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Hydroxyurea-induced cell death in human T lymphoma cells as related to imbalance in DNA/protein cycle and deoxyribonucleotide pools and DNA strand breaks

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The aim of this study was to elucidate the mechanism(s) behind the cellular toxicity of therapeutic concentrations of hydroxyurea (HU). Treatment of human T lymphoma cells (CCRF-CEM) with 60–100 μM of HU for 24 h decreased the growth rate by 90% due to accumulation of cells in early S phase. It induced a marked imbalance in both the DNA/protein cycle (as measured by two-parameter flow cytometry) and the deoxyribonucleotide (dNTP) pools. HU treatment did not enhance the frequency of DNA single-strand breaks (SSBs), as measured by the alkaline unwinding technique. Cell viability was unaffected. However, removal of HU led to 10–15% cell loss during the following 12 h period in parallel with increasing SSBs, and a rapid progression of cells through S and G_2 stages. The unbalanced DNA to protein content per cell and the dNTP pools were normalized 6–12 and 24 h after removal of HU, respectively. These results show that marked changes in the DNA to protein ratio and dNTP pools alone are not directly lethal, but when combined with a high replicative DNA synthesis rate, as found after removal of HU, apparently lead to elevated cell death.

Key words: Deoxribonucleotide pools, DNA damage, DNA/protein cycle, hydroxyurea, human T lymphoma cells.

Introduction

The mechanism(s) behind hydroxyurea (HU)-induced cell death has been studied extensively.[1–4] Early interphase cell death has been explained by the induction of free radicals resulting in DNA damage.[2,4] Cell death at later stages of growth is most likely related to an imbalance in the DNA/protein cycle[3,4] which leads to an increase in lysosomal and non-lysosomal hydrolytic enzyme activity.[3] The unbalanced DNA to protein ratio is due to inhibition of ribonucleotide reductase,[5–8] which causes a depletion of DNA precursors and, thus, a reduction in DNA synthesis. HU treatment gives little or no reduction in cellular protein synthesis and therefore cells with low DNA but high protein content accumulate. It has been suggested that the concomitant overproduction of hydrolytic enzymes would lead to degradation of DNA,[9] which would result in cell death. The mechanism behind the elevated hydrolytic enzymes is still unknown. It has been shown that a number of genes increase their expression when cell growth is arrested by, for example, HU. These genes have been designated *gadd* (growth arrest and DNA damage inducible).[10] The corresponding proteins have not yet been identified, but it is possible that expression of gadd genes is related to the increase in hydrolytic enzyme activity found after HU treatment.

Although some studies of the mechanism of HU toxicity have been performed at 200 μM, the majority of the investigations were done at 1 mM or higher concentrations, which is considerably higher than the serum concentration of HU obtained in treated patients, which was found to be about 200 μM.[11] In order to mimic the therapeutic situation we previously studied[12] the effect of HU on mouse lymphoma (S49) and human lymphoblastoid (CEM) cells at concentrations (60 μM) comparable to values found in tumors after HU treatment.[11] The

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cells were exposed to HU for about one cell generation (24 h), which has been suggested to give maximum cytotoxicity in human tumor therapy.[11] No cell death was found after this incubation time, even after prolonged exposure (48 h) to HU (unpublished results). Surprisingly, removal of HU resulted in a 10–30% cell loss during the following 12 h period, with no over-representation of any cell cycle phase. In parallel, there was an increase in DNA precursor pools, and a rapid progression through S and G_2 stages of the cell cycle. Our hypothesis was that this rapid passage through the cell cycle in combination with DNA damage leads to various mistakes in macromolecular synthesis and assembly resulting in cell death. In order to further elucidate this mechanism(s), we directly determined the cellular protein to DNA ratio by a two-parameter flow cytometry (FCM) technique, the frequency of DNA strand breaks by alkaline unwinding, and the deoxyribonucleotide (dNTP) pools in relation to cell cycle progression and cell loss.

Materials and methods

Cell culture and experimental conditions

CEM wild-type cells and Ag1 (generously supplied by B Ullman, Oregon Health Science University, OR, USA), a $HGPRT^-$ mutant of CEM cells (the Ag1 cells were originally obtained from MS Hershfield, Duke University, Durham, NC, USA), were grown in suspension culture in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated horse serum. The details of cell culture conditions have been described previously.[13–16] The number of cells was determined by a Coulter counter and the proportion of dead cells was calculated using dye exclusion with trypan blue. HU (Sigma, St Louis, MO, USA) was dissolved in distilled water and used fresh.

The cells were treated with HU for 24 h, followed by centrifugation at 500 *g* at room temperature, washed once in complete medium without HU and resuspended in the original volume of the complete medium without HU. The control cells were treated according to the same washing procedure.

Determination of cellular DNA and protein content

Cells were fixed in ethanol overnight and then washed in Tris-buffer (Tris 0.1 M, NaCl 0.07 M, EDTA 0.005 M, pH 7.5). For cell flow calculation purposes (see below), DNA was stained with ethidium bromide.[17] For the cell cycle related protein study, DNA was stained with DAPI and protein with Sulforhodamine 101 (SR 101; Aldrich Chemie 28491-9) for 30–60 min according to Stöhr *et al.*[18] Control of staining conditions and internal standardization using human lymphocytes from peripheral blood was performed as described elsewhere.[19]

The fluorescence emissions from DAPI and SR 101 were analyzed in a modified Partec PAS II flow cytometer (Partec GMBH Munster, Germany) equipped with a mercury arc lamp. In this system, the cells sequentially cross two spatially separated regions illuminated with green- and UV-excitation light, respectively.[20] In this way, the effects of overlapping emission spectra and of energy transfer, which can give artifacts of measurements, are minimized and both the DNA and protein content are analyzed with high precision. Evaluation of the two-parameter histograms was performed by the data analysis program of the PAS II.

The mean protein contents of the different populations of G_1, early and late S phase, and G_2 + M cells were related to the internal standard (human lymphocyte). The mode value of the protein content of each population was determined from the middle point of the protein values seen in the two-dimensional histogram (see Figure 3). Since the protein curves of all cell types in the cell cycle are close to a normal distribution (data not shown), the measured mode values correspond approximately to mean values.

Calculation of cell cycle flow and cell loss

Cell cycle flow was calculated as described previously[21] assuming a growth fraction of 1.0. At unbalanced cell flow, a minimum value of cell loss could also be calculated.

Determination of deoxyribonucleotide triphosphate

The cells were harvested and centrifuged at ambient temperature, and washed with chilled, isotonic phosphate buffered saline. Nucleotides were extracted with 0.6 M trichloroacetic acid and neutralized with tri-*n*-octylamin:freon as described elsewhere.[22] Deoxyribonucleoside triphosphates were

determined by high-performance liquid chromatography after prior oxidation of ribonucleotides.[23,24]

DNA strand breaks

DNA strand breaks were measured by the unwinding technique as described previously.[25]

Results

Cell growth, cell cycle flow and cell loss

Two different CEM cell lines were used (CEM wild-type and Ag1) since they show some differences in growth kinetic properties and HU sensitivity (with the wild-type cell showing about 2-fold higher sensitivity). These differences were unrelated to the HGPRT$^-$ mutation in Ag1, but were due to the frequently observed heterogeneous growth properties of closely related cell lines, which have been passaged for long periods *in vitro*. Cell growth of untreated wild-type and Ag1 cells was exponential during the time interval studied with a doubling time of about 30 h. When the cells were exposed to HU for 24 h (60 μM for CEM cells and 100 μM for Ag1 cells), the growth rate was inhibited by 90% (Figure 1). Replacement of medium with fresh medium prolonged the inhibition of cell growth of HU-treated cells and inhibited the growth of control cells for about 10 h (Figure 1). The cell number doubled 24 h after removal of the drug.

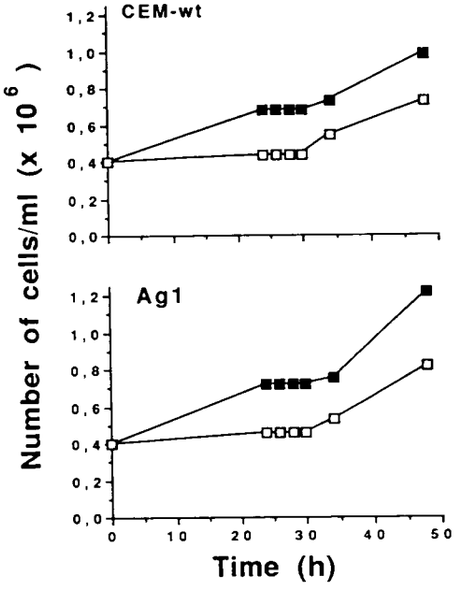

Figure 1. Total number of untreated (■) and HU-treated cells (□). HU was added at time 0 and removed after 24 h. The results shown are from typical experiments ($n = 4$).

Table 1. Mean cell outflow from G_1, S phase and $G_2 + M$ of untreated and HU-treated cells during the following time periods: 0–24, 24–26, 26–28, 28–31, 31–36 and 36–48 h

Cell line	Time (h)	Cell outflow rate (%/h)					
		G_1 −HU	S +HU	S −HU	$G_2 + M$ +HU	$G_2 + M$ −HU	G_1 +HU
CEM	0–24	100	84	100	4	100	32
	24–26	81	525	0	7	116	0
	26–28	0	0	0	69	0	116
	28–31	63	0	17	91	0	63
	31–36	38	18	16	56	13	123
	36–48	98	145	227	120	245	71
Ag1	0–24	100	115	100	11	100	44
	24–26	0	849	0	55	17	0
	26–28	43	0	38	112	0	137
	28–31	0	0	36	127	19	150
	31–36	68	445	65	447	47	443
	36–48	177	102	182	225	134	59

The results are expressed as a percentage of untreated cells in the time period 0–24 h. Representative results of four experiments.

Cells treated with HU were arrested in early S phase of the cell cycle (Figure 2). When the drug was removed from the medium, these cells started to pass through the S phase and reached mitosis after about 7–12 h (Figure 2). In order to describe the cell cycle kinetics after removal of HU in more detail, we also calculated the cell outflow from the various cell cycle stages (Table 1). The presence of HU for 24 h completely inhibited the outflow from the S phase and also partly from the $G_2 + M$ stage, while the outflow from G_1 was almost unaffected. When HU was removed, the outflow from the G_1, S and $G_2 + M$ stages immediately increased to values corresponding to the flow rate of the untreated control or even higher (Table 1).

Since the total number of HU-treated cells was unchanged up to about 10 h after removal of the drug, while the number of G_1 cells increased, cell division had occured in connection with cell loss. From cell flow calculations it is possible to estimate the minimum value of cell loss.[21] Cell loss appeared at a few hours after removal of the drug and was 7.5% for CEM cells and 16% for Ag1 cells, with a maximum in the time interval 4–7 h after removal of HU (Table 2). No cell loss was found in un-

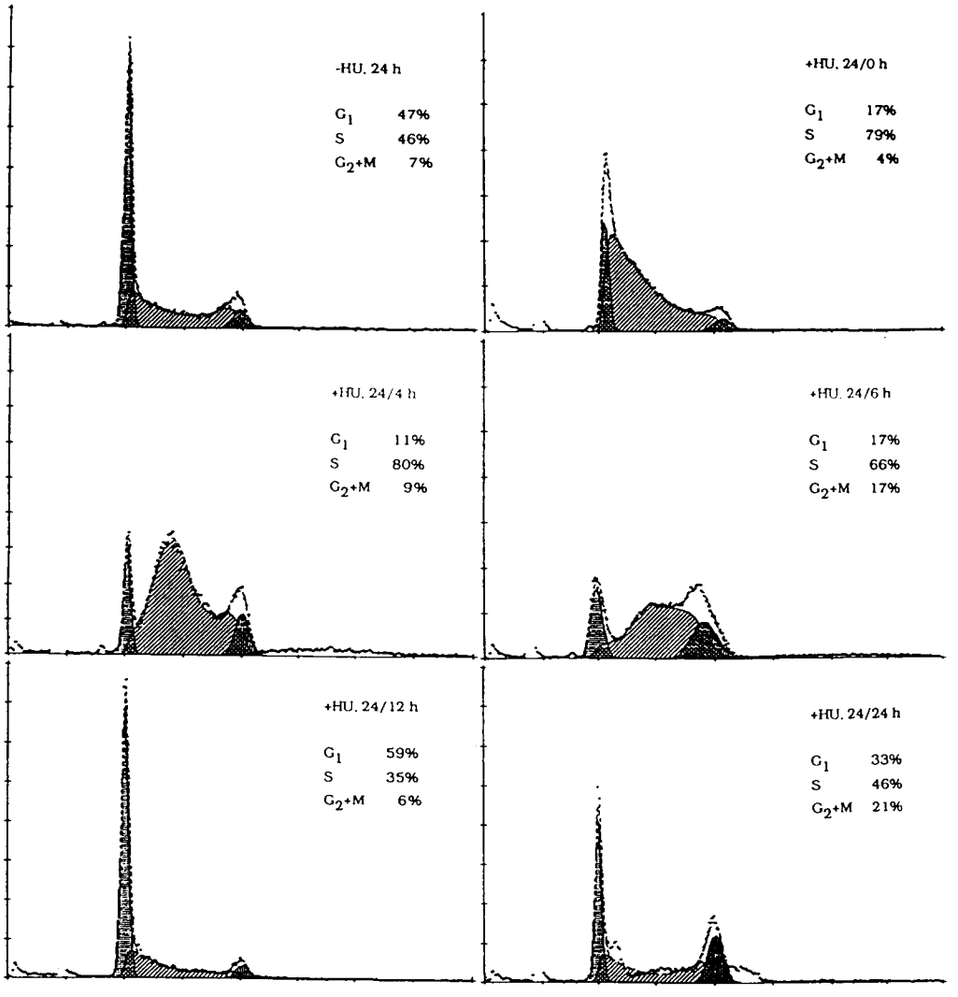

Figure 2. Example of DNA histograms of untreated and HU-treated CEM and Ag1 cells. HU was added at time 0 and removed after 24 h. The peak to the left represents G_1 cells, while the peak to the right represents G_2 + M cells. The S cells are between the two.

Table 2. Cell loss after removal of HU obtained by cell flow calculations (see Material and methods)

Time (h)	CEM		Agl	
	−HU	+HU	−HU	+HU
0–2	0	0	0	0
2–4	0	3	0	6
4–7	0	4.5	0	11
7–12	0	0	0	0
12–24	0	0	0	0

The amount of cell loss is expressed as a percentage of the number of cells at the time of removal of the drug. Representative results of four experiments.

treated control cells. Cell viability, as measured by the trypan blue exclusion test, was unchanged up to 24 h in the presence of HU. Prolongation of the exposure time up to 48 h did not affect the viability (data not shown).

Cellular DNA and protein content

Typical histograms of correlated DNA and protein analyses of untreated and HU-treated CEM and Ag1 cells are shown in Figure 3. From such histograms the mean protein content of G_1, S and G_2 + M cells was estimated as described in Material

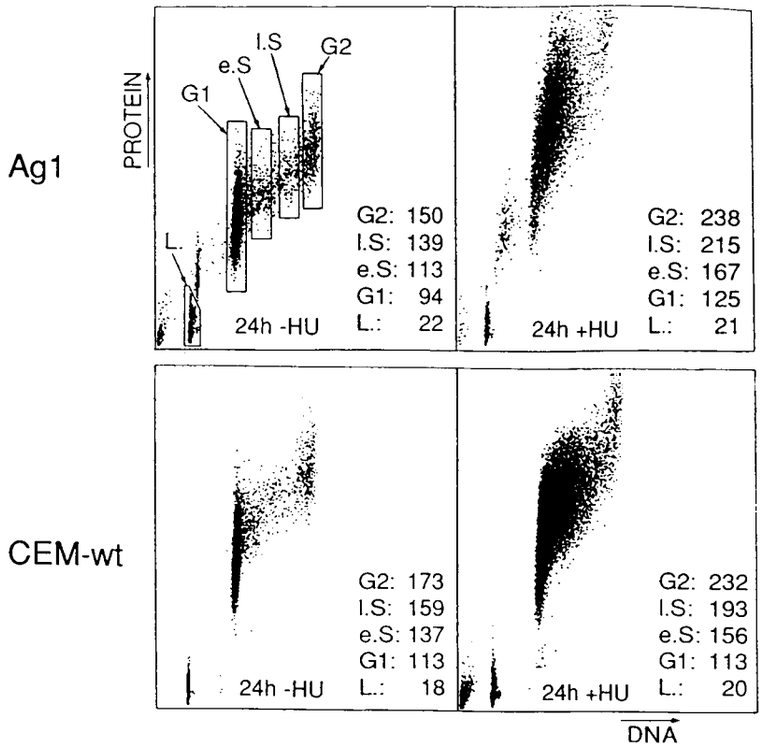

Figure 3. Flow cytometric analysis of untreated (−HU) and HU-treated (+HU) Ag1 and CEM cells. Cells were stained for protein and DNA as described in Materials and methods and than measured by two-parameter FCM. Five populations were defined based on their protein and DNA content: internal standard cells of human lymphocytes from peripheral blood (L.), G_1 (G1), early S (e.S), late S (I.S) and G_2 + M (G2). The results shown are from typical experiments ($n = 4$). The mean protein values of the different populations expressed in arbitrary units can be seen in each histogram. Based on these values, the protein content of cells/protein content of standard cells ratio was calculated. The results are shown in Figure 4.

and methods, and the results are summarized in Figure 4. Exposure of cells to HU for 24 h increased the mean cellular protein content of cells by about 30% in all cell cycle stages except for wild-type G_1 cells. The mean protein content of early S phase cells of both cell types corresponds to the mean protein content of their G_2 cells. When HU was removed, the mean cellular protein content decreased and reached normal values after about 6–12 h.

Deoxyribonucleoside triphosphate levels

The effects of HU on the level of the four deoxyribonucleoside triphosphates in CEM and Ag1 cells are shown in Table 3. dATP and dGTP decreased by about 50%, while dTTP increased by 25–50%. dCTP was only slightly reduced. After removal of the drug, all four deoxyribonucleoside pools increased 2- to 3-fold up to 10 h and then returned back to almost normal values at 24 h. The concentration of the four deoxyribonucleoside triphosphates in the untreated cells were unchanged throughout the experiment (data not shown). These results are in agreement with previous studies[12,16] and have been discussed in detail.

DNA strand breaks

Treatment with HU for 24 h did not affect the frequency of DNA single-strand breaks (SSBs) in CEM and Ag1 cells as compared with controls (Figure 5). However, after removal of the drug, the number of SSBs increased by about 150–200% up to 4 h and then returned to almost normal values at 10 h.

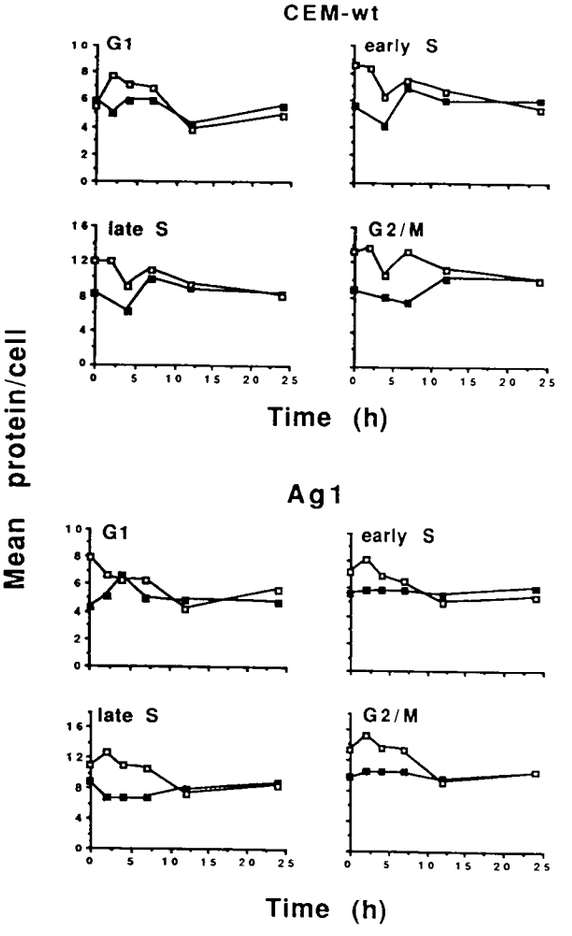

Figure 4. Cellular protein contents of untreated (■) and HU-treated (□) cells as estimated from the flow cytometric analysis shown in Figure 3 (see also Material and methods). The values are the protein content of cells/protein content of standard cells ratio, calculated as described in Figure 3. The values are arbitrary units and calculated as described in Figure 3. Cells were treated with HU for 24 h (time 0), washed once, and resuspended in HU-free medium and grown for further 24 h. The results shown are from typical experiments ($n = 4$).

Table 3. Deoxyribonucleoside triphosphate levels in CEM and Ag1 cells 24 h after treatment with HU, and at 5, 10 and 24 h after removal of the drug

Time (h)	dCTP	dTTP	dATP	dGTP
CEM				
Control	8	41	29	18
24	6	51	17	10
24/5	18	97	65	36
24/10	29	ND	20	70
24/24	14	ND	ND	37
Ag1				
Control	5	30	23	18
24	6	46	10	15
24/5	10	37	25	18
24/10	13	46	36	24
24/24	5	26	20	14

The values are expressed as nmol/10^9 cells, ND = not determined.

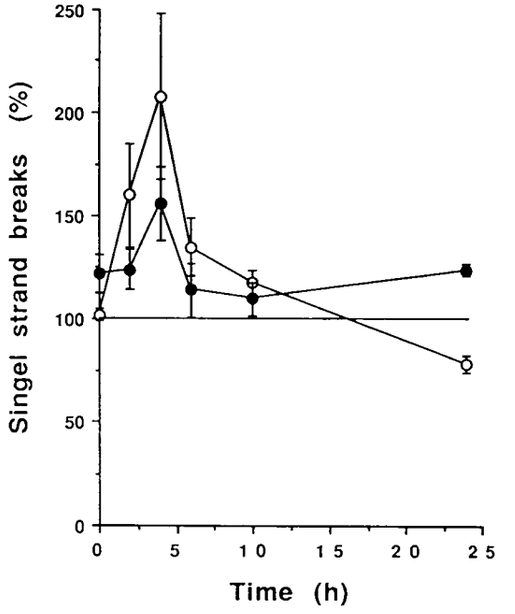

Figure 5. DNA SSBs of HU-treated CEM (○) and Ag1 (●) cells as a percentage of untreated cells. Cells were treated with HU for 24 h (time 0), washed once, and resuspended in HU-free medium and grown for a further 24 h. Mean values ± SE of two experiments.

Discussion

Recent studies on the cellular action of HU show that several mechanisms of cellular damage exist. Apart from the appearance of free radicals,[2] which probably leads to inactivation of enzymes, cross-linking of DNA and alteration of membrane functions, a state of unbalanced growth, i.e. disassociation of the DNA/protein cycle due to inhibited DNA synthesis, also seems to be of importance, particularly at later times of growth (one or two generations). The following causal relationships between unbalanced growth, DNA damage and cell death have been suggested: inhibition of DNA synthesis without alteration of protein synthesis leads to overproduction and accumulation of hydrolytic enzymes (acid phosphatase, endonucleases) resulting in damage to various cell constituents.

Accumulation of DNA damage, if not repaired, cause lethal changes in chromatin structure and, thus, cell death.[9] It has been reported that an imbalance in deoxyribonucleotide (dNTP) pools also induces endonucleases leading to DNA breaks and cell death.[26] Abolition of the DNA/protein imbalance, either by adding deoxyribonucleosides (dNR)[3] or by inhibiting protein synthesis[27] improved cell survival. Addition of dNR also normalized the hydrolase activities.[3]

In the present study, cells were treated with a concentration of HU (60–100 μM) for 24 h, which inhibited cell growth by 90%. Marked imbalances in the DNA/protein cycle and dNTP pools were found. In spite of these changes, cell viability was unaffected. Cell viability was not affected even after prolonged exposure up to 48 h (unpublished results). This is in contrast to a recent study[3,4] where cell death, as measured by trypan blue exclusion, increased by 20–30% after treatment with 200 μM HU for 24 h. The unchanged cell viability in the present study is observed in parallel with no or a very limited increase of DNA SSBs. However, it cannot be excluded that DNA damage other than SSBs is induced, e.g. base damage, DNA cross-links[2] and/or double-strand breaks. It is concluded that an imbalance in the DNA/protein cycle and/or dNTP pools is, at least in CEM wild-type and Ag1 cells, not *per se* lethal. However, the HU-induced imbalance in the DNA/protein cycle may be essential for the appearance of DNA strand breaks and cell death after removal of HU. When HU was removed, the replicative DNA synthesis rate reached values within a few hours which correspond to untreated cells. This may depend on increased concentrations of enzymes and protein factors needed for replication accumulated during exposure to HU. In combination with the increased dNTP pools, these proteins may give a high rate of progression of cells through the cell cycle. If DNA contains damage (base damage, cross-links) after 24 h exposure to HU, which is likely, replication of such DNA at normal or higher rates probably induces more damage and, thus, a reduced fraction of surviving cells. We found that the frequency of SSBs increased markedly a few hours after removal of HU followed in time by cell disintegration. Therefore, it is likely that the observed increase of SSBs is caused by the rapid replication of the damaged DNA. However, it cannot be excluded that part of the SSBs originated from DNA degradation of dying cells as a result rather than a cause of cell death. A number of toxic compounds kill cells by programmed cell death (apoptosis).[28,29] This type of death occurs in different stages. Cell and nuclear volume decrease followed by increasing cellular density. Both the cytoplasm and nuclei are then disintegrated into small membrane-bound bodies followed by fragmentation of DNA. The initiation of apoptosis may also depend on the influx of calcium ions and the progress of protein synthesis. In our study, however, it is not likely that apoptotic cell death occured, since the density of the dead cells was reduced.[12]

In previous studies, the degree of unbalanced growth was determined by measuring independently the total DNA content and either the total protein content or the mean cellular volume of whole cell populations.[3,4,30] In this study, the state of unbalanced growth was characterized by DNA and protein analysis in single cells using two-parameter FCM. This technique makes it possible to relate the protein content of cells to their cell cycle stage. In this way, it can be demonstrated how cells from different stages of the cell cycle are affected with respect to protein metabolism. A prolongation of early S phase with an unaffected protein synthesis rate would cause an increase in the protein content in these cells and, if these cells progress further, also in late S phase and G_2 + M cells. This was found for both cell types tested. If progress from G_1 to S is unaffected and if no or a very limited number of cells divide, no increase in the protein content of G_1 cells would be found. This was the case for the wild-type cells, but not for the Ag1 cells. They increased their protein content as much as for the other cells in the cell cycle. Since the cell division rate and the flow rate from G_1 to S phase is comparable to the wild-type cells, it may indicate stimulated protein synthesis or a decreased protein degradation in Ag1 G_1 cells.

The decrease in protein content in wild-type cells by time after removal of HU is caused by a continuos inflow of G_1 cells with normal protein content. The corresponding decrease in protein content of the Ag1 cells is probably more complex. As the G_1 cells enter S phase, proteins are degraded, reaching values of untreated cells. Thus, the HU-induced unbalanced growth of mutant cells is probably not only due to inhibited DNA synthesis, but also to an alteration of protein metabolism. These differences in HU response between closely related cell lines which are derived from the same initial cell culture demonstrate the problem of obtaining representative results when comparing different cell systems. However, the main biochemical and cellular effects of HU were very similar in the two cell lines, but apparently there is variability in the way

these effects alter the detailed growth regulation of the two cell types. At present we do not know if these differences are of genetic or epigenetic origin.

In summary, our results indicate that HU-induced cell death, after drug treatment at therapeutic concentrations, appears after rather than during the period of exposure. The toxicity is due to the combined effects of sublethal HU-induced DNA damage, an imbalance in the DNA/protein cycle and a fast replicative DNA synthesis rate. This type of toxic mechanism is probably also valid for other drugs with similar cellular effects as HU. We are now attempting to correct the imbalanced protein to DNA ratio by combining HU treatment with the application of a protein synthesis inhibitor. Furthermore, characterization of HU-induced dead cells with regard to their DNA fragmentation pattern is also in progress.

Acknowledgments

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