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THE ROLE OF THIOLS IN LETHAL AND MUTATIONAL RADIATION DAMAGE

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Summary. - *Over the last few decades, free radicals have been increasingly implicated in biological processes including radiation effects, ageing, carcinogenesis, initiation and progression of various diseases, toxicity of chemicals and drugs. In this field Radiation Biology has played an important role in the development of both technical and cultural background, because it was very soon recognized the radical nature of processes following exposure to ionizing radiation. Several studies have pointed out the importance of both radicals, reacting with cellular targets, and endogenous thiols, mainly represented by glutathione, in controlling radiation responses of living cells. Experimental supports for such a role mainly rest on observations made on cell lines depleted of glutathione content because of a genetic defect or as result of a pharmacological manipulation. We present a study on the influence of endogenous and exogenous thiols on the correlation between lethal and mutational damage in mammalian cells. Survival (S) and induction of HPRT- mutation (M) were measured in cells irradiated with X-rays either after treatment with BSO or in the presence of MEA or GSH. In control experiments log of S is linearly correlated to M. Incubation with 1 mM BSO reduces cellular GSH content and produces an increase in radiosensitivity with regard to both lethal and mutagenic effects. In the presence of MEA a concentration dependent radioprotective effect can be observed on both end-points. GSH added to cells immediately or 90 min before irradiation only displays a slight protective effect on lethality. The yield of mutant cells is not significantly affected when GSH is added immediately before irradiation. Instead, when GSH is added 90 min in advance and the cells incubated at 37 °C, the mutation frequency is almost completely reduced to the background level. The conclusion that can be drawn from the data is that while the modified competition model for the role of thiols in cellular radiosensitivity can possibly justify the results obtained with cells treated with BSO and/or MEA, the results obtained treating the cells with exogenous GSH show that in responses implying a long chain of events, post-irradiation biochemical and biological processes appear to be of prevailing importance.*

Riassunto (Ruolo delle sostanze tioliche negli eventi letali e mutageni indotti dalle radiazioni ionizzanti). - *Negli ultimi trenta anni si è sempre più affermato il ruolo dei radicali liberi in processi biologici quali la cancerogenesi, l'invecchiamento, lo sviluppo di alcune sindromi genetiche e la tossicità cellulare sia da agenti chimici che fisici. Un notevole sviluppo delle conoscenze in questo settore è stato dato dalla Radiobiologia in quanto, fin dall'inizio, è stato riconosciuto come fondamentale il ruolo dei radicali liberi indotti dalle radiazioni ionizzanti nel determinare la risposta cellulare. Molti studi hanno messo in evidenza l'importanza sia dei radicali primari, che possono reagire direttamente con il bersaglio, sia dei tioli endogeni, soprattutto il glutatione (GSH), nel modulare la radiosensibilità cellulare. Evidenze sperimentali di questo ruolo vengono soprattutto da cellule prive di GSH intracellulare o per difetti genetici o come risultato di una manipolazione farmacologica. In questo manoscritto viene presentato uno studio riguardante l'influenza di tioli endogeni ed esogeni sulla correlazione esistente tra eventi letali ed eventi mutageni in cellule di mammifero. La sopravvivenza (S) e l'induzione di mutazioni (M) HPRT- sono state misurate in cellule esposte a raggi-X sia dopo trattamento con butionina-sulfossimina (BSO) sia in presenza di cisteammina (MEA) o GSH. Il trattamento con BSO 1 mM riduce il contenuto intracellulare di GSH e produce un aumento della radiosensibilità sia per quanto riguarda l'effetto letale che quello mutageno. In presenza di MEA si osserva sia sulle mutazioni che sulla sopravvivenza un effetto radioprotettivo concentrazione-dipendente. L'aggiunta di GSH immediatamente o 90 min prima dell'irraggiamento produce solo un debole effetto protettivo sulla letalità cellulare. Il numero dei mutanti indotti non è significativamente influenzato dall'aggiunta di GSH immediatamente prima*

dell'irraggiamento mentre invece l'incubazione per 90 min a 37 °C provoca la quasi completa rimozione dell'effetto mutageno. In conclusione, mentre il modello di competizione del ruolo dei tioli nella radiosensibilità cellulare può giustificare i risultati ottenuti con cellule trattate con BSO e/o MEA, quelli relativi al trattamento con GSH esogeno mostrano che, in quelle risposte biologiche che implicano una lunga catena di eventi, il ruolo prevalente viene svolto dai processi biochimici e biologici che intervengono a seguito del trattamento radiante.

Introduction

Sulfur radicals of interest in radiation biology are those deriving from thiol compounds, *i.e.* from the aminoacid cysteine which is by far the main thiol in living matter. However, there is normally little free cysteine in cells or in the extracellular fluids, since all cysteine and cystine are in bound form either in proteins or in glutathione (GSH). Several evidences indicate that sulfur radicals which are involved in the response of living cells to noxious agents such as radiation and drugs, mainly originate from GSH.

GSH was first detected in yeast about hundred years ago and is the most abundant natural low molecular weight thiol being present in virtually all cells in millimolar concentration [1-5]. A general scheme of GSH metabolism and transport is presented in Fig. 1. GSH can permeate from the cells to the outside but not the opposite. In fact the repletion of intracellular GSH, by resuspending cells in media containing GSH, takes place by a process involving enzymatic degradation of the external GSH, uptake of the products, and intracellular resynthesis. High extra- and intra-cellular concentrations of GSH function as regulatory system for γ-glutamic-synthetase and transport of γ-glutamyl-aminoacids.

GSH participates in many metabolic cellular functions. It is coupled to the energy and oxidation reduction status of the cell. It regulates the activity of several enzymes and is known to be the prosthetic group and cofactor in a number of enzymatic pathways. It is involved in the metabolism of leukotriene and prostaglandines. It plays an important role in cell growth and replication. It is a limiting step in the synthesis of deoxiribonucleotide from the ribonucleotide acting as hydrogen donor in the reaction catalyzed by the GSH transhydrogenases. These GSH-dependent enzymes have important functions related not only to DNA synthesis but also to protein synthesis, structure, degradation and activity.

GSH and cellular radiosensitivity

The hydrogen donation mechanism, where oxygen and sulpydryl are in competition for radiation induced radicals, was postulated by Alexander and Charlesby [7]. This model, which is indicated as the "radical repair model", describes the role of intracellular thiols in radiation sensitivity and in the oxygen effects [6-9].

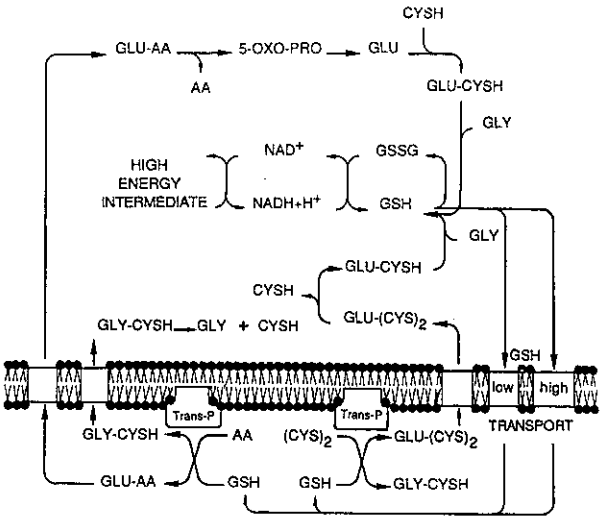

Fig. 1. - Overall view of glutathione metabolism. Glutathione is synthetized intracellularly by consecutive actions of γ-glutamyl-cysteine synthetase and GSH-synthetase. The first reaction is feedback inhibited by GSH. The breakdown of GSH is catalyzed by γ-glutamyl-transpeptidase (trans-P) an enzyme localized on the external surface of the cell membranes. The transport of GSH from inside to outside of the cell is catalyzed by two active processes possessing low and high affinities respectively to the substrate. The trans-P catalyzed at least three types of reactions: (i) transpeptidezation where the glutamyl moyety is transferred to an aminoacid as acceptor to form a glutamyl-aminoacid, (ii) autotranspeptidezation where the glutamyl is tranferred to GSH itself, (iii) hydrolysis where the acceptor molecule is water to form a molecule of glutammic acid. All three reactions have as secondary product the formation of a molecule of cysteinyl-glycine. Intracellular GSH is converted to GSSG by selenium containing GSH peroxidas which tatalyzed the reduction of H_2O_2 or other peroxides. GSH is also converted to GSSG by transhydrogenation and is formed by reaction of GSH with free radicals. Reduction of GSSG to GSH is mediated by the GSSG reductase which uses NADPH.

Conflicting evidences have been reported on the role of cellular SH-groups in controlling radiation response of mammalian cells in culture indicating that such simple correlations cannot be established [10-13]. Nevertheless, a modulating role of endogenous thiols in cellular response to radiation and in the oxygen effects is suggested.

Cellular thiol fluctuations in human beings include genetically controlled variations. Fibroblasts from patients affected by 5-oxoprolinuria [14] show low level of intracellular GSH, poor plating efficiency, reduced oxygen enhancement ratio (OER), and increased sensitivity to cytotoxicity, DNA single strand breaks (ssb) and micronuclei induction by hypoxic irradiation [15].

Attempts to artificially manipulate the intracellular SH content by treatment with suitable drugs, such as N-ethyl-maleimide (NEM) and iodo-acetamide (IAM), go back to early fifties [16]. However both drugs are so toxic that their use in mammalian cell experiments is severely limited. In recent years, thiols reagents with low toxicity and good specificity such as diamide (DAM), diethylmaleate (DEM) and buthionine sulfoximine (BSO) became available [17, 18]. The latter compound

is phosphorilated by ATP in living cells, and specifically inhibits γ-glutamylcysteine synthetase by irreversibly binding to the enzyme active site. The drug was synthetized by Griffith and Meister in 1979 [18], and shown to be a potent and specific inhibitor of the GSH synthesis *in vitro* as well as *in vivo*. Using BSO the intracellular GSH content can be drastically reduced without killing the cell. The relationship between radiosensitivity and biochemical/metabolic alterations occurring in drug treated cells is not well established [19-24].

Over the years, many experimental observations have been made which are consistent with the radical repair model. In many studies, cell survival was the end-point. However the model concerns the initial radical reactions, while survival, determined days after radiation treatment, reflects the integrated effect of numerous, subsequent biochemical reactions, which may modify the effect of the earlier processes involving radicals. Therefore survival may not be an appropriate end-point when the effects of such reactions are studied. On the contrary when the whole cellular response is considered, the use of short term end-points, such as DNA ssb production and repair, may be misleading [25].

Correlation between survival and mutation

For our studies on the influence of thiols on the cell response we chose as end-points the survival and the induction of hypoxantine phosphoribosil transpherase (HPRT-) mutations.

One of the difficulties in comparative mutagenicity studies of different chemical and physical agents is the evaluation of dose at the target cellular sites. In the case of physical agents, such as ionizing radiation, uncertainties on the actual dose are small but, when chemical agents are used, the concentration of such agents at the critical sites may be drastically affected, e.g. by permeability barriers. In general these difficulties might be circumvented by correlating the mutagenic effect of several agents not with the exposure or dose but with some other induced measurable effects, e.g. DNA modifications or survival levels. Several experiments, published in the last decade [26-32] have shown that there is a linear correlation between the logarithm of the cell surviving fraction and the frequency of mutants induced by radiation.

The survival/mutation (S/M) relationship must be independent of the nature and the number of lesions, provided this is not too large and does not explicitly include the dose. The chance that any random unrepaired or misrepaired DNA lesions can induce a mutational event or cause cell inactivation should be dependent on the relative frequencies of the initial lesions and on the corresponding probabilities that each lesion leads to inactivation or mutation.

The technique used in our experiments is the standard one for both survival and mutation induction as already reported [31].

Table 1. - *GSH cellular content*

Treatment	μg/mg protein	(%)
No treatment	8.3 ± 2.1	100
1 mM BSO for 18 h	0.073 ± 0.08	0.9
5 mM MEA	10.5 ± 2.1	120
50 mM MEA	8.6 ± 1.7	104
50 mM GSH	9.5 ± 1.6	114
BSO + GSH	0.4 ± 0.12	4.8

V-79 cells were grown in MEM + 10% FCS

When cells were treated with 1 mM BSO for 18 h the content of intracellular GSH decreased drastically (Table 1), and less than 1% of the original concentration could be found. This decrease affects both survival and mutation frequency (Fig. 2), but, while the lethality increases by a factor of 1.2, the mutagenic response rises by a factor of 2.1. The consequence is that, for the same survival level, a higher number of mutants can be detected in figure 6.

In cells treated with BSO we have followed the recovery of GSH (Fig. 3) [33] which is quite slow. After 24 h the intracellular concentration of GSH is still 10-20 % of the original value. On the contrary the radiosensitivity of the cells is back to normal only two hours after the removal of BSO (data not shown). This discrepancy between the GSH recovery and the radiosensitivity seems to indicate that at least part of the sensitizing effect is not directly correlated with the GSH concentration at the moment of irradiation. A second possible explanation is that the artificial depletion of thiols may not proceed uniformly in different cell compartments. In isolated V79 nuclei the depletion of GSH was considerably lower than in the whole cells [34]. However, nuclear GSH amounts to less than 1% of the total GSH. Therefore the measurement of the total GSH content after BSO treatment will hardly reflect the extent to which this thiol is depleted from the nucleus.

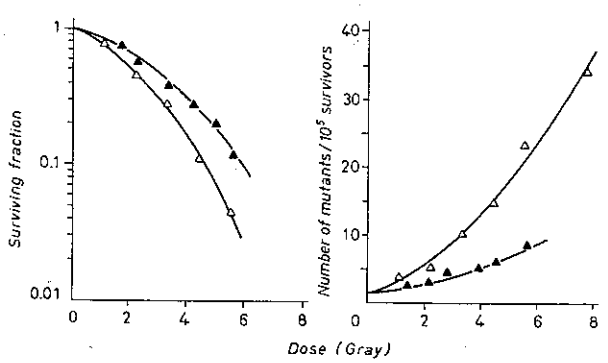

Fig. 2. - Lethal and mutagenic effects of different doses of X-ray on V79 cells treated (Δ) or not (▲) with 1 mM BSO for 18 h at 37 °C.

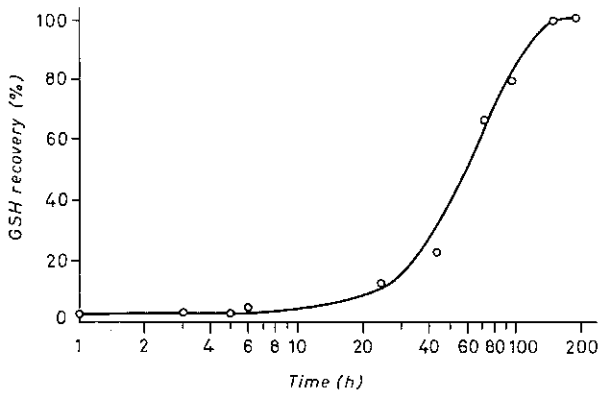

Fig. 3. - Glutathione recovery in V79 cells pretreated with 1 mM BSO for 18 h. After treatment the drugs were removed, the cells washed twice with fresh prewarmed medium and incubated for different lenghts of time at 37 °C. GSH concentration was determined using Tietze method [33]. GSH cellular content: untreated cells 6.5 μg/mg protein, BSO treated cells 0.11 μg/mg protein.

Subsequently we have investigated the effect of increasing concentration of thiols on both lethal and mutagenic events. We used two compounds: cysteamine (MEA) and the GSH at different concentrations. While MEA can freely diffuse into the cell, GSH cannot. In addition, relatively high concentrations of the latter compound can inhibit the transpeptidase enzymes localized on the external surface of the cell membrane, thus preventing the internalization of Cys-Glut from which new molecules of GSH can be synthesized.

In Fig. 4 the effects of two concentrations of MEA (5 and 50 mM) on lethality and mutation induction are reported. A concentration dependent protective effect can be detected both on survival and mutation. However, from the quantitative point of view, the effect on the two end-points is not similar. The effect on lethality is large for both MEA concentrations with dose modifying factors (DMF) of 2.3 and 3.6 respectively. Mutation induction is only reduced by a factor of 1.2 and of 2 at 5 mM and 50 mM MEA, respectively. At 3 mM MEA (data not shown), while the protective effect on survival is still relatively large (DMF = 1.8), no protective effect can be detected on mutation induction. The protective effect on both end-points can be detected only when MEA

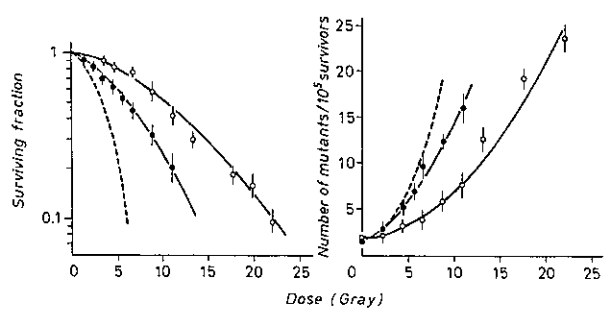

Fig. 4. - Cell killing and mutation induction in V79 cells irradiated in the presence of 5 (●) or 50 (○) mM cysteamine. The dashed lines represent the responses obtained irradiating the cells in the absence of the drug.

is present at the moment of irradiation, irrespective of the temperature and/or time between the addition of the compound and irradiation itself.

These results indicate that lethal and mutational damages exhibit different susceptibilities to the modifying action of MEA. Whether this is related to a common or different types of damage is still an open question. Plotting the surviving fraction as a function of the mutation frequency as shown in Figure 6, a larger amount of mutants are present, at the same survival level, compared to that found in cells irradiated in the absence of the drug. This effect is independent of the drug concentration. The same S/M relationship holds also for cells irradiated in the presence of 3 mM MEA where a protective effect is found only on survival (data not shown).

Different results are obtained by irradiating the cells in the presence of different concentrations of reduced GSH. Adding GSH immediately before irradiation to cells kept at room temperature or 90 min before irradiation to cells kept at 0 °C, no effects on both survival or mutation induction can be observed. This result is not surprising considering that GSH does not enter the cell. In the attempt to increase the intracellular GSH level, cell monolayers were incubated at 37 °C in growth medium in the presence of 50 mM GSH. After 90 min incubation the intracellular GSH concentration was still close to the control value (Table 1) and the survival levels (Fig. 5) did not show any significant difference as compared to those obtained by adding GSH immediately before irradiation (with only a small protection in the high dose range). In contrast, the cellular response to the GSH pretreatment with respect to the mutation induction was rather surprising (Figs 5 and 6). In fact, complete protection from the mutational effects induced by ionizing radiation was found. We tried to investigate this effect more closely by using different experimental conditions. Low concentrations of GSH (1 or 5 mM) have no effect on mutation frequency, while a full protective effect can be obtained with GSH concentrations of 10 mM or higher. Furthermore, in cells incubated with 50 mM GSH for the same length of time at 0 °C, no effect can be detected. These results indicate that the protective effect of GSH is an active process, possibly mediated through the cell membrane by an enzymatic system requiring preincubation in growth medium at optimal temperature. Moreover, the protective effect is observed only when the extracellular concentration of GSH exceeds the intracellular one.

To have more information on this effect a combined treatment with BSO, to decrease the intracellular GSH level, followed by a treatment at 37 °C with MEA or GSH was carried out. The addition of 10 mM MEA immediately or 90 min before irradiation produces a protective effect on both end-points removing the sensitation produced by BSO treatment. This effect does not depend on temperature and incubation time, confirming that the protection exerted by MEA is due to radiation chemical action and ruling out any possible metabolic involvement of the drug.

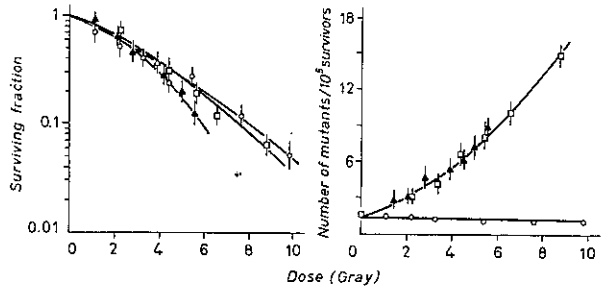

Fig. 5. - Effect of exogenous glutathione on cell survival and mutation induction. (□) Cells treated with 50 mM GSH added immediately before irradiation performed at room temperature. (○) Cells treated with 50 mM GSH at 37 °C for 90 min before irradiation, performed at room temperature. (▲) Cells treated with 50 mM GSH for 90 min at 0 °C before irradiation, performed at the same temperature.

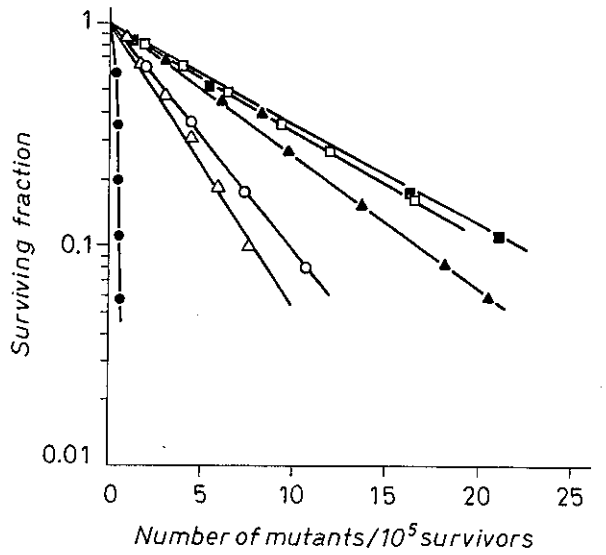

Fig. 6. - Relationships between the logarithm of surviving fraction and the mutation frequency from the data reported in Figs 2, 4 and 5. Untreated cells (Δ), cells treated before irradiation with 1 mM BSO (▲), cells irradiated in the presence of 5 mM (□) or 50 mM (■) cysteamine, cells irradiated in the presence of 5 mM GSH added just before irradiation (○) or 90 min earlier followed by incubation at 37 °C (●).

In contrast, GSH can exert its protective action against the BSO increased radiosensitivity and mutation frequency only when it is present in culture medium for 90 min at 37 °C (Fig. 7) at concentrations as low as 5 mM. Again the addition of GSH immediately before irradiation or incubation for the same length of time but at 0 °C do not produce any radioprotecting effect.

Similar results but only on cell survival have been reported by Clark *et al.* [35, 36]. These authors have shown that the increased radiosensitivity induced in CHO, V79 and A549 cells by GSH depletion can be reversed by the exogenous addition of GSH. A possible explanation of this effect might involve the extracellular metabolism of GSH. Exogenously added GSH can be metabolized by the action of extracellular membrane-bound enzymes GSH transpeptidases; as a consequence SH-containing metabolites of GSH may diffuse into the cells producing a sufficient increase in the low molecular weight SH compounds to restore the normal radiosensitivity. Several evidences do not support this interpretation. The first one is that high concentrations of GSH inhibit the transpeptidase activity so that a very low increase in the thiols concentration follows the GSH treatment (Table 1). The second evidence comes from mutation induction experiments with cells treated or not with BSO and/or with high concentrations of GSH. The last one is that inhibition of transpeptidase activity by specific inhibitors before, during and after irradiation, does not eliminate exogenous GSH induced recovery [37].

Facts and models

Our data point out the complex frame of thiols influence both on lethal and mutational damage indicating that controlled variations of NPSH do not allow to establish univocal quantitative relationships.

The "radical repair" model is currently the most accepted being supported by several experimental evidences. It has been confirmed that GSH is an hydrogen donor as good as other low molecular weight thiols. There are not substantial arguments against the possibility that the H-donation reaction can really occur in living cells. However, according to the model cellular thiols should mainly affect anoxic radiosensitivity. Nevertheless biological data indicate that GSH depletion brings about in some cell lines also an increase of the oxic radiosensitivity.

Biaglow *et al.* [39] have proposed a more comprehensive model, which includes the "radical repair" model, but also emphasizes the role of GSH in the aerobic radiation response (Fig. 8). In particular, with regard to oxic

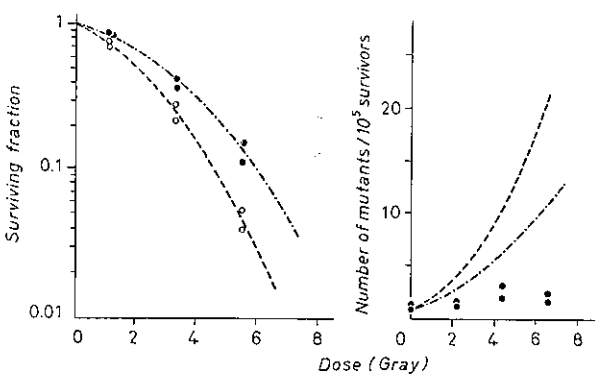

Fig. 7. - Effect of exogenous glutathione on survival and mutation induction in cell pretreated with 1 mM BSO. The cells were treated with BSO for 18 h at 37 °C then washed twice with medium and incubated for 90 min at 37 °C in presence of 50 mM GSH (●). Cells treated with BSO but not with GSH (○). The dashed lines represent the responses obtained irradiating untreated cells (—·—), or with cells treated only with BSO (——).

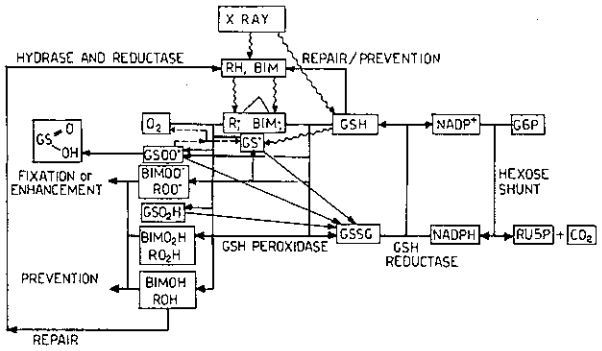

Fig. 8. - Hypothetical role of endogenous GSH in protection against radiation damage to biologically important molecules (BIM). With regard to oxic damage, the model postulates that GSH can chemically reduce, by hydrogen donation, peroxyradicals (BIMO2, RO2) while the resulting hydroxy-peroxides (BIMO2H, RO2H) can then be reduced by GSH-peroxidase. These intermediates must in turn be dehydrated and be reduced back to the original molecule. The model takes into account also the reaction of GSH with oxygen. However no mechanism can completely account for the total damage caused to a cell by radiation and it should be stressed that endogenous GSH does not totally prevent cellular lethality under aerobic or hypoxic conditions. Both R and BIM radicals may react directly with GSH or hydrogen donors to produce the original molecule. This process will chemically repair or prevent additional chain reactions by removing reactive radicals. On the other end oxygen will form adducts to fix the damage, thereby enhancing the radiation effects. The hexose monophosphate shunt provides the reducing equivalents necessary to maintain GSSG as GSH. (Diagram redrawn, adapted and enlarged from Biaglow *et al.* [40]).

damage, the model postulates that GSH can chemically reduce peroxy-radicals, while the resulting hydroxy-peroxides can be reduced by GSH-peroxidase. The model does certainly exhibit a better correspondence to the experimental evidences [40], although there are still aspects that are not taken into consideration. We refer to the problem of GSH radicals reaction with oxygen. In fact it is known that thiols irradiated with oxygen react with it. Since in living systems GSH exists in substantial concentration, significant amounts of GS· radicals should be formed together with target radicals. This is commonly accepted when the radical scavenging ability of thiols is invoked to justify part of their protective action. It seems reasonable, therefore, to postulate that, in oxic conditions, GS· radicals or in more general terms RS· radicals, also react with oxygen according to their rate constant and concentration. The reaction of thiyl radicals with oxygen would then be in competition with that of target radicals with the same oxygen. Therefore, it cannot be excluded that thiyl radicals reacting with oxygen might reduce the availability of oxygen for the damage fixation reactions.

Our results, obtained with BSO and/or MEA-treated cells are in agreement with such an extended model. It must be taken into account, in fact, that peroxy-radicals formed in DNA are considered to be highly mutagenic. Moreover it has been found that transferases, with bound GSH, in the histone fraction within the nucleus are critical in reducing hydroperoxides produced by radiation [41]. Thus the increase or decrease of thiol intracellular level may modulate the cellular oxic response. The protective or sensitizing effects can be mainly ascribed to the radiation chemical effects of thiols which must be present at the moment of irradiation and close to the cellular targets.

Similar explanation does not hold for the effect exerted by exogenous glutathione on both survival and mutation that occur outside the cells and depend on temperature, time and conditions of growth. This suggests that the GSH-induced cell recovery may be initiated solely by its interaction with the cell membrane and that this interaction may be an enzymatically catalyzed event. Several hypothesis can be suggested. One requires that the catalytic reaction of the cell membrane-bound enzyme would act to reverse radiation induced membrane damage. An example of such reaction is the GSH peroxidase reduction of organic hydroperoxides formed in unsaturated fatty acids. However this hypothesis seems to be unjustified in view of the experiments carried out by Wolters and Konings [42]. These authors have shown that the effect on radiosensitivity, both under oxic and anoxic conditions, induced by modulation of GSH is similar in cells containing different levels of polyunsaturated fatty acids. Alternatively a possible explanation might involve alterations of the membrane in the presence of high extracellular concentrations of GSH.

Althought unusual the effects induced by GSH are not unique in radiobiology; in fact pretreatment with prostaglandin E1, which cannot diffuse inside the cell, can induce in cultured cells a large radioresistence [43].

Conclusions

As conclusive remarks we believe that in order to satisfactory understand the role of thiols in cellular radiosensitivity, one must take into account not only what thiols do in absence of oxygen but also what they do in its presence. A comprehensive picture of the events occurring in oxically irradiated living matter cannot be proposed without providing a proper allocation for the reactions leading to the formation and decay of thiol peroxy-radicals. Finally our results on mutation induction might indicate a possible biochemical role of GSH in the induction of an error free repair system. However more experimental evidences on GSH direct or indirect involvement in the DNA synthesis and repair are needed.

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