre

Institut de la Santé et de la Recherche Médicale Unité 247, Institut Gustave Roussy, 94805 Villejuif Cedex, France

ABSTRACT

Different biological aspects of a novel 2-chloroethyl nitrosourea derived from cysteamine, N'-2-(chloroethyl)-N-N-2-(methylsulfinyl)ethyl-N'-nitrosourea (CMSOEN2), were studied. Drug-induced cytotoxic effects, uptake kinetics, DNA damage, and O6-alkylguanine-DNA alkyltransferase activity were determined in 3 melanoma cell lines: the murine B16 and 2 human metastatic-derived cell lines (M4 Beu and M3 Dau). These cell lines represent two different classes of anticancer agents, likely by saturating receptor sites (sulfhydryl groups) of this specific DNA repair enzyme. Nevertheless, in preliminary studies, the most resistant to the cytostatic drug effects showed little or no ability to form DNA lethal cross-links. These results correspond to the higher O6-alkylguanine-DNA alkyltransferase activity found in human-derived cell lines compared with that present in murine B16 cell lines. This study confirms that the cell content in this repair DNA protein is certainly one of the important factors implicated in the variability of response to 2-chloroethyl nitrosoureas treatment observed in a number of established malignant cell lines. It has been shown that pretreatment of derived cell lines with methyldrug agents (N-methyl-N-nitrosourea, N-methyl-N'-nitro-N-nitrosoguanidine or O6-methylguanine used as a free base, increased cytotoxic effects of this class of anticancer agents, likely by saturating receptor sites (sulfhydryl groups) of this specific DNA repair enzyme. Nevertheless, in preliminary Phase I and II clinical trials, 2 patients who had been treated with multiple chemotherapies including alkylating agents [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, 5-(3,3-dimethyl-1-triazeno-imidazole-4-carboxamide, platinum derivatives] presented complete or partial remission after CMSOEN2 treatment. Our results raise the question of the exact relation between the Mer phenotype determined in derived murine or human cultured cells and that directly observed on surgically excised tumors in cancer patients. The original Mer phenotype could be modified by cell culture conditions since it has been shown that O6-alkylguanine-DNA alkyltransferase activity is widely distributed between normal and tumor tissues without any real difference.

INTRODUCTION

Research on nitrosourea metabolism in our laboratory has shown that 2-chloroethyl nitrosocarbamoylcystamine, an isomeric mixture of (2-chloroethyl) nitrosoureas, is rapidly metabolized in animals (1–3). Among the 4 main plasma metabolites identified, 2 of them, CMSOEN2 and CMSOEN2, presented very promising anti-cancer properties against a variety of murine tumors (4, 5). Their anti-tumor activity spectrum includes in particular i.p. or i.e. grafted L1210 leukemia in ascitic form and solid tumor s.c. implanted in mice, such as B16 melanoma, glioma, Lewis lung, and colon carcinoma. The biodistribution and the metabolism in animals of CMSOEN2 and CMSOEN2 labeled with 14C in 3 positions have been recently investigated (6, 7). On the basis of their toxicological and pharmacological properties in various animal species (mice, rats, and monkeys) they were submitted to Phase I clinical trials at Institut de Cancérologie et d’Immun-Génétique (ICIG) (Villejuif, France). Although cytotoxicity of (2-chloroethyl)nitrosoureas was mainly attributed to DNA damage and particularly to interstrand DNA cross-linking (8–14), the molecular basis of cell resistance observed with this class of chemotherapeutic agents is not yet definitively established. The variability in the response of human neoplasms to (2-chloroethyl)nitrosoureas, but also to other alkylating drugs, has led many investigators to study their mechanism of action at the cellular level (15–17). Because of the good activity of CMSOEN2 and CMSOEN2 against B16 melanoma murine tumor and the present lack of an effective treatment in humans of this malignancy of poor prognosis, we report here some studies dealing with several aspects of the biochemical effects of CMSOEN2 upon melanoma cells of different origin: the B16 murine cell line and M4 Beu and M3 Dau cell lines derived from melanoma metastasis. In the present study, cytotoxicity, cell uptake, incorporation into macromolecules, DNA damage induced by drug treatment, and O6-methylguanine-DNA-methyltransferase activity (Mer phenotype) were determined. MATERIALS AND METHODS

Chemicals. CMSOEN2 (Fig. 1) was prepared in our laboratory and labeled with 14C on the carbon-2 of the chloroethyl moiety as previously described by Madelmont et al. (6) (5 mCi/mmol). [3H]- and [14C]-thymidine (45–50 mCi/mmol) were obtained from CEA (Saclay, France). [14C]-MNU (1.75 Ci/mmol) was purchased from Amersham.

Cell Culture. The human melanoma M4 Beu (melanotic) and M3 Dau (amelanotic) cells were established in the laboratory of Dr. J. F. Dore (Leon Berard Center, Institut de la Santé et de la Recherche Médicale Unité 218, Lyon, France) from metastatic biopsy specimens and maintained in cell culture for 10 years (18). The transplantable B16 melanoma originating in C57BL/6J Ico mice was obtained from ICIG (Villejuif, France) and adapted to growth in culture (19). Stock cell cultures were maintained as monolayers in 75-cm2 culture flasks in Eagle’s minimum essential medium (Gibco) supplemented with 10% fetal calf serum, vitamin solution, sodium pyruvate, nonessential amino acids, l-glutamine, and penicillin-streptomycin. The cells were grown in a humidified 37°C incubator containing 5% CO2. Doubling times were 24 h for M4 Beu and M3 Dau cells and 15 h for B16 cells.

Drug-induced Cytotoxicity. For colony formation assays, cells (50 to 2000) were plated into plastic Petri dishes and grown for 20 h. Various...
concentrations of CMSOEN2 dissolved in sterile water were then added. After a 2-h drug exposure, the medium was removed and the cells were grown for an additional 10 to 14 days. The dishes were rinsed with PBS, fixed with methanol, and stained with crystal violet. Colonies (>50 cells) were counted. Surviving fraction was calculated as the ratio of cloning efficiencies of treated and untreated cells. The plating efficiencies of untreated cells were absent 70, 40, and 80% for M4 Beu, M3 Dau, and B16 cells, respectively.

Radioactivity Cellular Uptake and Incorporation into Macromolecules. Exponential growth phase cells were incubated at 37°C in 90-mm plastic Petri dishes with culture medium containing 50 μM [14C] CMSOEN2. At regular time intervals, the labeled drug-treated cells were rinsed with ice-cold PBS, then gently scraped in buffer with a rubber policeman. The cell suspension was counted (hemocytometer) and centrifuged, and the pellets were suspended in 1 ml of PBS. Two equal aliquots of the cell suspension were poured onto Whatman GFB glass fiber filters, which were washed 3 times either with 5 ml of PBS for cell radioactivity uptake determination or 5 ml of 15% trichloroacetic acid for measurement of radioactivity incorporation into macromolecules. Filter radioactivity was counted after addition of 1 ml of Soluene and 10 ml of Instafluor in a Tri-Carb 4530 liquid scintillation spectrometer (Packard, Downers Grove, IL). Cell volumes were determined in a Coulter counter (Coulter Electronics, Hialeah, FL) according to the manufacturer's instructions. Radioactivity amounts, expressed as pmol drug equivalent per μl cell, were calculated from the cell volume, the specific activity of the drug, and the radioactivity of the filters.

Assay of DNA Damage by Alkaline Elution. Determination of DNA interstrand cross-links and strand breaks was performed by alkaline elution (20). Briefly after DNA labeling with [14C]- or [3H]thymidine, experimental [14C]-labeled cells either remained without any treatment or were exposed to drug for 2 h (25 to 100 μM) followed by a 3-Gy X-ray irradiation, or were directly X-ray-irradiated. The [3H]-labeled internal standard cells were all 3 Gy X-ray irradiated to study DNA lesion formation or removal kinetics. [14C]CMSOEN2-treated cells were postincubated in drug-free medium from 0 to 24 h. For elution analysis, cell suspension in PBS-EDTA solution, pH 7.4, were layered on 0.8-μm pore-size polycarbonate filters (Nuclepore Corp., Pleasanton, CA); lysed with a 0.02 M EDTA solution, pH 9.7, containing 2% sodium dodecyl sulfate and 0.5 mg/ml proteinase K (Boehringer-Mannheim, Meylan, France); and washed with a 0.02 M EDTA solution, pH 10. DNA was eluted from filters at a flow rate of 35-40 μl/min with a 0.02 M EDTA solution containing 0.1% sodium dodecyl sulfate and adjusted to pH 12.1 with tetrapropylammonium hydroxide. Fractions were collected every 90 min. The radioactivity of fractions, filters, and washings was determined and results expressed using the computation model described by Ewig and Kohn (10).

Determination of O6-Methylguanine-DNA Methyltransferase Activity. Cells were harvested by trypsinization, washed twice in PBS, and then suspended (10⁶ cells/ml) in a buffer containing 70 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.6), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. Protease inhibitors were added to the cell suspension (20 μg/ml each of antipain, leupeptin, and aprotinin), and the cells were disrupted by sonication at 0°C. Cell extracts were centrifuged at 12,000 × g for 10 min at 4°C, and the transferase activity was measured by incubating aliquots of the supernatant with [3H]MNU-treated DNA, prepared as already described, and by measuring the disappearance of O6-methylguanine from this substrate (21). Briefly, the incubation mixture contained (for a final volume of 100 μl) 70 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 5 μg of [3H] MNU-treated DNA (100 nmol of O6-methylguanine), and increasing amounts of cell extracts. Incubation was carried out for 20 min at 37°C, the substrate was then acid hydrolyzed, and the remaining O6-methylguanine was measured by high-pressure liquid chromatography (22).

RESULTS

The CMSOEN2 sensitivity of the 3 cell lines was determined by the colony-forming test. Cell survival in a drug concentration range of 10 to 100 μM, was related to the origin of the cell line used (Fig. 2). The B16 murine melanoma cells were much more susceptible to the cytotoxic effect of CMSOEN2 than its 2 human counterparts. The least sensitive were the human M4 Beu cells, while M3 Dau cells showed an intermediate sensitivity. By comparing equitoxic doses, M4 Beu cells and M3 Dau cells appeared to be, respectively, about 9-fold and 5-fold more resistant than B16 cells. These data led us to investigate whether cytotoxicity could be dependent on a different cell drug and/or metabolite uptake.

Using CMSOEN2 labeled with 14C on the chlorocthyl part (50 μM), a time course of cell uptake and incorporation of drug-derived radioactivity into macromolecules was determined for each cell line (Fig. 3). Total radioactivity uptake at 37°C was rapid during the first 2 h of incubation, then increased slowly for the next 6 h. Surprisingly the least sensitive M4 Beu cells concentrated the radioactivity more readily than did the 2 other lines. After a 2-h drug exposure, the mean cellular concentrations expressed as drug equivalents/μl cell volume were 700, 260, and 150 pmol for M4 Beu, M3 Dau, and B16 cells, respectively. The very susceptible murine B16 cell line in the colony-forming assay showed the lowest uptake; once more the human amelanotic melanoma M3 Dau cell line occupied an intermediate position. After 6 h, the cell/medium distribution ratios were about 26, 15, and 9 for M4 Beu, M3 Dau, and B16 cell lines, respectively. The kinetics of radioactivity incorporation into the acid-precipitable material perfectly mimicked that of total drug-derived radioactivity uptake. It was noteworthy that after 30 min as well as after 15 min almost all cellular radioactivity was bound to acid-precipitable macromolecules, the most resistant cells incorporating the highest amount of drug equivalents (Fig. 3).

In order to correlate cell sensitivity to DNA repair damage,
we compared DNA alkaline elution rates of 14C-labeled cells, which received only a 3-Gy irradiation or a 2-h drug treatment (100 µM) without postincubation in drug-free medium (t = 0 h) plus a 3-Gy irradiation (Fig. 4). Treated M4 Beu and M3 Dau cells did not show a significant decrease in their DNA elution rate, which paralleled that of control cells. By contrast, DNA of similarly treated B16 cells eluted from the filter at a slightly lower rate. This phenomenon was more pronounced after a 12-h drug-free postincubation and at this time no evidence of cross-link formation in DNA was obtained for M4 Beu cells, but a reduction in the elution rate was noted for M3 Dau cells. Compared with M4 Beu cells and, to a lesser extent, M3 Dau cells, a marked decrease in DNA elution rate was observed in murine B16 cells. Analysis of the curves confirmed the high frequency of drug-induced cross-links in B16 cells that accumulated during the 12 h postincubation in drug-free medium. After a 12- to 24-h postincubation, there was an apparent elimination of cross-links (Fig. 5). The DNA cross-link formation rate in M3 Dau cells, although much lower than that found in murine cells, was also maximum after a 12-h postincubation. In the case of M4 Beu cells, whatever the postincubation time, DNA elution rate was similar to that of DNA control cells. Using this assay, DNA cross-link frequency was in decreasing order as follows: B16 > M3 Dau > M4 Beu. It was, however, difficult to analyze and compare accurately the kinetics of DNA strand break formation in each cell line. Indeed, the fact that DNA cross-link frequency differed widely from one cell to another could mask the exact number of drug-induced strand breaks. Nevertheless, the plots (Fig. 4) of elution rate of treated and unirradiated cells without postincubation period (t = 0 h) showed an evident increase of DNA elution in the 3 cell lines. At the time of drug removal from the medium, DNA B16 cell elution rate was likely to be underestimated by the early appearance of DNA cross-links. It was clear through that CMSOEN$_2$ induced DNA strand breaks in all 3 cell lines. Despite that, the interpretation of the DNA strand break or removal frequency was neither misleading nor critical for hu-
DNA damage from CMSOEN on malignant melanoma cells

Fig. 5. Plots of DNA interstrand cross-link frequency versus time of drug-free postincubation. M4 Beu (■), M3 Dau (○), and B16 (▲) melanoma cells. Results expressed as Gy equivalent.

Fig. 6. Removal of O' Me-Gua from [3H]MNU alkylated DNA by M4 Beu (■), M3 Dau (○), and B16 (▲) melanoma cell extracts.

DISCUSSION

Preclinical screening including several murine models showed the good activity of CMSOEN, a newly synthesized 2-chloroethylnitrosourea, against a variety of ascitic or solid tumors implanted in mice (4, 5). The s.c. grafted B16 melanoma, in particular, proved to be very sensitive to the anticancer properties of the drug. However, in the preliminary Phase I and II clinical trials, a variability in the drug response rate for patients suffering from advanced malignant melanoma was observed. In order to define more precisely the mechanism by which CMSOEN acted at the cellular level, we studied its effect on several biological and biochemical parameters in melanoma lines of different origin maintained in cell culture: the murine B16 and 2 human melanoma M4 Beu and M3 Dau cell lines. In the colony-forming assay, the murine B16 cells exhibited the highest sensitivity to drug treatment with a surviving fraction close to 0.1% at the 50 μM dose. These data were consistent with the excellent CMSOEN activity observed with experimental tumor model in vivo. The relative drug resistance to treatment showed by the 2 human-derived cell lines, especially M4 Beu cells, could not be explained by a defect in cell incorporation of drug and/or metabolites. Indeed, we found that drug-derived radioactivity was taken up by cells and incorporated into macromolecules at a rate inversely proportional to that expected from the survival curves obtained from the colony-forming assay. Therefore, no correlation appeared between the extent of cell killing and the drug or metabolite concentrations. We do not intend to develop here the mechanism by which 2-chloroethylnitrosoureas are supposed to act at the DNA level. Several well-documented reports have been published in this field (8, 11, 13). Investigations on CMSOEN-induced DNA damage and repair using the alkaline elution method showed that drug cell sensitivity was essentially related to DNA cross-link frequency and thus confirmed the importance of this type of DNA lesion in the manifestation of the cytotoxic effects produced by chloroethyl agents. From our data, it was evident that the 3 melanoma cell lines studied did not possess the same ability to repair certain DNA lesions. To our knowledge, comparison of the extent of DNA damage and the repair mechanism of murine and human malignant melanoma cells have not yet been published. Using other murine or human cell line strains, several authors have shown that O'-alkylguanine-DNA alkyltransferase activity was directly implicated in the repair of DNA damage induced by different alkylating drugs, including methylyating agents (N-methyl-N'-nitroso-N-nitrosoguanidine, MNU), 2-chloroethylnitrosoureas [1,3-bis(2-chloroethyl)-1-nitrosourea], and other chloroethylnitrosoureas such as derivatives of the 2-chloroethylnitrosourea series (May & Baker Ltd., London 39565) (23–26). Of the cell lines studied, only murine B16 melanoma presented an important defect in the repair ability of DNA lesions produced by alkylating agents. As stated by Lindahl (27, 28), human cells are more proficient at repairing DNA damage than cells derived from mice or other short-lived animals. A similar observation was also made by Harris et al. (29) in a study that compared the extent of DNA repair in normal murine (spleen, thymus, bone marrow) and human lymphocytes treated with methylating carcinogens. Day et al. (30) defined a classification between Mer and Mer* phenotypes in human cell lines on the basis of their ability to counteract the DNA biochemical lesions induced by this class of anti-cancer agents. Although numerous published data exist on the effect of alkylating drug DNA upon murine and human-derived cell lines maintained in culture (31, 32), little information is available regarding the O'-alkylguanine-DNA alkyltrans-
ference activity in human normal and tumoral cell or tissue extracts. Waldstein et al. (33), in a study dealing with the level of this DNA repair protein in chronic lymphocytic leukemia and its ability to remove O6-methylguanine, have found a 7-fold greater activity in B lymphocytes of patients than in the counterparts of normal individuals. Wiestler et al. (34) and Myrnes et al. (35) compared the O6-alkylguanine-DNA alkyltransferase activity in human brain tumors and corresponding peripheral normal tissue and did not find particular differences between normal and pathological brain enzyme concentration. Moreover, expression of the protein in other normal tissues differed slightly from one patient to another. As an exception to the above-described findings, significantly lower levels of repair protein in some histological types of brain tumors (gliomas) were described (one case of hepatocellular carcinoma and one of hypernephroma). A recent review of D'Incalci et al. (36) pointed out the discrepancies observed by several authors between the Mer phenotype of human transformed or derived cell lines, and those found in tumors removed after surgery. According to them, about 30% of human cells maintained in culture posses the Mer~ phenotype. These data suggest that culture conditions could perhaps favor the selection or the development of cell populations either overproducing O6-alkylguanine-DNA alkyltransferase or lacking this repair protein (37). The murine B16 line was selected for its high drug sensitivity in preclinical screening (5). M4 Beu and M3 Dau melanoma cells were included in this study as human models, the 2 cell lines showing different biological characteristics (18, 38). Interestingly, among 10 patients suffering from advanced malignant melanoma and submitted to CMSOEN2 treatment in preliminary clinical trials, 2 of those having received heavy prior chemotherapy, including alkylating agents such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide, and platinum derivatives, demonstrated good drug sensitivity. According to the WHO classification, one patient presented a partial response (brain metastasis) and the other a complete response (skin, liver, and bone metastasis). These results seem perhaps contrary to the concept of an inductive mechanism of O6-alkylguanine-DNA alkyltransferase production by certain chemical agents (36), but a survey of the recent literature concerning the tumor resistance to a number of anticancer drugs shows that this problem is far from being elucidated. The present data underline the difficulty of correctly correlating the in vitro and in vivo situations and also the great complexity of research in this field. However, the Mer phenotype of a tumor is obviously one of the biochemical parameters that could be responsible for resistance to 2-chloroethylnitrosoureas. An early knowledge of the tumor phenotype responsible for 2-chloroethylnitrosourea resistance could assist in the selection of the treatment having the best chance of success in patients suffering from certain types of cancer diseases (39, 40). Another promising topic of investigation would be the design of specific inhibitors able to selectively lower or suppress O6-alkylguanine repair in Mer~ tumoral tissues.

ACKNOWLEDGMENTS

We thank Jean-Michel Dupuy and Nadine Gallais for technical assistance, Dr. Jean-Pierre Magnin for English corrections, and Jacqueline Lefrançois for preparing and typing the manuscript.

REFERENCES

DNA DAMAGE FROM CMSOEN1 ON MALIGNANT MELANOMA CELLS


DNA Damage Induced by a New 2-Chloroethyl Nitrosourea on Malignant Melanoma Cells

Denise Godeneche, Maryse Rapp, Alain Thierry, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/18/5898

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.