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Modification of potentially lethal damage in irradiated Chinese hamster V79 cells after incorporation of halogenated pyrimidines

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Abstract. Radiosensitization of exponentially growing and plateau phase Chinese hamster V79 cells by incorporation of halogenated pyrimidines (HP) was investigated for different culture conditions that influenced repair. For this purpose cells were grown for 72 h with 0, 1, 2 and 4 µM of chloro-(CldUrd), bromo- (BrdUrd) or iodo-deoxyuridine (IdUrd) and were subsequently irradiated with gamma-rays from a 137Cs source, either in exponential growth or in plateau-phase. Cell survival after irradiation was determined by clonogenic assay. In exponentially growing cultures thymidine-replacement in the DNA of the cells after incubation with 4 µM of CldUrd, BrdUrd and IdUrd was 22±5, 32±7 and 12±7%, respectively. In plateau-phase cultures the percentage thymidine replacement in the DNA of the cells after incubation during growth with 4 µM of CldUrd, BrdUrd and IdUrd was 27±5, 33±8 and 10±7%, respectively. Linear-quadratic analyses of the radiation survival curves were performed. In exponentially growing cells a marked increase by a factor 2–3 of the value of α was observed. The β term significantly increased only in cells which were grown in the presence of BrdUrd and which were trypsinized and replated immediately after irradiation. In plateau-phase cells which were trypsinized and plated immediately after irradiation both α and β increased up to a factor 2–3 with increasing incorporation of halogenated pyrimidines. In plateau phase cells which were allowed to repair potentially lethal damage (PLD) for 6 h and subsequently trypsinized and plated, α increased by a factor 3–4. In these latter conditions changes in β were smaller. In exponentially growing cells in which repair was allowed after irradiation by plating prior to the treatment, the α values decreased for all the HP drugs tested as compared to the α of cells plated immediately after irradiation. In contrast, delay of plating for plateau phase cells yielded increased α values not only when compared with the α of plateau phase cells plated immediately after treatment but also when compared with the α value of radiosensitized exponentially growing cells. The increase of α might be interpreted as an enhancement in the expression of PLD. The larger contribution of fixation of PLD might be due to initial DNA damage and/or to inhibition of PLD repair resulting from incorporation of HP. The increase of β might be attributed to enhanced interaction or to fixation of sublethal damage (SLD). In view of clinical applications of HP it is of interest that sensitization is not abolished in plateau-phase cells.

1. Introduction

Incorporation of halogenated pyrimidines (HP), chloro-, bromo- and iodo-deoxyuridine (CldUrd, BrdUrd, IdUrd) into DNA is known to sensitize cells to ionizing radiation (Lett et al. 1964, Szybalski 1974, Iliakis et al. 1987a, 1989, Miller et al. 1992a,b). The induced radiosensitization increases with the degree of thymidine-replacement (Erikson and Szybalski 1963, Phillips et al. 1989). The mechanism of radiosensitization by the HP has been suggested to be either an increase in the amount of DNA damage induced by radiation, (Kinsella et al. 1984, Iliakis et al. 1989, 1991, Ling and Ward 1990, Webb et al. 1993, Jones et al. 1995), an influence on repair of sublethal damage (SLD), and/or an enhanced expression of potentially lethal damage (PLD) (Iliakis et al. 1987a,b, Wang and Iliakis 1992, Wang et al. 1994). Since different processes are involved in these phenomena several mechanisms might contribute to radiosensitization.

Additional complexity of the mechanisms causing HP-induced radiosensitization is suggested by results of Iliakis and co-workers, who showed that the degree of sensitization can be lower in plateau-phase cells as compared to proliferating cells (Iliakis et al. 1987a, Iliakis et al. 1992, Wang and Iliakis 1992, Wang et al. 1994). These authors hypothesized that alterations in chromatin conformation as cells enter a resting G0-like phase, stabilizes chromatin and enables repair of lesions that would have been fixed in proliferating cells.

HP have been suggested to provide an advantage in radiotherapy as radiosensitizers of cells in rapidly growing tumours, in particular in clinical conditions in which critical normal tissues show limited proliferation and, as a consequence, take up less HP. Labelling depends on the growth fraction, cell loss, cell cycle time and potential doubling time (Steel 1977, Rodriguez et al. 1994). Of special importance for sensitization is the rate at which non-cycling cells are recruited into the proliferative compartment during exposure to HP and a course of radiotherapy. However, even in rapidly growing tumours, cells may, after proliferative cycles, move into a non-proliferative stage. This might compromise the degree of radiosensitization if resting cells are less affected by HP, or are better able to cope with
additional damage by repair of PLD. Further information and insight in the mechanisms involved is therefore of interest for the selection and optimization of treatment schedules in clinical applications.

Sensitization of cells by HP has been commonly described in terms of various parameters of survival curves, either using the single-hit multitarget model with the extrapolation number \( N \) and the final slope given by \( D_0 \) as parameters, or the linear-quadratic model (LQ) describing lethal events as a function of the dose \( D \) with two parameters: \( f(D) = \alpha D + \beta D^2 \). This model is based on well accepted biophysical concepts, involving the assumption that lethal damage can be induced by single-particle tracks and by accumulation of multiple-particle induced damage. It has been found to describe the low-dose region of the survival curves better than the single-hit multitarget model. Furthermore the LQ-model has been shown to describe, adequately, fractionation effects for normal tissue tolerance and for experimental tumours. The LQ-model also has the advantage that it requires only two parameters to describe survival curves. It allows the separate analysis of changes in effectiveness in the low dose range, mainly determined by the linear term and in the high dose range determined mainly by the quadratic term (Barendsen 1982, Joiner 1993). An additional advantage of the LQ model is that its parameters can be discussed in terms of specific mechanisms of cell inactivation by radiation (Barendsen 1990, 1994). Linear-quadratic analyses of HP-induced radiosensitization have been reported for exponentially growing human tumour cells and for four different experimental rodent cell lines (Miller et al. 1992a,b, van Bree et al. in press). An increase of the \( \alpha \)-component was observed, corresponding to an enhanced lethal damage at low doses. The \( \beta \)-component, which is assumed to depend on the interaction of sublethal lesions (SLD), was hardly affected by the observed radiosensitization. In both studies the most radioresistant cell lines were more sensitized than the radiosensitive lines.

As mentioned earlier, in the literature data have been presented concerning differences in radiosensitization between exponentially growing and plateau-phase cells and in the repair of additional lesions produced by HP uptake (Iliakis et al. 1987a, Wang and Iliakis 1992). Analyses of these data were not based on the LQ model, but nevertheless a large contribution of repair of PLD was shown. A further study of sensitization of cells in plateau phase and of PLD repair in HP sensitized cells using the LQ formula is evidently of interest in order to assess quantitatively the influence of HP in the range of low doses of 0–3 Gy, important in radiotherapy.

In the present communication radiosensitization is reported of exponentially growing and plateau-phase Chinese hamster V79 cells, studied in conditions with and without repair of PLD, respectively.

2. Materials and methods

2.1. Cell culture

V79 Chinese hamster cells (Joshi et al. 1977) were grown as monolayers in Costar tissue culture flasks (75 cm\(^2\)) in minimal essential medium (GIBCO-BRL) supplemented with 10% foetal bovine serum, glutamine and penicillin (MEM-s) at 37 °C in an atmosphere of 2% CO\(_2\) in air. About \( 5 \times 10^4 \) cells were plated in 4 ml MEM-s in a 30 mm culture dish when exponentially growing cells were needed, or \( 7 \times 10^5 \) cells were plated in 2 ml MEM-s when plateau-phase cells were needed at the time of irradiation. After plating this number of cells, in exponentially growing cultures, cells went through 5–6 cell divisions and in plateau phase cultures, cells went through 4–5 cell cycles before irradiation treatment. The cells were incubated for 72 h in MEM-s in the presence of 0, 1, 2 or 4 \( \mu \)M of CldUrd, BrdUrd or IdUrd, while 2.5 \( \mu \)M thymidine was added to mimic the average level of thymidine in rodent plasma (Rodriguez et al. 1994) and to minimise HP-incorporation fluctuations due to variations of thymidine concentrations of the serum (Limoli and Ward 1993). The distribution of cells in the various phases of the cell cycle was monitored by flow cytometry. In exponentially growing cultures at the time of irradiation treatment, cells were 45, 25 and 30% in \( G_0+G_1 \), S, and \( G_2+M \) phase respectively, and in plateau-phase cultures at time of treatment over 95% of the cells were in \( G_0+G_1 \) phase.

2.2. Determination of percentage thymidine-replacement

Exponentially growing and plateau-phase cells were plated at the densities mentioned earlier and treated with CldUrd, BrdUrd and IdUrd as described above. Percentage thymidine-replacement was measured by the technique described by Belanger et al. (1987) and modified by Miller et al. (1992a). In short, the cells were trypsinized, pelleted and lysed in water. After isolation from the lysate, DNA was digested in order to liberate nucleosides. The digest was analysed by high-pressure liquid chromatography. Percentage thymidine-replacement was calculated as the concentration of incorporated HP \( \times 100 \) divided by the sum of the concentrations of thymidine and incorporated HP. The ratio of the concentration guanidine and the sum of the concen-
trations of thymidine and incorporated HP was calculated for each sample. This ratio was used to verify that the recoveries of HP and thymidine were equivalent regardless of the percentage of replacement into DNA.

2.3. Radiation survival curves

After incubation with CldUrd, BrdUrd or IdUrd, exponentially growing cells were trypsinized and replated in appropriate dilutions in 6-well macroplates (Greiner) 4 h prior to irradiation. For an appropriate comparison with procedures applied to cells in plateau-phase, a separate set of cultures with cells in exponential growth were irradiated first and immediately after irradiation trypsinized and plated in appropriate dilutions in macroplates. Plateau-phase cells were irradiated, and 0 or 6 h after irradiation trypsinized and replated in appropriate dilutions in 6-well macroplates. Irradiations were performed with gamma-rays from a 137Cs source, yielding a dose rate of about 1 Gy/min. Seven days after inoculation in the 6-well macroplates the colonies were fixed and stained in 6% glacial acetic with 0·05% crystal violet. Colonies of 50 cells or more were scored as originating from a single clonogenic cell.

Surviving fractions \((S(D)/S(0))\) after dose \(D\), corrected for toxicity of CldUrd, BrdUrd and IdUrd alone, \((S(0))\), were calculated and survival curves were analysed using BMDP (Los Angeles, USA) statistical software by means of a fit of the data by multiple regression, according to the linear-quadratic formula:

\[
S(D)/S(0) = \exp\left(-\alpha D - \beta D^2\right)
\]

3. Results

3.1. Percentage of thymidine-replacement and effects on proliferation after culturing cells in the presence of HP

The 72-h exposure to the CldUrd, BrdUrd and IdUrd did not result in reduced cell numbers, neither in exponentially growing cultures nor in plateau phase cultures at the end of the incubation periods (data not shown). Plating efficiency decreased to 85, 77 and 65% after incubation with 1, 2 and 4 \(\mu\)M of CldUrd respectively, but declined less for BrdUrd and IdUrd (data not shown).

Determination of the percentage thymidine-replacement after drug exposure showed that the incorporation of halogenated pyrimidines is concentration-dependent (Figure 1). The percentage of thymidine-replacement in the DNA of cells which, after 72 h growth in the presence of HP, are still exponentially growing was not very different from the percentage of thymidine-replacement in the DNA of cells which had reached plateau phase after 72 h. In exponentially growing cultures thymidine-replacement in the DNA of the cells after incubation with 4 \(\mu\)M of CldUrd, BrdUrd and IdUrd was 22·3, 32·7 and 12·7% respectively. In plateau-phase cultures the percentage thymidine-replacement in the DNA of the cells after incubation during growth with 4 \(\mu\)M CldUrd, BrdUrd and IdUrd was 27·5, 33·8 and 10·7% respectively.

3.2. Radiation dose survival curves of cells in exponential growth plated before or after irradiation

A few examples of the survival curves obtained are shown in Figure 2, illustrating radiation dose-survival curves of exponentially growing V79 cells irradiated before or after plating and of plateau-phase V79 cells with or without plating delay after irradiation. The four curves in Figure 2A differ both in the linear term determining the initial slope (\(\alpha\) parameter) and in the quadratic term mainly determining the continuously curving high dose region (\(\beta\) parameter). As can be deduced from the \(\alpha\) and \(\beta\) values, exponentially growing cells without IdUrd are only slightly more sensitive if plated immediately after irradiation as compared to cells plated before irradiation. The extent of radiosensitization of these cells grown in the presence of 4 \(\mu\)M IdUrd, a factor of about 2 in \(\alpha\) and a factor 1·3 in \(\beta\), is not significantly different for the cells plated before or after irradiation.
3.3. Radiation dose survival curves of plateau-phase cells plated immediately and 6 h after irradiation and compared with proliferating cells

In Figure 2B a few survival curves for plateau-phase cells are illustrated. As comparison of the data for 0% thymidine replacement with data from Figure 2A shows, $\alpha$ values for plateau-phase cells plated immediately after irradiation are smaller by a factor 2 and $\beta$ values are larger by a factor of about 1.5 than for exponentially growing cells. The values of both $\alpha$ and $\beta$ of plateau-phase cells without HP plated 6 h after irradiation are smaller than the corresponding values of plateau-phase cells plated immediately after irradiation, showing a relatively small influence of repair of PLD. It can further be deduced from Figure 2B, that radiosensitization of plateau-phase cells by 4 $\mu$m IdUrd causes an increase by a factor 2 in $\alpha$ and $\beta$ if trypsinization and plating is performed immediately, while for delayed plating $\alpha$ increases more strongly.

3.4. Linear-quadratic analyses of radiosensitisation with different HP in exponentially growing and plateau-phase cells: effect on the linear parameter $\alpha$

Radiosensitization was obtained in exponentially growing and in plateau-phase cells for all three halogenated pyrimidines. The values of $\alpha$ and $\beta$ derived by linear-quadratic analyses of survival curves of exponentially growing cells and plateau-phase cells are presented as a function of the percentage thymidine replacement in Figures 3 and 4 respectively. The increase of $\alpha$ for exponentially growing cells after incubation with 4 $\mu$m of HP was equal to factors between 2 and 3 (Figure 3A and B). The plating conditions, i.e. plating before or after

Figure 2. Radiation dose-survival curves of exponentially growing (Panel A) cells without HP (open symbols) and after incubation with 4 $\mu$m IdUrd (closed symbols), and plateau-phase (Panel B) cells without HP (open symbols) and after incubation with 4 $\mu$m IdUrd (closed symbols). Lines represent polynomial fits to the data. Each point represents the mean value of nine different experiments ± s.e.m. Linear-quadratic parameters of these cells are: Exponentially growing cells plated immediately after irradiation: Without HP: $\alpha=0.18 \pm 0.02$ $\beta=0.017 \pm 0.003$ $\square$. 4 $\mu$m IdUrd: $\alpha=0.38 \pm 0.04$ $\beta=0.023 \pm 0.007$ $\blacksquare$.

Exponentially growing cells plated prior to irradiation: Without HP: $\alpha=0.15 \pm 0.02$ $\beta=0.013 \pm 0.003$ $\square$. 4 $\mu$m IdUrd: $\alpha=0.29 \pm 0.03$ $\beta=0.016 \pm 0.004$ $\bullet$.

Plateau-phase cells plated immediately after irradiation: Without HP: $\alpha=0.09 \pm 0.02$ $\beta=0.026 \pm 0.004$ $\square$. 4 $\mu$m IdUrd: $\alpha=0.17 \pm 0.02$ $\beta=0.062 \pm 0.005$ $\blacksquare$.

Plateau-phase cells plated 6 h after irradiation: Without HP: $\alpha=0.07 \pm 0.02$ $\beta=0.020 \pm 0.002$ $\square$. 4 $\mu$m IdUrd: $\alpha=0.30 \pm 0.03$ $\beta=0.024 \pm 0.004$ $\bullet$. 
irradiation, had no influence on the factor of increase of the value of $\alpha$. For all three HP in the exponentially growing cells which were plated before irradiation the $\alpha$ increases with thymidine replacement. The data are not sufficient to conclude whether a maximum is attained, although the results for CldUrd and BrdUrd with cells plated immediately after irradiation might indicate that a maximum of about 0.5 Gy$^{-1}$ is approached already after incubation with 1 $\mu$M.

For plateau-phase cells which were trypsinized and plated immediately after irradiation (Panel 3C), an
increase of $\alpha$ by a factor of about 2 is obtained for IdUrd, but for CldUrd the increases are smaller. By contrast, for plateau-phase cells plated after a delay of 6 h (Panel 3D) a larger increase of $\alpha$ by a factor 3 to 4 is derived after incubation with 4 $\mu$M Cld-Urd, BrdUrd and IdUrd. IdUrd is the most potent radiosensitizer at low thymidine-replacement.

3.5. Linear-quadratic analyses of radiosensitization with HP in exponentially growing and plateau-phase cells: effect on the quadratic parameter $\beta$

The results obtained for the parameter $\beta$ of the quadratic term, which dominates responses at larger doses, are subject to rather larger uncertainties than the values of $\alpha$. For exponentially growing cells a
significant increase in $\beta$ is obtained only for BrdUrd in cells that were plated immediately after irradiation (panel 4A). For cells plated prior to irradiation $\beta$ values were not significantly influenced by either of the HP (panel 4B). In plateau-phase cells plated without delay a significant increase in $\beta$ of a factor 2 to 3 is obtained for all three HP (panel 4C), but for delayed plating the increase is less pronounced for all HP (panel 4D).

4. Discussion

The major aim of the reported experiments is to evaluate differences in sensitization by the three halogenated pyrimidines ClD Urd, BrdUrd and IdUrd in V79 cells which have incorporated these compounds in DNA and subsequently either remain in the proliferating phase or pass into a resting phase. In particular repair of potentially lethal damage was assessed because of its relevance to radiation responses of tumours as well as normal tissues.

4.1. Incorporation of HP

HP incorporation does reduce the plating efficiency compared to controls, mainly with ClD Urd and less with BrdUrd, but in the assessment of parameters of survival curves this is taken into account. Uptake of HP differed between the compounds tested. Data on incorporation of HP presented in Figure 1 show the expected increase with the concentration in the medium for exponentially growing cells, with less incorporation of IdUrd by a factor of about 2–3 as compared to ClD Urd and BrdUrd. The lower incorporation of IdUrd may be explained by the larger radius of the iodine atom (Elkind and Whitmore 1967).

4.2. Radiosensitization with HP

Both increased fixation of PLD and increased repair of PLD (reviewed by Iliakis et al. 1992), have been suggested to depend on trypsinization and culture conditions. In addition these conditions might also influence the modification of cellular sensitivity by HP. Because of this possibility the sensitization by HP has been assessed for exponentially growing and plateau-phase cells plated immediately after irradiation or after allowing the cells to repair PLD.

4.2.1. LQ analyses of radiosensitized exponentially growing cells. In Figures 3 and 4, results are presented of the analyses by the linear-quadratic formula plotted as a function of the % of thymidine replacement. In exponentially growing cells HP-induced-radiosensitization is mainly due to an increase in the linear parameter $\alpha$. The quadratic parameter only increased when BdUrd was used as the radiosensitizer and the cells were replated immediately after irradiation. The data on the $\alpha$ obtained for radiosensitized exponentially growing cells are in agreement with results reported by Miller et al. (1992a,b) who observed a clear increase in the $\alpha$ of two tumour cell lines growing in vitro and were sensitized with BrdUrd or IdUrd. However they observed a small increase in the $\beta$ in only one of the cell lines after sensitization with BrdUrd (Miller 1992b). The effect might be cell line dependent. On the other hand the value of $\beta$ is especially, in cases of high values of $\alpha$, subject to large uncertainties as shown by the relatively large error bars in Figure 4.

In Figure 3A the relation between $\alpha$ of exponentially growing cells trypsinized and replated immediately after irradiation, and the increasing % of thymidine replacement is presented as linear. However, after incubation with 1 $\mu$M of ClD Urd or BrdUrd or 2 $\mu$M of IdUrd, almost maximal increases of the $\alpha$ were observed suggesting that a saturation in radiosensitization was reached, associated with the trypsinization and replating procedure immediately after irradiation. When the cells were plated before irradiation a clear linear relation between $\alpha$ and thymidine replacement could be obtained. In these cells PLD repair could occur, because the cells are not disturbed after irradiation. After the lower % of thymidine replacement, this repair could probably occur more easily than after a high % of thymidine replacement by HP.

4.2.2. LQ analyses of radiosensitized plateau-phase cells and comparison with exponentially growing cells. In plateau-phase cells which were trypsinized and replated immediately after irradiation both the linear and the quadratic parameters, $\alpha$ and $\beta$, changed. The increase of the $\alpha$ was less than a factor 2 while the increase of $\beta$ was about a factor 2–3. In delayed plated plateau-phase cells, the $\alpha$ further increased while the $\beta$ returned to values as found in cells without HP. The factor of increase of the $\alpha$ was much greater than in plateau-phase cells without plating delay and also much greater than in radiosensitized exponentially growing cells. These results on plateau-phase cells seem to be different from results of Wang and Iliakis (1992) and Iliakis et al. (1987a). They reported a decreased radiosensitivity in IdUrd or BrdUrd containing plateau-phase CHO and C3H10T1/2 cells plated after a delay as compared with cells that were immediately plated after irradiation. This decreased radiosensitization was observed at radiation doses of 4 Gy and higher. Our
LQ analyses of survival curves of radiosensitized V79 cells demonstrated decreased radiosensitization after doses of 4 Gy and higher, resulting in lower $\beta$ values. However, increased radiosensitization was observed after doses of 1–3 Gy resulting in higher values of $\alpha$ for delayed plated plateau-phase cells as compared with immediately plated cells.

4.3. *Mechanisms of HP-induced radiosensitization*

Different mechanisms might be involved in the radiosensitization induced by halogenated pyrimidines in exponentially growing compared with plateau-phase cells. Wang *et al.* (1994) already suggested that in exponentially growing cells increased DNA damage production was the major component of radiosensitization while in plateau-phase cells radiosensitization occurred through inhibited repair and/or enhanced fixation of potentially lethal damage. The increase of the $\alpha$ values for exponentially growing cells as found in this study, indicates an increase in the number of directly lethal events due to the HP. This is in agreement with observations of Webb *et al.* (1993) and Jones *et al.* (1995) which suggest that an important mechanism of radiosensitization involves an increase of effective DNA double strand breaks (dsb). Miller *et al.* (1992a,b) have suggested that radiation-induced damage in cells which have HP incorporated into the DNA after low-LET irradiation resembles the damage produced by high-LET radiation.

In plateau-phase cells plated immediately after irradiation the increase of $\alpha$ might be due to the same mechanism as involved in exponentially growing cells. In these cells also an increase of $\beta$ was observed indicating that accumulation of sublethal lesions contributed significantly (Barendsen 1990). Due to the immediate plating after irradiation this sublethal damage might be fixed.

The greatest increase in $\alpha$ was found in delayed plated plateau-phase cells. This radiosensitization can be interpreted as an enhanced fixation of potentially lethal damage due to immediate DNA damage and/or to damaged DNA repair function in these cells expressed during the interval before delayed plating. The value of $\beta$ in these cells returned to values as found in cells not containing HP. This demonstrates that sublethal damage has been repaired in HP-containing plateau-phase cells.

5. *Concluding remarks*

In most earlier studies of radiosensitization by HP, attention has not been focused on differences in repair and associated changes of the linear and quadratic parameters, derived from analyses of survival curves between proliferating as compared with resting cells. From the results in this study it can be concluded that radiosensitization of mammalian cells by HP is due to a complex of mechanisms involving repair and fixation of damage, acting differently in dependence on the proliferative state of the cells and on cell handling after irradiation. The direct comparison between immediate and delayed plating of plateau-phase cells and between plateau phase and exponentially growing cells shows significant quantitative differences. The data on the linear and quadratic parameters described in this paper provide various new insights in the interpretation of radiosensitization of delay plated plateau-phase cells. In particular it is shown that the sensitization by HP of non-cycling cells is not abolished by enhanced repair of PLD. The possibility that HP increase the $\alpha$ in proliferating as well as in resting cells suggest possible advantages for clinical applications especially at low radiation doses, used in radiotherapy. Further studies with other cell lines are required to elucidate whether, quantitatively, contributions to the increased lethality are generally applicable. Insight into the modification of these contributions is required if suggestions about therapeutic applications are to be derived.

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