Radioresistance in cells with high content of metallothionein

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Summary. An endogenous cytoplasmic protein, metallothionein (MT) apparently gave rise to radioresistance in 2 different cell lines. A dose reduction factor of 1.9 was achieved in MT-containing cells. MT accounted for a 3–4-fold increase of total sulfhydryl groups in the resistant cell strains, compared to the non-resistant lines from which they were derived. The protein is rich in cysteine (30%), an amino acid known to give radioprotection when administered exogeneously. Glutathione levels and cell-cycle phase distribution showed no marked difference between resistant and corresponding non-resistant cells.

Metallothioneins (MT) are proteins with molecular weights of 6000–7000 and high metal content (cadmium, zinc or copper). A most distinctive aspect of the MT is the high content of cysteine (approximately 30% of the amino acid residues) in the absence of disulfide linkages. The physiological role of MT is largely unknown, although it is considered to be of some importance in the metabolism and storage of zinc and copper[2–5]. Induction of MT is probably a protective mechanism in mammals against the toxic cadmium ion[6,7] and possible other heavy metals[8]. Recent studies extend the hypothesis of the detoxifying properties of MTs to include a potential protection against alkylating agents[9,10].

Since cysteine and certain cysteine derivatives are radioprotectors[11], we have investigated the effect of ionizing radiation on the growth of 2 cell strains with a high concentration of the cytoplasmic MT. The effect of radiation on these cells was compared to that on the corresponding 'wild-type' cell lines containing no MT.

Materials and methods. The 2 different cell lines used were 1 human epithelial line (designated HE-cells) derived from normal skin and 1 mouse fibroblast line (designated Cl 1D-cells) derived from L-cells. Substrains of both cell lines previously made resistant to, and maintained in, 100 µmoles Cd^{++}/l culture medium are designated HE_{100} and Cl $1D_{100}$, respectively[12,13]. These substrains have high intracellular contents of MT. In terms of sulfhydryl groups the MT provides 90 nmoles SH/mg cell protein in HE_{100} cells and 110 nmoles SH/mg in the Cl $1D_{100}$ cells[10]. In the non-resistant cells no MT is found, using the technique reported, with calculations on the basis of metal content in the relevant eluate fractions from a Sephadex G-75 filtration of the cell cytosols. Cl 1D and Cl $1D_{100}$ cells are deficient in thymidine kinase (TK) (ATP: thymidine-5′-phosphotransferase, EC 2.7.1.21) shown by incubation of cell cytosol with ^{3}H-thymidine and measurements of thymidine-monophosphate (TMP) at intervals. They are resistant to 30 µg/ml of 5-bromo-deoxyuridine (BrdU). The Cl 1D cell line was exposed to Cd[13] because it was of interest to study whether a non-epithelial cell line in culture could gain the Cd-resistance and the cytoplasmic MT content that was gained by HE cells[12]. Furthermore, the Cl 1D cell line is suitable for cell hybridization in selective media. Lack of TK is not known to have any influence on Cd-resistance and did not, apparently, affect the MT-induction in these cells. The Cd-resistant substrains with cytoplasmic MT developed quite analogously from their parent HE and Cl 1D cell lines. With respect to uptake and egress of Cd[14] and resistance against cis-dichlorodiammineplatinum[10] and chlorambucil[15], also, the HE_{100} and Cl $1D_{100}$ cells behave very similarly. The Cd-resistant strains seem to be genetically altered, in that they are still resistant to 100 µmoles Cd/l after 4 weeks' growth in Cd-free medium. At that time they contain no measurable intracellular Cd, but have the ability to reproduce their remarkable level of cytoplasmic MT (2–3% of the total cell protein) within 24 h. (Results to be published).

The doubling time of the Cl 1D and Cl $1D_{100}$ cells is about 25 h and that of HE and HE_{100} cells about 24 h. All cells were maintained as monolayers in Falcon tissue culture flasks (75 cm^2) in Dulbecco's modified Eagle's medium as described[14].

The colony-forming ability of the Cl 1D and Cl $1D_{100}$ cells was used to test their sensitivity to ionizing radiation. (As HE and HE_{100} cells do not form distinct colonies under these conditions, such studies were not done with these cells). 600 cells of each strain (Cl 1D and Cl $1D_{100}$) were plated on Lux 35 mm tissue culture dishes and allowed to adhere for 3–4 h covered by a culture medium layer of 2.5 mm before being irradiated. During the irradiation procedure the 35 mm dishes with their plastic cover of 1 mm thickness were kept in 140 mm dishes whose 1.5 mm thick plastic covers were sealed with Sellotape to keep the atmosphere constant (5% CO_2 in air, culture medium with pH 7.3). When out of the 37 °C incubator for irradiation all culture dishes were kept in a room at 22 °C for exactly 10 min. Colonies appeared in 5–6 days; they were fixed, stained and then counted under a microscope. Distinct colonies of 10 or more cells were considered to originate from viable cells.

Cl 1D and Cl $1D_{100}$, as well as HE and HE_{100} cells, were also tested for radiation sensitivity by counting the number of cells at certain intervals during a total incubation period of 96 h after irradiation. 20,000 cells were plated in 16 mm diameter wells (Costar) 24 h prior to irradiation, which took place when the cells were attached as a monolayer covered by a culture medium layer of 3 mm; the culture trays with 1 mm thick plastic covers were kept in thin sealed plastic bags, and otherwise treated as described above. Cells were harvested by trypsinisation at 0, 24, 48, 72, 96 h and counted (Coulter Counter).

For both types of experiments radiation was generated by a Philips Müller MG 300 Roentgen unit at 240 kV and 10 mA. No external filter was used; the unit's internal filtration was comparable to 0.2 mm Cu. The dose rate was 2.2 Gy/min; FD was 40 cm. Radiation was measured using an ionization chamber.

Determination of SH-groups and glutathione (GSH) was done in cell cytosols prepared by ultrasound sonication and ultra-centrifugation of cells harvested by trypsinisation and rinsed in ice-cold phosphate buffered saline. The analyses were performed according to established techniques[16,17].

In order to study the cell cycle phase distributions, flow cytometric DNA measurements were performed in an IC

Total SH and GSH in the cell cytosol (105,000 × g, 1 h) given as nmole/mg cell protein

	Total SH	GSH
HE	50	29
HE_{100}	127	21
Cl 1D	36	12
Cl $1D_{100}$	152	13

P11 (Phywe AG, Göttingen, FRG) after preparation of the cells by a conventional method[18,19].

The amount of metallothionein was calculated on the basis of the metal (Cd, Zn, Cu) content determined by atomic absorption spectrophotometry according to the user's manual (Instrumentation Laboratory aa/ac spectrophotometer 257) in the relevant eluate fraction from a Sephadex G-75 (Pharmacia) gel filtration of the cell cytosols.

Results and discussion. The colony-forming ability of Cl 1D and Cl $1D_{100}$ cells after different doses of radiation is shown in figure 1. This colony formation experiment was repeated twice, each time including at least 5 radiation doses in the range of 3-13 Gy. 4 parallel cultures from each cell strain were exposed to each dose. Since these 3 experiments were performed at different times and under slightly different conditions, their results could not be pooled. However, all 3 experiments showed a very similar relationship between resistant and non-resistant cell strains. It is the results from one of these experiments which are shown in figure 1. With a radiation dose of 3 Gy this experiment gave percent survivals of 39 ± 4 (Cl 1D)/74 ± 2 (Cl $1D_{100}$). The corresponding pair of values from the other 2 sets were 42 ± 4/67 ± 2 and 44 ± 5/62 ± 7 when 3 Gy were used. With a radiation dose of 5 Gy the percent survivals were 18 ± 4/34 ± 3 in the experiment given in figure 1, 24 ± 3/46 ± 4 in the 2nd experiment, while this dose was not used in the 3rd one. A feature of the survival curve for the metallothionein-containing cells was the enhancement of the shoulder region. For doses up to 9 Gy there was a statistically significant difference between Cl 1D and Cl $1D_{100}$ cells (t-test, $p < 0.01$ (1 Gy), $p < 0.005$ (3, 5 and 7 Gy), $p < 0.025$ (9 Gy)). Within this region the remarkable dose reduction factor of 1.9 was achieved. Such a modification of the curve shape may be an indication that the biophysical properties of the targets are altered by the prolonged Cd-pretreatment involved (e.g. target size and target separation), and/or that the pretreatment renders a cell capable of modifying the expression of radiation damage. MT may represent such a modification of targets, for instance through absorbance of radiation energy by the mercaptide bonds and by scavenging of free radicals formed by the irradiation.

Figure 2 shows the effect of increasing radiation doses on the number of Cl 1D cells (fig. 2a) and the corresponding Cd-resistant strain (fig. 2b) over a 96-h period of cellular growth. 2 additional experiments were performed under almost the same conditions. They gave quite similar differences in radiosensitivity between the cells with and without cytoplasmic MT. In analogous experiments the growth of HE and HE_{100} cells was also determined. These studies gave similar growth curves. After 96 h 20 Gy had reduced the number of HE cells to 20% of controls while HE_{100} cells still constituted 64%.

GSH and the total concentration of SH groups were also

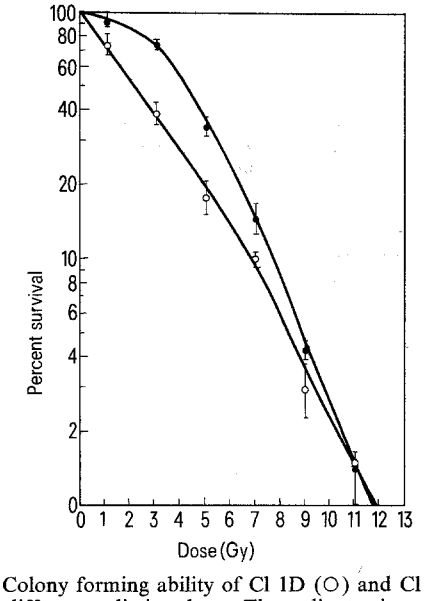

Figure 1. Colony forming ability of Cl 1D (○) and Cl $1D_{100}$ (●) cells after different radiation doses. The ordinate gives the percent survival of cells (logarithmic scale); the abscissa gives the radiation dose. Each point represents the mean (±SD) of 4 parallels. The fitted curves are in accordance with a 3rd degree polynom.

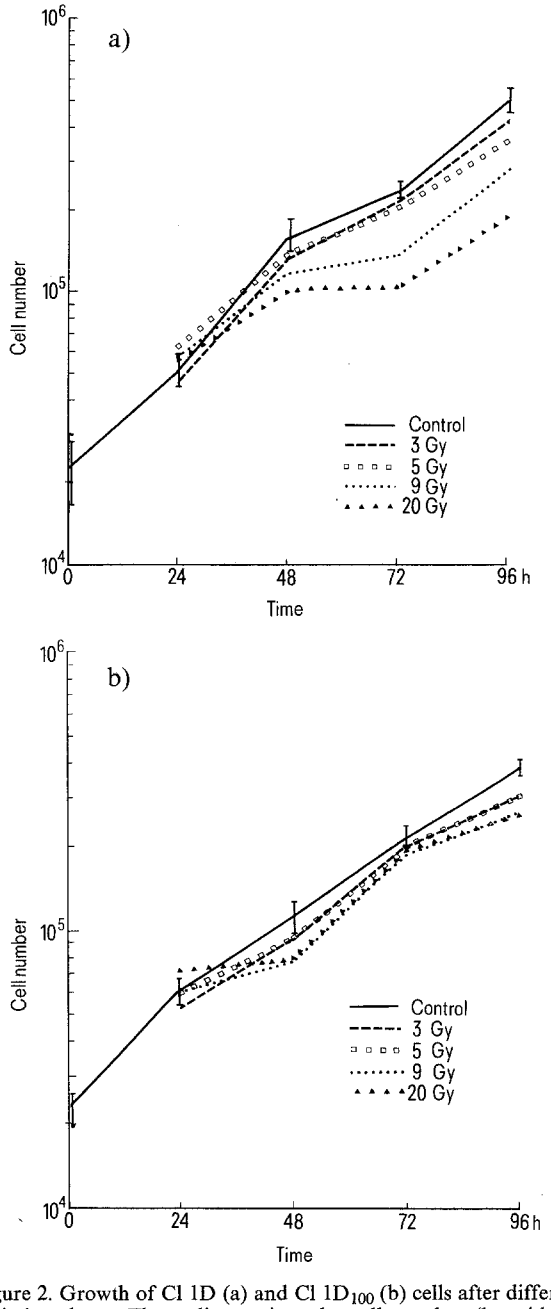

Figure 2. Growth of Cl 1D (a) and Cl $1D_{100}$ (b) cells after different radiation doses. The ordinate gives the cell number (logarithmic scale); the abscissa gives the growth time after irradiation. Each curve represents 1 radiation dose; each point represents the mean of 12 parallels, for 'control' cultures ± SD.

determined in the cytosol of all 4 cell strains. The results are given in the table. The Cd-resistant cell strains had about the same content of GSH as the 'wild-type' line. Consequently, induction of GSH did not explain the observed radioprotection. The concentration of total SH was increased 2.5 times for the HE_{100} cells and 4.2 times for the Cl $1D_{100}$ compared to the corresponding HE and Cl 1D cells. However, the amount of MT calculated on the basis of the metal content, accounted for the increase in SH groups in the Cd-resistant strains.

Flow cytometric DNA measurements in non-irradiated cells showed only small differences of the cell cycle phase distribution between the 'wild-type' cell lines and their corresponding MT-containing substrains during periods of exponential growth. The Cl 1D cells showed 62.7% of the cells in G_1, 21.8% in S and 15.5% in G_2. For Cl $1D_{100}$ the phase distribution was 59.8% (G_1), 20.1% (S), 20.0% (G_2); and for HE and HE_{100} cells 56.0% (G_1), 26.2% (S), 17.8% (G_2) and 58.5% (G_1), 23.2% (S), 18.2% (G_2), respectively. Thus, the radio-resistance observed apparently did not result from the accumulation of MT-containing cells in a radio-resistant phase.

Our results show that the murine Cl $1D_{100}$ cell line, with high intracellular levels of MT, has increased resistance against ionizing radiation. Growth curves for the clearly different human HE_{100} cell line indicate that these cells have also acquired resistance. MT accounts for the 3-4-fold increase of SH-groups in these cells compared to the corresponding non-resistant lines. Thus, for the 1st time it has been shown that radioprotection apparently can be provided by an endogenously produced protein, namely the extremely cysteine-rich metallothionein.

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Cysteine-induced effect on amino acids in neonatal rat brain

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Summary. In neonatal rat brain 6 h after s.c. administration of L-cysteine, an increase was observed in most of the amino acids with the exception of glutamic acid, aspartic acid, phenylalanine, sarcosine, glutamine, hydroxyproline and phosphoethanolamine compared to age-matched saline controls. Cysteine was not present at detectable levels in control brain but was found to be 0.38 to 0.52 µmole/g of fresh brain tissue in 2- and 4-day-old rats respectively after cysteine treatment.

Certain amino acids like glutamic acid, aspartic acid, cysteine sulfinic acid, cysteic acid and cysteine produced lesion in the brain when administered s.c. or p.o.[2,3]. Lesions induced by these compounds, with the exception of cysteine, are confined to specific regions of brain, such as the arcuate hypothalamic nucleus. Cysteine induces much more widespread lesions than the more acidic amino acids such as glutamic acid. Olney et al.[4] have postulated that cysteine-induced brain damage may be the result of conversion of cysteine to cysteine sulfinic acid and cysteic acid, which have been classified as neuroexcitatory amino acids by Curtis and his associates[5]. Efforts to produce a cysteine-type of lesion in 12-21-day-old mice have met with little success[4], despite that, the enzyme cysteine oxidase, which is responsible for the conversion of cysteine to cysteine sulfinic acid[6], was found at a higher level in that age group than in neonatal rats (1-4 days old), which is a vulnerable age for such damage. The present study was undertaken to determine whether cysteine enters the brain when injected s.c. and how it would affect the pool of other amino acids[7].

Methods. Pregnant Wistar strain rats in late gestation (18-20 days) were bought from Charles River Colony at Willmington in Massachusetts, and litters were delivered in our facilities. 2 and 4-day-old neonatal rats were used because earlier studies showed that young infants are more susceptible to cysteine-induced brain lesions than older rats. The dosage (1.2 mg/g) of L-cysteine was chosen on the basis of earlier studies[3], which showed that this dose produces widespread lesions in the central nervous system (CNS), with minimal mortality.

2 groups of rats (ages 2 and 4 days) were injected s.c. with a solution of L-cysteine in physiological saline (1.2 mg/g, neutralized to pH 7.0 with 1 N NaOH). They were decapitated 6 h later which was a sufficient time to produce maximum lesions[3]. Brains were removed and chilled in ice; immediately a pool of 6 brains from each group were weighed and homogenized in ice-cold 6% perchloric acid[8], in a ratio of 1 g fresh tissue to 5 ml of perchloric acid. The homogenate was centrifuged at 0 °C and the supernatant collected separately. The resultant pellet was washed twice by resuspending it in perchloric acid. The washing solution and supernatant were mixed and titrated to pH 4.0 with 2 N