Dependence on Treatment Time of Melphalan Resistance and DNA Cross-Linking in Human Melanoma Cell Lines

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ABSTRACT

In the human melanoma cell line MM127, the melphalan survival curve was linear and exhibited reciprocity with respect to concentration and treatment time. The survival curve of an allogeneic line, MM253c1, exhibited a shoulder and, on a concentration × time basis, was resistant to 1-hr compared with 4-hr treatment. This type of resistance, which was not found using chlorambucil, nitrogen mustard, or methyl methanesulfonate, could be overcome by simultaneous hyperthermia (42°) but not by treatment with thymidine or caffeine. Both lines had similar levels of DNA interstrand cross-linking (perchlorate renaturation method) after 1-hr treatment, but MM253c1 cells were able to repair most of this damage during the next 23 hr. The cross-links formed in MM253c1 cells after 1 hr were predominantly heat sensitive and photoresistant, whereas those formed in MM127 cells were heat resistant and photosensitive. These results suggest that melphalan formed repairable (possibly diguaninyl or adeninyl-guaninyl) cross-links in MM253c1 cells during the first hr of treatment and nonrepairable, possibly diguaninyl cross-links in MM127 cells at all stages of treatment. It appears therefore that the mode of action of melphalan and the effect of synergistic agents may not be identical in all cells.

INTRODUCTION

Studies of human and rodent cell lines resistant to the bifunctional antitumor agent melphalan (phenylalanine nitrogen mustard) indicate that cytotoxicity results from the formation of DNA interstrand cross-links (2, 17, 28), resistance to killing being attributed to the ability of the cell to reduce such damage by inhibited cell (5) or nuclear membrane (21) transport, or by mechanisms as yet unknown (18). Despite the many different DNA alkylation sites reported for monofunctional agents (13, 26), only diguaninyl derivatives have been detected in double-stranded DNA using nitrogen or sulfur mustards (1, 3, 13). No information is available for melphalan, but the lack of rapid cross-link repair (18, 23) invites comparison with the reduced ability of many mammalian cells to exercise N-3 adenine substitution accounts for 10 to 20% of the total DNA alkylation levels required to achieve toxicity with these agents (1). The absence of melphalan labeled sufficiently to distinguish guaninyl from adeninyl adducts and the failure of previous workers to obtain any stable product from the reaction of melphalan with DNA (1) prompted exploration of indirect methods derived from known base-specific properties, namely, photosensitised oxidation of guanine (24, 29) and heat depurination of alkylated adenine (12).

We now report that brief melphalan treatment of a particular human melanoma cell line results in the formation of repairable, nontoxic DNA interstrand cross-links which are chemically distinguishable from the unrepaired, toxic cross-links formed in this cell line by prolonged treatment and in another melanoma cell line by either brief or prolonged treatment.

MATERIALS AND METHODS

The origin and general properties of the human melanoma cell lines MM253c1 and MM127 have been described (19, 20, 30). Cells were cultured in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) containing 10% fetal calf serum, 100 IU penicillin/ml, 100 µg streptomycin/ml, and 10 µM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid. Routine assays (10, 19) for Mycoplasma were negative.

Cell survival was determined as described previously (10) by treating cells seeded 24 hr earlier (2 x 10⁶/10-mm well) with the cultures with complete medium to terminate treatment, and [³H]thymidine labeling of colonies formed 8 days after treatment.

Uptake of [³H]melphalan by cell monolayers was determined as previously described (18). The concentration ratio (ratio of intracellular concentration to extracellular concentration) was calculated using measured cell diameters of 23.4 µm for MM253c1 and 18.2 µm for MM127.

For determination of DNA interstrand cross-linking using the renaturation assay (8, 14, 18), cells (5 x 10⁶/60-mm plate) were prelabeled for 24 hr with [²¹⁴C]thymidine (0.4 µCi/ml, 57 Ci/mmol; Radiochemical Centre, Amersham, Buckinghamshire, United Kingdom) or [methyl-³H]thymidine (0.4 µCi/ml, 40 Ci/mmol; Radiochemical Centre). The cultures were washed once and incubated in medium at 37° for 30 min, and the ³H-labeled cells were treated with melphalan using the times and doses specified. At the end of the treatment period, cultures were washed once and refed fresh medium. Cells were harvested (trypsin), and the treated cells were mixed with the controls, washed in Tris buffer (0.1 M NaCl-10 mM Tris-2 mM EDTA, pH 8), and kept at 0°. Cells (10⁶) in 20 µl Tris buffer were mixed with 50 µl NaCO₃ (0.05 M, pH 10.5) containing 0.5% Sarkosyl and heated at 75° for 5 min. After addition of 0.5 ml 7 M NaClO₄, 1 ml EDTA, and 0.5% Sarkosyl (pH 7.5), the solution was sheared 3 times in a Pasteur pipet, heated at 75° for 5 min, and immediately cooled in ice water for 10 min. After 24 hr at 4°, 50-µl duplicates were removed for digestion with S₁ nuclease (200 units; Sigma Chemical Co., St. Louis, MO) in 1 ml of buffer containing native calf thymus DNA (100 µg/ml), heat-denatured DNA (10 µg/ml), 0.1 M KCl, 0.1 mM ZnSO₄, and 25 mM sodium acetate (pH 4.5). After 60 min at 37°, the solutions were precipitated with cold 10% trichloroacetic acid and washed onto glass-fiber discs with ethanol for liquid scintillation counting. The heat treatment to destroy adenine cross-links was carried out prior to renaturation assay.
RESULTS

Melphalan Survival Curves. The melphalan dose responses for survival of MM127 cells using different treatment times were almost linear (Chart 1A). As anticipated from the 60-min half-life of melphalan under these conditions due to spontaneous hydrolysis (18), 4-hr treatment was almost as toxic as continuous treatment and only twice as toxic as a 1-hr treatment (Chart 1A). The MM253c1 line was much more resistant than MM127 at all treatment times. In addition, the survival curves of the former line had pronounced shoulders, and the difference between the 1-hr and longer treatment times was much greater (Chart 1B). The latter effect was evident as both an increased shoulder ($D_0$ 6.1 $\mu$M) and slope ($D_0$ 4.8 $\mu$M) for the 1 hr treatment compared with the other treatment times (2.6 and 1.9 $\mu$M, respectively).

A more detailed temporal response study using a single melphalan dose showed that extreme resistance of MM253c1 cells occurred only during the first hr of treatment (Chart 2A). Continuation of treatment progressively reduced the survival level but not in the exponential decay fashion as observed for MM127 cells or as anticipated from the hydrolysis of melphalan determined chromatographically (18) or by bioassay (Chart 2A). Plotting cell survival against the integrated product of melphalan concentration and treatment time provided a more direct comparison of the effect of treatment time. On this basis, MM127 cells were equally sensitive to 1-hr or 4-hr treatments, whereas MM253c1 cells still showed resistance to a 1-hr treatment (Chart 2B). Appropriate control experiments showed that the difference between the 2 cell lines was not due to time-dependent differences in pH, temperature, or conditioning of the medium.

As found previously using the uncloned MM253 cell line (18), MM253c1 cells accumulated [3H]melphalan rapidly during the first 10 min, reaching a concentration ratio of 3 which was maintained for the 60-min observation period. The rate of uptake and concentration ratio achieved by MM127 cells were identical to those of MM253c1 (results not shown).

Cell Survival Using Other Alkylating Agents. The treatment time dependence of survival of MM127 and MM253c1 cells was also compared using 2 other nitrogen mustard derivatives (chlorambucil and HN2) and a monofunctional alkylating agent (MMS). The MM253c1 1-hr survival curve for chlorambucil, a less reactive agent than melphalan and HN2, lacked a shoulder and was somewhat closer to the survival curve of MM127 cells compared with the survival response to the latter compounds (Chart 3A). Prolonged treatment led to an equally large decrease in survival in both cell lines. Apart from showing a small shoulder with MM253c1 cells, the relative survival of both lines following HN2 treatment was similar for brief or prolonged treatment (Chart 3B). Both cell lines exhibited shoulders when treated with MMS for 1 hr, but the difference in survival between the lines was not greatly affected by prolonged exposure to the drug (Chart 3C).

Combination of Melphalan with Other Treatments. Melphalan treatment was combined with nonlethal levels (50 to 100% survival) of 2 compounds reported to enhance the toxicity of alkylating agents in mammalian cells, caffeine (17) and thymidine (16). After allowing for the somewhat greater inhibition of MM127 cells by caffeine or thymidine used alone, these results indicated that melphalan survival was not affected in either line (Table 1). As a result of mechanistic studies (see below), the cells were treated simultaneously with melphalan and hyperthermia for 1 hr. This combination had a much greater effect on the survival of MM253c1 cells than on MM127 cells, as judged by decreases in $D_0$ and $D_0$ (Chart 3D, Table 1). Hyperthermia, on the other hand, enhanced survival equally in both cell lines (Table 1).

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2 The abbreviations used are: HN2, nitrogen mustard; MMS, methyl methanesulfonate.
in the proportion of rapidly renaturable DNA compared with controls (typically 20 to 25% of the total DNA). The level of cross-linking induced by melphalan in MM253c1 cells was slightly higher than that found previously in the parent line (18), presumably because of the additional step of heating at pH 10.5 prior to denaturation in sodium perchlorate. This procedure has been shown to "fix" cross-links by opening the imidazole ring and preventing depurination of N-7-alkylated guanine residues (11). In the present work, separate experiments showed that heating cell lysates directly in perchlorate at 75° for 30 min destroyed cross-linking, whereas heating first at pH 10.5 completely retained cross-linking during subsequent heating in perchlorate.

The melphalan dose response in MM127 and MM253c1 cells showed the greatest increase in cross-linking up to 16 µM, with a much smaller increase at 32 µM (Chart 4A). A 4-hr treatment produced only a 20 to 50% increase in cross-linking compared with a 1-hr treatment, the amount of cross-linking being similar in both lines (Chart 4C). When the cells were allowed to recover for 23 hr after a 1-hr treatment, cross-linking in MM253c1 cells fell to very low levels compared with that in MM127 cells (Chart 4B). This difference was also evident, although less pronounced, in cells treated for 4 hr (Chart 4D).

The thermal and chemical stability of the cross-links formed by 16 µM melphalan was compared in both cell lines. Heating 1-hr-treated MM127 cells for 2 min in Tris buffer before lysis at pH 10.5 had little effect, whereas the cross-linking in MM253c1 was almost completely abolished (Chart 5A). With a 4-hr drug treatment, approximately 50% of the cross-links were heat sensitive in both cell lines (results not shown). After a 21- to 24-hr recovery period in the absence of melphalan, the level of heat-sensitive cross-links was much lower in both lines (Chart 5C).

Preliminary studies of photosensitized destruction of cross-links showed that 2 µg methylene blue/ml was a sufficiently low

DNA Interstrand Cross-Linking. Alkaline elution was found to be unsuitable in this study for detecting cross-links, because the control DNA was highly fragmented. This was presumably due to apurinic sites generated during the treatments used to distinguish the 2 types of cross-link. A modification of the neutral perchlorate method (8, 14, 18) was therefore used. Melphalan-induced interstrand cross-linking was measured as the increase
The solution was diluted with 1 ml H2O and the absorbance was measured at the wavelength maxima indicated. •, deoxycytidine (274 nm); A, 7-methylguanosine (287 nm); D, deoxyguanosine (260 nm); O, thymidine (268 nm); Δ, 7-methylguanosine (287 nm); E, deoxyguanosine (280 nm); A, melphalan (290 nm).

level to avoid interference from laboratory lighting while allowing the desired reaction to proceed rapidly under intense illumination. It was also found that the rate of cross-link decomposition under these conditions was, like that reported for destruction of guanine residues (29), greatly increased by alkaline pH. Methylene blue and light had no effect on cross-linking when used separately. The photosensitivity under these conditions of 7-methylguanosine and the 4 deoxynucleosides was determined spectrophotometrically (Chart 6). Only deoxyguanosine and, to a lesser extent, 7-methylguanosine were affected by exposure to light for up to 4 min. Melphalan, present as the hydrolyzed diol at this pH, was also quite sensitive (Chart 6). The renaturation level of 14C-labeled control DNA (20 to 25% of the total label) decreased to 15 to 20% after methylene blue and light treatment, indicating that destruction of unsubstituted guanine residues did not greatly affect the rapid reassocation properties of the DNA.

Comparison of MM127 and MM253c1 using this method showed that 1-hr-treated MM127 but not MM253c1 cells contained cross-links sensitive to 0.5-min illumination in the presence of methylene blue (Chart 5B). Like the complementary thermal sensitivity, this difference was quantitative; illumination for 2 min further decreased the cross-linking in both cell lines. The cross-links formed after a 4-hr drug treatment were sensitive to a 0.5-min exposure in both lines. Cells treated for 1 hr and allowed to recover for 21 to 24 hr showed little difference in photosensitivity of the remaining cross-links (Chart 5D). Cross-links formed by HN2 in MM253c1 cells (3 μM for 1 hr) were also sensitive to a 0.5-min exposure (results not shown), indicating that loss of cross-links in the above experiments need not have resulted merely from destruction of the aromatic ring of the melphalan molecule.

DISCUSSION

The melphalan survival curves of the MM253 parent line (10, 18) and early passages of the MM253c1 clone (18) had no shoulder, had D0 values (1.1 μM) considerably less than those of current passages of MM253c1, and were not resistant to 1-hr treatment (10). The MM253c1 line was also more resistant to 1-hr hyperthermia than MM253 (10). These differences cannot yet be explained, but could be associated with the spontaneous increase observed (19) in the DNA content and chromosome number of MM253c1 during prolonged culture. The corresponding properties of MM127 cells (10, 30) have remained unchanged.

The excessive resistance of MM253c1 cells to 1-hr treatment and the nonreciprocal C × t relationship extended to physiologically obtainable levels (27) of melphalan. Thus, 90% cell survival after 1-hr treatment with 5 μM melphalan was reduced to 10% after prolonged treatment. One implication of this result, that the toxicity of prolonged treatment would also be achieved by dose fractionation over shorter periods, has been verified in preliminary experiments, but no schedule has yet been found which improves upon the toxicity of the prolonged, single dose.

Measurement of total DNA interstrand cross-linking after 4-hr treatment showed only slightly more damage in MM127 compared with MM253c1 cells, thus excluding drug transport as a mechanism of resistance. After 21-hr recovery, however, this difference was much greater, and therefore, the overall resistance of MM253c1 cells could be attributed to more proficient repair of such cross-links. As anticipated from the melphalan decay curve, most of the DNA cross-linking occurred during the first hr of treatment, and again, the level of damage was similar in both cell lines. A difference in drug transport was also excluded by direct measurement of melphalan uptake. The high degree of resistance of MM253c1 cells to 1-hr treatment could therefore have been explained by ability to substantially repair 1-hr damage. Further studies were needed, however, to determine
whether MM253c1 differed from MM127 cells in the type of cross-link formed or in the type of repair system present.

The 2 complementary methods used for studying the nature of melphalan-induced cross-links were based upon known properties of alkylated DNA (12, 13, 24, 29), the base specificities for the present purpose having been also demonstrated in a general sense in this work and in DNA-sequencing studies (7, 15). The results from analysis of cross-links formed after 1 hr could therefore be interpreted as showing that MM253c1 cells form predominantly N-7 guanine cross-links (heat resistant and photosensitive) over the whole of the effective treatment time. These cross-links may be removed by a slow process, such as spontaneous depurination. MM253c1 cells would be considered to form predominantly N-3 adenine cross-links (heat sensitive and photostable) in the first hr and to subsequently repair this damage. Continued treatment of MM253c1 cells, on the other hand, would increase the proportion of N-7 guanine, nonrepairable, cross-links.

While this hypothesis explains both the survival and cross-linking results, several uncertainties remain. (a) The lack of qualitative differences between adeninyl and guaninyl cross-links and the variability of the renaturation method at very low cross-linking levels have so far prevented conclusive analysis of the nature of cross-linking in cells treated for 4 hr and in cells allowed to recover for 24 hr. Attempts to use the alkaline elution or sedimentation methods (19, 23) were unsuccessful, because extensive strand cleavage of heated or illuminated control DNA occurred under alkaline conditions. This was due presumably to the many apurinic sites expected after such treatments. (b) The photosensitivity of melphalan itself and differences between alkylating agents in steric and other effects may mean that the base specificities of the model systems, which are based on untreated or methylated DNA, are not fully applicable to the properties of melphalan-induced cross-links. It should also be noted that, although the photooxidation experiments were carried out using cell lysates, which should contain naked DNA, profilavine-light treatment of intact HeLa cells was reported to result in pyrimidine rather than purine damage (22). Such questions may be difficult to resolve directly in view of the previous lack of success in isolating adducts from melphalan-treated DNA (1).

The reason why the nature of the DNA cross-linking should differ in these cell lines after a 1-hr treatment is not yet clear. Previous (19) and current studies using methylaing agents indicate that the MM253c1 line, as a variant of the Mer- phenotype (4), is deficient in the ability to excise O\(^{\alpha}\)-methylguanine. Therefore, it is highly unlikely that 1-hr resistance arises from repair of O\(^{\alpha}\)-alkylguanine monoadducts as found for the cross-linking agent 1-(2-chloroethyl)-1-nitrosourea in Mer\(^{\alpha}\) phenotypes (6). MM253c1 cells have a similar DNA content but a shorter doubling time (24 hr) compared with MM127 cells (48 hr), and, since a HN2 analogue was reported to preferentially alkylate adenine in single-stranded DNA (20), it is possible that, in MM253c1 cells, guanine cross-linking in G\(_{-}\) and G\(_{2}\)-phase DNA occurs more slowly and that repairable, possibly mutable, adenine cross-linking in S-phase DNA occurs more rapidly than in MM127 cells. Thus, the total cross-linking would initially be the same in both lines.

The failure of thymidine and caffeine to display synergism for melphalan toxicity is perhaps not surprising in view of the complex nature of alkylation damage and the widely differing ability of human tumor cells to repair at least one kind of lesion (4). Hyperthermia, however, had been shown previously to be synergistic with prolonged melphalan treatment in the MM253 parent line (10) and to inhibit DNA replication and repair of UV damage in human melanoma cell lines (9). The absence of a reverse effect using hyperthermia tends to rule out changes in drug transport or melphalan reactivity. It is therefore possible that 1-hr hyperthermia inhibits the subsequent cross-link repair in MM253c1 cells.

Apart from highlighting the individual variation in human cell lines, the relevance of these findings to in vivo effects cannot be assessed until more cell types are examined; of 4 other human melanoma lines, 2 have shown time-dependent resistance in preliminary studies. It is possible that either brief or prolonged melphalan treatment may be desirable, depending on the C \(\times\) t response of the normal and tumor cells in each individual.

**REFERENCES**


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