DNA DAMAGE AND REPAIR INDUCED BY RADIATION

Ann. Ist. Super. Sanità
Vol. 25, N. 1 (1989), pp. 59-68

RADIATION DAMAGE AND CHROMATIN STRUCTURE

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Summary. - *The recent advances made in the knowledge of chromatin structure have important implications in molecular and cellular radiobiology. There are now many lines of evidence that the chromatin organization can affect the production, the distribution and the repair of radiation-induced damage in DNA. Experiments with polynucleosomes show that DNA double strand breaks (dsb) are not randomly distributed along the DNA molecule. Rather, they are preferentially localized in linker regions, while core regions are more resistant. Isolated DNA is about 4-fold more susceptible to dsb than DNA irradiated as a part of polynucleosomes. This differential radiosensitivity is apparently due to the close association of DNA with proteins. The analysis of DNA single strand breaks production and repair in a human erythroleukemic cell line that can be induced to differentiate* in vitro, *showed that the repair kinetics in differentiated cells appears significantly slower than in undifferentiated ones. This can be interpreted as a decrease in the genome accessibility to repair enzymes due to the presence of more structured regions in chromatin after differentiation. It appears that a high degree of genome compactness could imply, on one hand, a high DNA radioresistance and, on the other hand, a slow DNA repair so that the identification of chromatin domains which are critical, from the structural point of view, in determining cellular effects such as cell killing and mutation, should take into account a sort of balance between the amount of damage and the extent of repair.*

Riassunto (Danno da radiazioni e struttura della cromatina). - *I recenti sviluppi delle conoscenze sull'organizzazione strutturale del genoma eucariotico hanno aperto nuove prospettive di ricerca nella radiobiologia molecolare e cellulare. Vi sono attualmente diverse evidenze sperimentali che sottolineano l'importanza dell'organizzazione strutturale della cromatina nella produzione, distribuzione e riparazione del danno radioindotto sul DNA. Esperimenti effettuati su sistemi modello, quali polinucleosomi irradiati* in vitro, *hanno mostrato che le doppie rotture sul DNA sono non uniformemente distribuite lungo la molecola ma preferenzialmente localizzate nelle regioni internucleosomali, mentre le regioni intranucleosomali risultano più resistenti. Inoltre il DNA isolato da polinucleosomi e successivamente irradiato è circa 4 volte più sensibile allo stesso tipo di danno. Questa diversa radiosensibilità è determinata dall'associazione del DNA con la componente proteica della cromatina. L'analisi della produzione e riparazione di singole rotture sul DNA di una linea cellulare eritroleucemica umana che può essere indotta a differenziare* in vitro *ha mostrato che la cinetica di riparazione del danno è significativamente più lenta in cellule differenziate. Questo comportamento può essere interpretato come una minore accessibilità del genoma agli enzimi della riparazione dovuta alla presenza di regioni maggiormente strutturate nella cromatina di cellule differenziate. Appare quindi che regioni più aperte del genoma presentano, da un lato, una maggiore radiosensibilità e, dall'altro, una più rapida riparazione. Pertanto, l'identificazione di quelle regioni che sono maggiormente critiche, da un punto di vista strutturale, nel determinare effetti cellulari, come letalità e mutagenicità, deve tener presente che l'organizzazione del genoma influenza sia l'ammontare del danno prodotto che l'entità della sua riparazione.*

Introduction

It is well established that DNA is the main cellular target of ionizing radiation. A description of radiation action, as well as that of other harmful agents, on eukaryotic cells must consider that the DNA is closely associated with proteins. The great advances in the knowledge of chromatin structure made in the last decade have provided an interesting framework for a better understanding of the mechanisms involved in DNA damage production and repair [1, 2].

The currently accepted model of the genome takes into account different levels of organization: the nucleosome, the 34 nm "solenoidal" fiber, and further levels of

folding that provide the packaging of the fiber itself [3, 4]. This organization is flexible and undergoes various structural changes related to the cellular functions. During cell division, euchromatic regions become tightly packed in mitotic chromosomes while events such as replication and transcription involve partial relaxing of structure.

The organization of the fiber in loops, associated to the nuclear matrix, appears to play an important structural and functional role [3], forming domains that can provide a structural compartmentalization of the nucleus. It has been proposed [5] that such a compartment, rich in specific DNA binding sites, would facilitate the formation of the appropriate DNA-protein complexes required for the control of transcription, replication and repair by reducing the search volume within the nucleus [6-9]. These sites can change with differentiation and specialization of the cellular pattern of expression.

The influence of chromatin organization on the production and distribution of DNA damages is an important subject that deserves particular consideration in toxicological studies. That chemical agents can produce DNA damages preferentially in more open regions of chromatin is not surprising in view of the greater accessibility to the target molecule [10-12]. On the other hand, for some physical agents such as ionizing radiation, since there are no reasons to suppose that the spatial distribution of energy deposition events could be affected by the target structural organization, it was assumed for long time that the subsequent damages are randomly distributed throughout the cellular DNA. Studies of damage production on cellular DNA, measured in terms of single (ssb) or double (dsb) strand breaks, showing that the size distribution of the DNA fragments is nearly random, seems to support such an assumption. However, the techniques currently used to evaluate the damage in high molecular weight DNA are unable to distinguish between a random distribution of the damage and different radiosensitive regions randomly distribuited throughout the genome.

Experimental evidences

Early experiments [13] aimed at investigating the radiolysis of chromatin in aqueous solutions indicated that the proximity of the proteins to the DNA could have a protective effect. In recent years some studies, which have analyzed the damage production in particular regions of the genome, have shown that chromatin structure plays an important role in determining the damage distribution and repair after treatment with ionizing radiation. This kind of evidences comes from studies pointing to different levels of genome organization, performed using different biological systems such as isolated chromatin fragments or cells in different metabolic states.

Mee and Adelstein [14] recently reported that isolated chromatin can be a good model for radiation damage investigations. They irradiated high molecular weight chromatin extracted from V79 cells and found a linear relationship between the number of dsb or ssb and the dose, with a ratio of nearly 1:10 between the amounts of such lesions. The yields for both ssb and dsb are very close to those reported in the literature for the initial number of breaks in irradiated cells, suggesting that the mechanisms of damage induction in isolated chromatin may be comparable to those operating in the intact cells.

Heussen *et al.* [15] examined the extent of X-ray damage in chromatin either in low ionic strength buffer or condensed by Mg or H1-histone depleted. At the concentration of 1 mM $MgCl_2$, chromatin assumes the "solenoid" configuration while at low ionic strength the individual nucleosomes are well separated and the diameter of the whole nucleosome chain is about 11 nm [16]. They used chromatin fragments, extracted from V79 cells, large enough to form the "solenoid" configuration with regular subunit structure similar to that present in the nuclei. The fluorometric assay (FADU) they used is extremely sensitive even if it does not give an absolute measurement of the number of ssb. These authors found that radiation damage decreased with increasing $MgCl_2$ concentration. At 1 mM, chromatin presented less than half the radiation damage found at low ionic strength, while an increase of 1.3 fold was observed in H1-depleted chromatin. In terms of chromatin structure, these results mean that radiation sensitivity increases from 34 to 11 nm fiber and further with the dissociation of H1 from linker DNA. The authors proposed that the enhanced radiation sensitivity found in H1-depleted chromatin could be due to a greater susceptibility of the unprotected linker regions to radiation damage.

Studying the interphasic death of rat thymocytes caused by X-rays, Yamada *et al.* [17] found that chromatin was subjected to double strand cleavage in the internucleosomal DNA regions, as pointed out by the progressive release of nucleosomes and their oligomers during post-irradiation incubation at 37 °C. Other groups, using the same cell type, also found a release of monosomes and attributed the cleavage to nucleases activated by radiation [18-20]. Gelderblom *et al.* [21], studying the sensitivity to micrococcal nuclease of rat liver nuclei, irradiated under conditions in which endogenous nucleases are inhibited, reported that for doses up to 200 Gy, no preferential radiosensitization of linker regions could be detected. However, the micrococcal nuclease assay is not specific for radiation damaged sites. Therefore, the absence of such an effect does not rule out the possibility of preferential radiation-induced alterations since they could occur too infrequently along the DNA chain to be visualized in this assay. Moreover, radiation damaged DNA could mantain its normal relationship to the histones and other chromosomal proteins and hence its nuclease-sensitive sites [22].

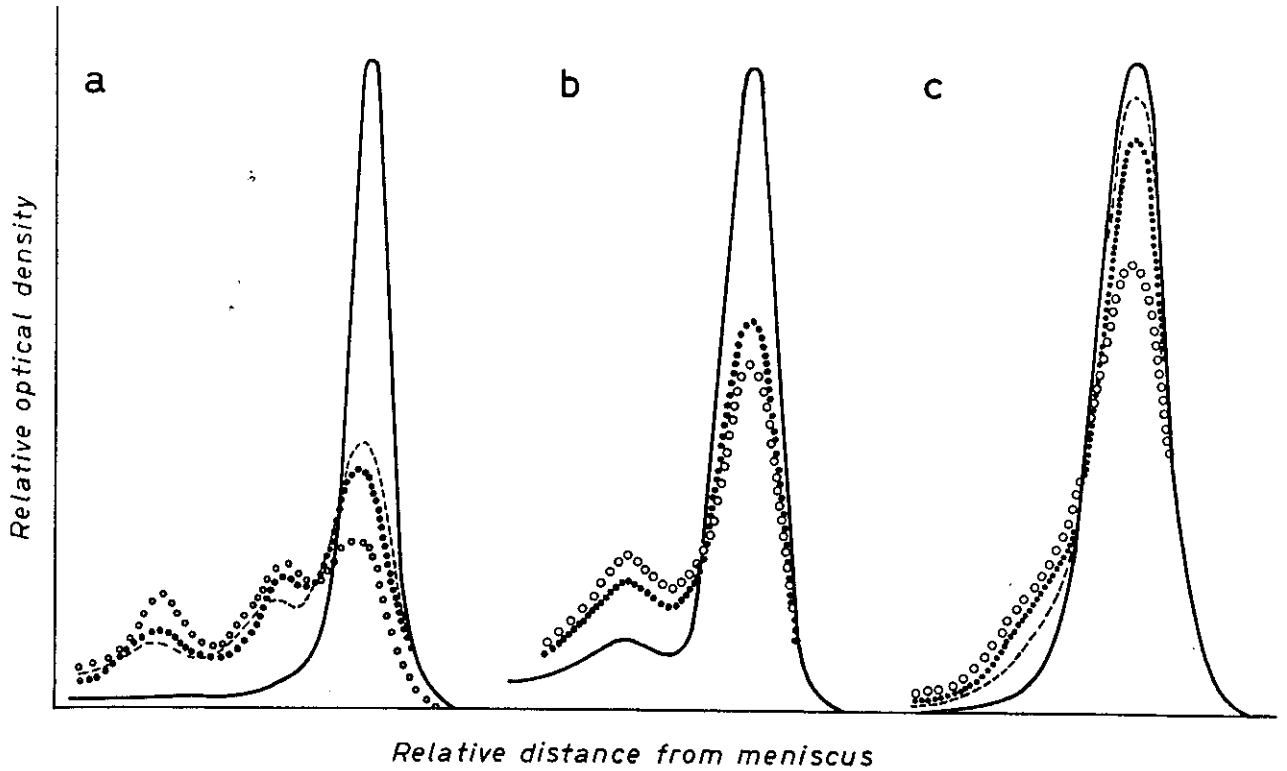

Fig. 1. - Sedimentation profiles of polynucleosomes irradiated with several doses of X-rays. The samples were loaded on a 5-30% neutral sucrose gradient and centrifuged at 4 °C for 17 h at 31000 rpm in a SW41 rotor. At the end of the run the gradients were scanned by using an ISCO-UA5 multi-wavelenght absorbance monitor at 260 nm. (a) trinucleosomes, (b) dinucleosomes, (c) mononucleosomes, (——) no irradiation, (– –) 3.3 kGy, (●●●) 5 kGy, (ooo) 6.6 kGy.

The experimental evidences already mentioned are rather contradictory, probably due to the techniques used, and do not give any direct and conclusive indications about the damage distribution along the DNA.

We approached this problem using a more simple model system, consisting of isolated polynucleosomes from hen erythrocytes, to investigate the yield and the distribution of one of the most critical lesions, the DNA dsb, produced by ionizing radiation. Polynucleosomes are not as representative of the eukaryotic genome as large chromatin fragments, but they retain the repetitive structure of core particles spaced by linker DNA and can be useful to investigate the influence of this level of organization in determining the DNA radiosensitivity. We analyzed the fragmentation in isolated nucleosomes and their oligomers as well as in DNA extracted from them using the sedimentation technique [23].

When trinucleosomes were irradiated, layered on top of neutral sucrose gradients and sedimented, a discrete pattern in the sedimentation profiles was observed. Increasing the dose led to a progressive accumulation of fragments in the regions corresponding to the lower oligomers (Fig. 1a). Similar behavior was found when dinucleosomes were irradiated (Fig. 1b). For mononucleosomes, only a limited broadening in the low molecular weight region of the sedimentation pattern was produced (Fig. 1c). These results seem to indicate that polynucleosome fragmentation is not random but occurs preferentially in the linker regions. However, the double strand breaks in the core regions could be hidden by the histones holding together the DNA fragments. To test this possibility, trinucleosomes were treated immediately after irradiation to remove both proteins and possible RNA contamination and the isolated DNA was sedimented (Fig. 2a). The profiles showed, also in this case, a discrete fragmentation pattern, confirming a specific localization of dsb when the DNA was irradiated as part of polynucleosomal structure. On the contrary, a different sedimentation pattern was obtained irradiating the DNA after isolation from trinucleosomes (Fig. 2b). The distribution of the DNA molecules was the one expected for randomly introduced dsb. We evaluated the mean number of dsb per molecule from the sedimentation profiles. Samples showing a discrete pattern were analyzed by Gaussian fitting and the fraction of intact molecules was measured by the normalized area under the relevant peak. For random patterns the analysis was performed in terms of molecular weight distribution, using a modification of the fitting procedure reported by Fox [24]. The results are reported in Fig. 3. It appears that the DNA associated with proteins in polynucleosomes is 3-4 fold more resistant than isolated DNA. Moreover, the comparison between the number of breaks found in irradiated trinucleosomes and that found

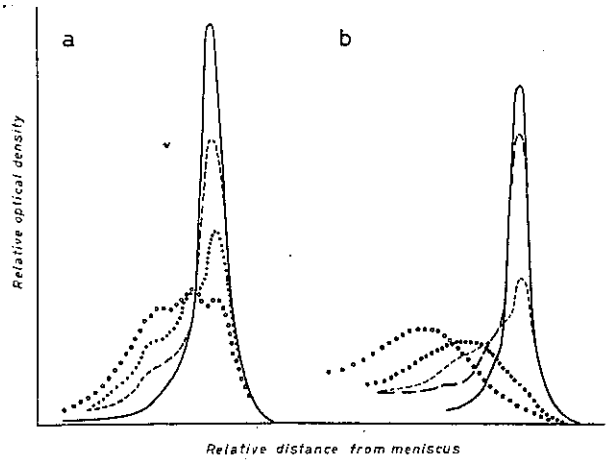

Fig. 2. - Sedimentation profiles of DNA (a) extracted from irradiated trinucleosomes or (b) irradiated after isolation from unirradiated trinucleosomes. The samples were loaded on a 5-30% neutral sucrose gradient and centrifuged at 4 °C for 27 h at 40000 rpm in a SW41 rotor, (———) no irradiation, (— — —) 1.6 kGy, (– – –) 3.3 kGy, (•••) 5 kGy, (ooo) 6.6 kGy.

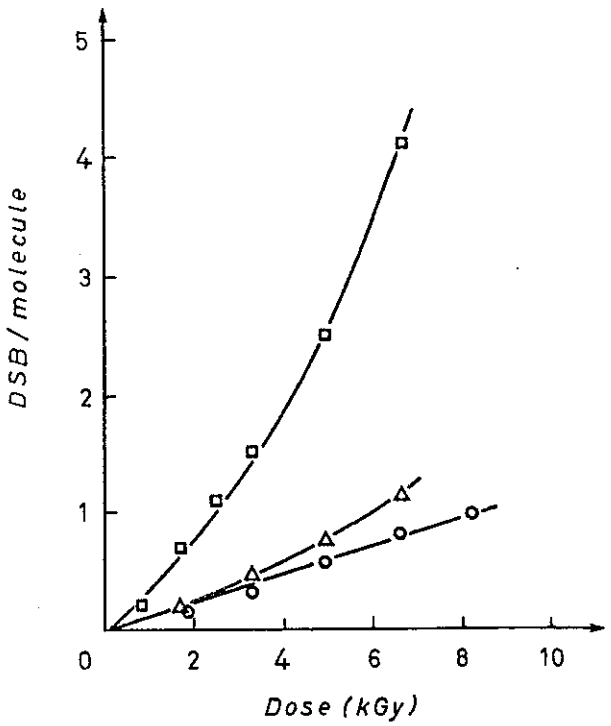

Fig. 3. - Dose-response curves for the number of double strand breaks induced per molecule in (O) trinucleosomes, (▲) DNA extracted from irradiated trinucleosomes and (□) DNA irradiated after isolation from unirradiated trinucleosomes.

in the DNA extracted from them after irradiation, indicates that only few breaks are hidden by the nucleosomal structure. These findings point out the importance of the association of DNA with proteins in determining the amount of damage and strongly suggest a non-random distribution of the damage itself.

Table 1. - *Relative efficiency (RE) of tγ thymine damage in various substrates (modified from [25])*

Mature chromatin	1.00
Nucleosome core	0.99
Nascent chromatin	3.32
DNA (mature)	6.45
DNA (nascent)	5.74
Heated nascent chromatin (48 °C, 30 min)	1.10

The main criticism against our experimental system is the need of using high doses to introduce detectable levels of damage. However, this is a direct consequence of the small mass of the target which, on the other hand, has the advantage to be very well characterized. A more general criticism is that the reported experimental evidence was all obtained by irradiating *in vitro* molecular systems, and the consequent extrapolation of the results to cellular systems can be questionable. However, similar indications have been obtained from works performed on cultured cells.

Eukaryotic chromatin at the replication fork transiently contains a reduced complement of histones and a low frequency of nucleosome core particles that increases to normal level rapidly during chromatin maturation. Warters and Childers [25] irradiating HeLa cells with increasing doses of gamma-rays, found different levels of thymine base damage (tγ) in mature and nascent chromatin DNA (Table 1). At all doses there was approximately 3.3 times more damage in nascent than in mature chromatin DNA. The authors postulated that the increased radiation sensitivity of nascent chromatin DNA could be due to its low histone concentration. Two more experimental evidences support this conclusion. The exposure of HeLa cells to 48 °C for 30 min resulted in 2.2 fold increase in nuclear protein content and reduced the radiosensitivity of nascent chromatin. Moreover, when irradiations were carried out on DNA extracted from nascent or from mature chromatin, no differences were detected in the damage production. The amount of such damage was about 6 times and 2 times more than that found in the DNA contained in irradiated mature and nascent chromatin, respectively. This is a further indication that the extent of DNA damage is related to the histone content of chromatin.

A more open organization in the chromatin structure is not only a characteristic feature of the replicating regions but also of regions transcriptionally active, as demonstrated by enzymatic digestion [26]. Chiu *et al.* [27-29] using electrophoresis technique followed by hybridization to specific probes for transcriptionally inert or active genes, studied the DNA damage production and repair in chromatin from different cell types. In a study performed on the radioresistant protozoan *Tetrahymena pyriformis* [27] they found that both the yield of γ-ray induced ssb and the repair rate were higher in actively

transcribing genes than in total DNA. The same trend was reported for rodent cells. In L929 mouse cell line, 5-6 times more damage was found in active gene DNA compared with that found in the total cellular DNA or in the transcriptionally inert centromeric heterochromatic DNA (Table 2) [28]. The same authors also reported that in Chinese hamster V79 cells active sequences contained within metaphase chromosomes mantain a radiation hypersentive structure (Table 3). The degree of hypersensitivity of active sequences is similar for mitotic and asynchronous V79 cells but, in both cases, is lower than that observed for asynchronous L929 cells [29]. They also studied the repair kinetics of the DNA damage. After 60 Gy, more initial breaks and more rapid repair kinetics were observed in active sequences than in total DNA.

A criticism to these experiments concerns the large supralethal doses delivered to the cells. Nevertheless, Warters *et al.* [30] recently demonstrate that even at doses which leave a significant fraction of viable cells, active DNA domains appear to be hypersensitive to radiation-induced ssb. Using the alkaline filter elution assay, they measured the ssb in transcriptionally active, transcriptionally inert and bulk DNA after doses of X-rays ranging from 2.5 to 10 Gy. Active sequences eluted more rapidly than bulk DNA (Fig. 4) and, at all doses, a larger amount of active DNA was also found in the first fraction (Table 4). For satellite DNA no differences were instead detected. Since DNA elutes as a function of size, the early fractions contain smaller DNA fragments than the later fractions, indicating that irradiation causes more damage in regions containing active sequences even at low doses.

Further suggestions about the role of the genome organization in determining the cell response to ionizing radiation arise from experiments performed on *in vivo* and *in vitro* differentiating cell systems. During differentiation the cells undergo morfological and functional modifications. Most of the genes are not expressed and only those devoted to specialized functions are in transcription. Moreover differentiated cells are not cycling and present structural genome modifications. Chromatin rearrangements have been detected by using intercalating agents in Friend erythroleukemia cells [31] and the presence of single strand breaks were found in the DNA of several differentiated cells, such as lymphocytes, neurons, erythroleukemic and muscle cells [32-35]. It was suggested that the DNA fragmentation might be a dinamic process, modulated by repair enzymes [36] and involved in the control of replication [37].

Wheeler *et al.* [35], using alkaline sucrose gradient sedimentation in zonal rotors, compared the ssb induction and repair in undifferentiated intracerebral 9L/Ro tumor cells (gliosarcoma), and terminally differentiated cerebellar neurons, irradiating *in vivo* male Fisher 344 rats. The experimental methodology required the use of relatively high doses for the damage evaluation. While the initial number of ssb seemed to be the same in both

Table 2. - *Single strand breaks in log phase L929 cells (modified from [28])*

Chromatin substrate	Hybridization probe	N. ssb/10 Gy per 4 x 10^8 Da
Total (^{3}H labelled)		0.85 ± 0.25
Active (mRNA)	Poly(A+)RNA	4.40 ± 0.50
Ribosomal chromatin	rRNA	6.10 ± 1.00
Centromeric	Satellite DNA	0.54 ± 0.58

Table 3. - *Single strand breaks in non-mitotic and metaphasic V79 cells (modified from [29])*

Cell type	Total DNA	Active DNA	Active/ Total
Nonmitotic	1.40 ± 0.70	3.2 ± 1.0	2.3
Metaphasic	0.84 ± 0.28	3.0 ± 0.6	3.6

Table 4. - *Percentage of DNA eluting in the first fraction (modified from [30])*

Dose (Gy)	Bulk DNA	Active sequences
2.5	1.4	3.5
5.0	4.4	8.8
7.5	9.4	14.6
10.0	13.8	22.3

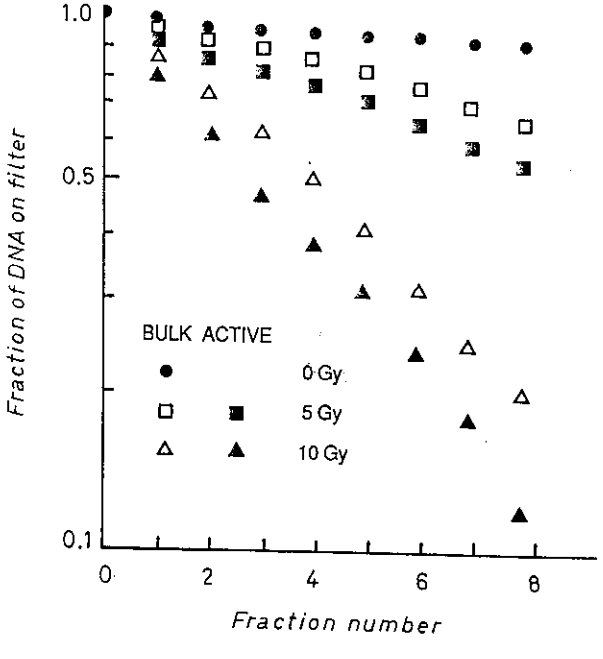

Fig. 4. - Alkaline elution kinetics of L929 cell DNA irradiated with 5 or 10 Gy X-rays. The DNA either eluted from or retained on polycarbonate filter was hybridized against poly(A+)RNA or mouse satellite DNA to probe transcribing or inert sequences (modified from [30]).

cellular systems, the repair kinetics showed interesting features. The process presented a fast and a slow phase at all doses. In tumor cells, the half-time for the fast phase was shorter than that found for neurons, and less damage remained to be removed during the slow phase. Moreover the half-time for the slow phase in cerebellar neurons always increased with increasing dose while in tumor cells the half-time remained constant up to 25 Gy, and increased at higher doses (Fig. 5). Since the change in the half-time of the slow repair kinetics indicates a saturable enzymatic process, it was concluded that the dose limit for such saturation is different in tumor and in neuron cells [38]. These saturation thresholds were reinvestigated in a more recent paper [39], where the authors used lower doses, but the results were questionable because of the detection limits of the sedimentation technique.

Recently, we investigated the DNA damage production and repair in a human erythroleukemic cell line (K562) [40] that can be chemically induced to differentiate *in vitro*. Using this cellular system we were able to compare directly the effects on the same cells before and after differentiation [41]. The process is irreversible and progressive modifications and appearance of new structures and functions can be observed in different cell compartments; 24 h after induction the cells stop dividing and 48 h later there are evidences of changes in their membrane properties [42, 43]. At about the same time, the cells start synthesizing fetal and embryonic hemoglobin. This synthesis represents the main biochemical characteristic of the pseudo-erythroid differentiation [44], and reaches its maximum after 5 days. Our experiments were performed 48 h after induction with 2 mM butyric acid, when the cells are definitely committed even if some differentiation markers are not completely expressed. We measured the X-ray ssb induction and repair using the alkaline elution technique that allows the study of the damage production at relatively low doses. Fig. 6 reports the dose-response curves, up to 7 Gy, for ssb production in undifferentiated and differentiated cells. No differences were detected between the two cell types, but it is necessary to point out that elution measures the damage in the bulk genome and can mask differences in sensitivity between active and unexpressed DNA sequences. Moreover, the measurements of ssb on differentiated cells were carried out on 60-70% of the total cellular DNA. The remaining 30-40% is lost during the lysis at pH 9.6 and is represented by DNA, which is acid precipitable, with a molecular weight close to 5 x 10^7 Da. The presence of nicks on cellular DNA is characteristic of the differentiation state of the cell as already mentioned.

We also measured the repair after a dose of 4 Gy and found a slower kinetics in differentiated cells, where a significant fraction of lesions was still present after 40 min of incubation (Fig. 7).

The repair results obtained using *in vivo* and *in vitro* differentiating systems can be interpreted either with the presence of different repair mechanisms or different levels of enzyme concentration, or with a different genome organization that can limit the accessibility of the repair

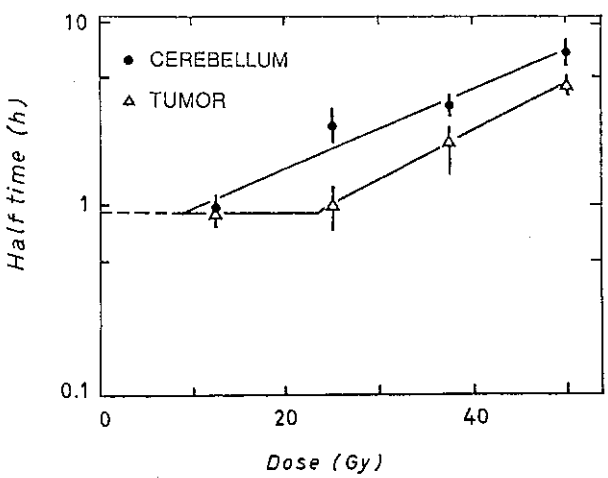

Fig. 5. - Half-time for the slow phase of the repair process in (●) neurons and (▲) tumor cells (gliosarcoma) after *in vivo* X-ray irradiation of male Fisher 344 rats (modified from [35]).

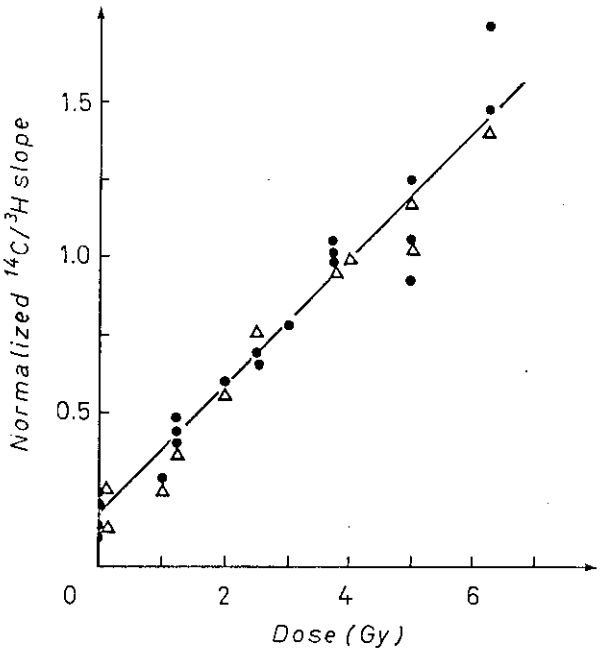

Fig. 6. - Dose-response curve for ssb production in undifferentiated (●) and differentiated (▲) K562 cells. Two cell populations, the first one labelled with ^{14}C thymidine and irradiated with different doses of X-rays, and the second one labelled with ^{3}H thymidine, irradiated with a fixed dose (4 Gy) and used as internal standard, were loaded on the same filter. The elution kinetics were obtained by plotting for each fraction the ^{14}C agains ^{3}H activity. The slope values corresponding to the samples irradiated with different doses were used as relative measure of damage. All the experimental procedures were carried out at ice temperature to prevent repair.

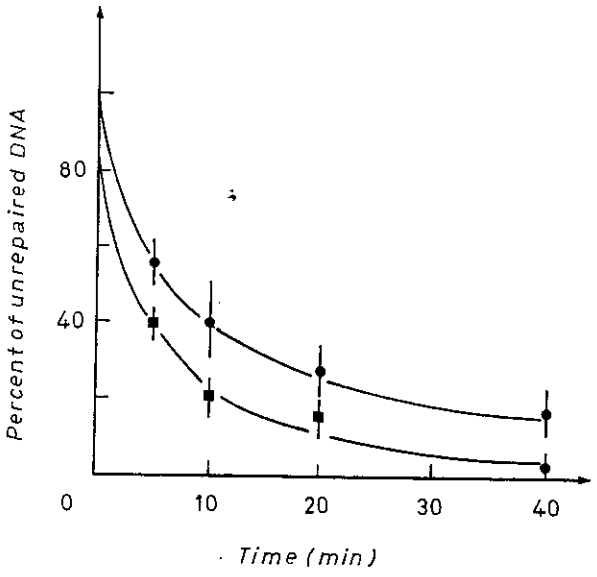

Fig. 7. - Repair kinetics of radiation induced ssb in undifferentiated (■) and differentiated (●) K562 cells. Both cell populations were irradiated at ice temperature with a fixed dose of X-rays (4 Gy). ^{14}C labelled cells were then incubated in medium at 37 °C to allow the repair while ^{3}H labelled cells were used as internal standard.

enzymes to the damaged sites. Terminally differentiated cells do not divide so that some inducible enzymes involved in replication and repair, such as alpha-polymerases, could be absent or only partially present in these cells. In our experiments only constitutive enzymes are presumably involved in the repair process since we considered short repair times and used such low doses that any enzymatic saturation effect should be excluded. There are no reasons to believe that differentiated and undifferentiated cells differ in their constitutive enzymes, so that our results can hardly be explained just in terms of differences in the enzymatic pool. Furthermore, it has been reported that the DNA contained in the nucleus of terminally differentiated cells results less sensitive to micrococcal nuclease digestion than that in undifferentiated cells [35]. In view of these considerations, the hypothesis of a different genome accessibility appears be a better explanation of the experimental evidence, even if other metabolic implications cannot be completely excluded.

Conclusion

A critical review of many experimental studies strongly suggests a relation between radiation damage and chromatin structure. Several lines of evidence, obtained with biological systems representative of different levels of genome organization, suggest the general view of a non-random distribution of the DNA damage. Structured chromatin regions suffer less damage than relaxed ones or isolated DNA and this appears to be related to the association with the protein component of chromatin. The mechanisms underlying this effect are not clear. They can include: chemical restitution (for example due to H· donation by protein thiolic groups), scavenging of free radicals or induction of a conformation that makes the target less exposed to free radical attack (for example through the exclusion of a portion of bound water molecules). The actual importance of the two latter mechanisms is linked to the role of the indirect effect of ionizing radiation in determining the biological damage, that is still an open question. Along with a non-uniform damage distribution, there is evidence for a differential repair of such damage. This is likely a consequence of the limited accessibility of the repair enzymes to the DNA in structured regions of chromatin. Therefore, the identification of chromatin domains that are critical, from the structural point of view, in determining cellular effects such as cell killing and mutation, should take into account the balance between the amount of damage and the extent of repair.

Review submitted on invitation by the Editorial Board of the *Annali*. Accepted for publication: October 1988.

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