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LASER-UV-MICROIRRADIATION (λ = 257 nm) OF CHINESE HAMSTER CELLS: EVIDENCE OF UV-INDUCED CHROMOSOME ABERRATIONS WHICH DO NOT ORIGINATE AT THE SITES OF PHOTOLESIONS IN THE CHROMATIN

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1. Introduction

Using a laser-uv-microbeam (λ = 257 nm) we have investigated the hypothesis that photolesions in the chromatin are the sites at which most uv-induced chromosome aberrations originate (1,2). The hypothesis follows from two assumptions:
(i) Photolesions produced in uv-irradiated chromatin are decisive for the production of most uv-induced chromosome aberrations.
(ii) All chromosome aberrations which depend on the formation of photolesions in the chromatin originate at the sites of the photolesions.
(ii) includes the possibility that an aberration, such as a chromatid break, occurs very near, but not exactly at the site of a chromatin lesion (3) as well as the possibility that primarily undamaged sister chromatids may be involved in the final aberration by recombinational repair processes (2). The mechanism of aberration production by photolesions is considered to include incomplete or faulty repair processes at the sites of these lesions (2). The percentage of lesions which finally lead to a chromosome aberration may be very small. It is important to note, that assumption (ii) does not necessarily follow from assumption (i) (see discussion).

The hypothesis has several consequences which can be tested by the use of a uv-microbeam. Most uv-induced chromosome aberrations should originate at the sites where uv-photons are absorbed in the chromatin. Photolesions in the chromatin should not have any important influence on the yield of aberrations produced in other parts of chromatin not containing photolesions. Chromatin lesions created by mutagens photochemically produced in the karyoplasm or cytoplasm (1) should contribute very little to the yield of aberrations. In such a case the site where it creates a chromatin lesion and finally a chromosome aberration may be significantly apart from each other.
A non random distribution of chromosome aberrations is compatible with the hypothesis, since the probability that a chromatin lesion will cause an aberration may be different in different chromosome segments.

Griggs and Bender (4) have obtained evidence in Xenopus cells that a certain class of DNA-photolesions, namely the pyrimidine cyclobutane dimer is responsible for most uv-induced chromosome aberrations. Bender et al. (2) have provided a model which suggests that the site of dimer formation is the starting point for the formation of uv-induced aberrations. With respect to this model the above predictions can be formulated in a more restricted form, substituting pyrimidine cyclobutane dimer for photolesion.

It is still a matter of controversy, however, whether this dimer should be considered the only important class of photolesions decisive for practically all uv-induced chromosome aberrations in all eukaryote cells (5, 6,7).
According to the general formulation of the hypothesis any photolesion introduced either in the DNA or in the protein moiety of the chromatin may be considered to contribute to the yield of uv-induced chromosome aberrations.

2. Experimental methods and rationale

Chinese hamster cells were microirradiated during interphase either in the nucleus or in the cytoplasm and chromosome spreads were obtained in situ after different incubation times.
The laser-uv-microbeam, the microirradiation procedure, cell material and culture conditions have been described elsewhere (8,9,10,11). UV-microirradiation alone did not induce sufficient numbers of chromosome aberrations at a convenient uv-dose (Fig. 1d). At higher doses the mitotic index decreased strongly so that the microbeam procedure became unsuitable. The yield of aberrations in cells microirradiated in the nucleus, however, was greatly increased by addition of caffeine (0.5 - 2 mM) to the culture medium during the incubation period (Fig. 1e). Caffeine is known to potentiate synergistically the effect of uv-irradiation on the formation of chromosome aberrations in rodent cells, when it is present during the subsequent S-phase after irradiation (13). The number of aberrations in control cells and in cells microirradiated in the cytoplasm was small and not significantly increased by caffeine at the indicated concentrations (Fig. 1a-c,6a). The hypothesis predicts that those interphase chromosomes which lie in the microirradiated part of the nucleus and receive uv-induced photolesions, should show aberrations at metaphase, depending on the number of photolesions and the stage of the cell cycle at irradiation. Those chromosomes or chromosome segments which are situated in the unirradiated part of a nucleus during microirradiation and bear few or no photolesions should show very few or no aberrations. An increase of the size of the irradiated nuclear area maintaining a constant total irradiation energy, with any distribution of interphase chromosomes, should result in very different distributions of aberrations in metaphase spreads, reflecting the distribution of photolesions.

3. Results

3.1. Distribution of chromatin photolesions within microirradiated nuclei.

For the interpretation of the results of a chromosome analysis of microirradiated cells it is important to know the distribution of chromatin photolesions within the nucleus obtained by uv-microirradiation. A method to measure this distribution directly was not available, but an evaluation of the distribution was obtained indirectly by theoretical considerations and the results of different experiments (8,12). All data available indicate that most of the total uv-dose absorbed by a cell is absorbed within the microirradiated area. Consequently most of the photolesions have to be produced there, while the number of photolesions produced outside this area by diffraction and dispersion of the uv-microbeam is small.

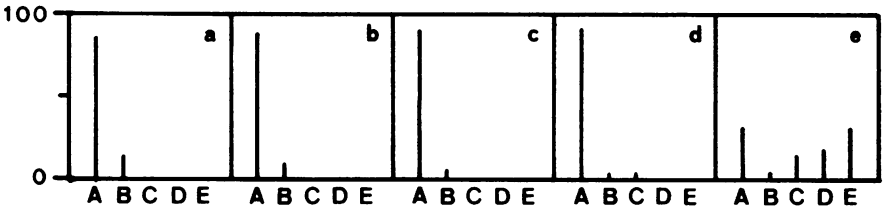

Fig. 1: Chromosome damage after laser-uv-microirradiation in the nucleus and the cytoplasm and posttreatment with/without caffeine.

Cells of a V-79-subline of the Chinese hamster (10) were microirradiated (λ = 257.3 nm) at one site and thereafter incubated in the presence/absence of caffeine. Colchicine was added 3 hours before in situ chromosome preparation which was performed 13 hours after irradiation. Scorable mitotic figures obtained were classified according to the following criteria:
A: No chromosome damage; B: only single defects (1-2 breaks, gaps);
C: some chromosomes severely damaged, the majority being intact;
D: most chromosomes damaged, at least one chromosome remaining intact; E: all chromosomes damaged up to complete disintegration.

Abscissa: type of damaged mitotic figure. Ordinate: proportion of each type (%).
The total number of mitotic figures classified as type A - E is given in parentheses.
a) No irradiation, no caffeine (114)
b) no irradiation, 1 mM caffeine (82)
c) microirradiation of the cytoplasm (1/8 sec), 1 mM caffeine (87)
d) microirradiation of the nucleus (1/8 sec), no caffeine (29)
e) microirradiation of the nucleus (1/8 sec), 1 mM caffeine (47).

The size of the fluorescent area induced by the microbeam in the object plane and the size of visible lesions produced in unstained nuclei of living cells, as well as in stained cell specimens is very similar (8,12). The size of the lesion detectable in the light microscope produced with the Ultrafluar 32x/Ph, used in all experiments described in the present paper, is approx. 1 µm in diameter. A localized type of chromatin damage obtained by the microbeam procedure is further indicated by electron microscopic observation (14) and by the finding that unscheduled DNA synthesis can be induced selectively at the irradiation site (Fig. 2) (15).
Autoradiographs of microirradiated nuclei which were not in S-phase during the subsequent incubation period with ^{3}HTdR show that the number of silver grains/µm^2 over the microirradiated site clearly increases with dose, but falls to the small level of control nuclei within a distance of 6 µm from the center of the microirradiated area (data not shown). The area covered densely with silver grains was found to be approx. 3.6 $\pm$ 1.0 µm in diameter. This area is somewhat larger than the size of the fluorescent spot suggesting that a significant number of DNA-lesions giving rise to DNA

repair synthesis is produced within the whole cone of uv-rays within the nucleus. A large part of the chromatin of microirradiated nuclei as shown in Fig. 2 did not perform any uv-induced unscheduled DNA synthesis. It has been shown that the amount of unscheduled DNA synthesis induced by microirradiation of the nucleus depends only on the total number of incident photons and not on the area of irradiation (15).
These data suggest that a considerable proportion of uv-induced DNA lesions produced outside the microirradiated area and giving rise to DNA repair synthesis, should be detected by the autoradiographic method. Our results indicate, therefore, that the large majority of such lesions is produced within a radius of less than 6 µm from the center of the irradiation site.

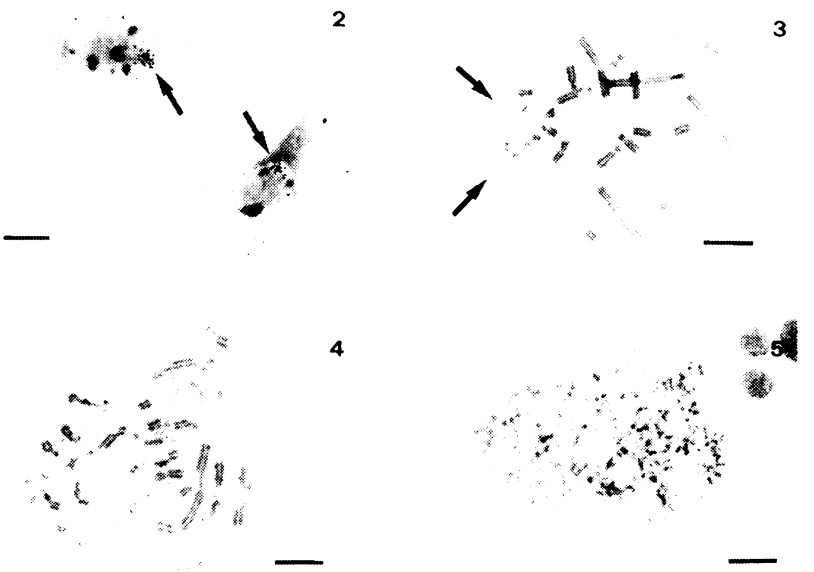

Fig. 2: Autoradiograph of two fibroblastoid Chinese hamster cells derived from lung tissue (11) after laser-uv-microirradiation of the nucleus and subsequent incubation with ^{3}H-Thymidine for 1 hour. Unscheduled DNA synthesis at the microirradiated site is indicated by silver grains(arrows).

Figs.3-5: Mitotic figures obtained after laser-uv-microirradiation of nuclei of V-79 cells and posttreatment with caffeine. (For experimental detail see Fig. 1)
Fig. 3: chromosome damage in a limited area (arrows) (type C), Figs. 4,5: generalized chromosome damage (type E). Fig. 5 shows an example of complete chromosomal disintegration

Bars indicate 10 µm.

3.2. Chromosome aberrations obtained in metaphase spreads from cells microirradiated in the nucleus.

Five types A - E of mitotic figures obtained from cells microirradiated in the nucleus and thereafter incubated in the presence of caffeine were tentatively distinguished (see legend of Fig. 1). After irradiation of one small part of the nucleus a certain number of mitotic figures showed a small number of damaged chromosomes (type C, Figs. 1e, 6). In the large majority of these cases the damaged chromosomes were clearly not randomly distributed over the whole metaphase spread, but more or less concentrated in one small area (Fig. 3). This type of damage has not been observed after uv-irradiation of whole cells (11) and is in agreement with the hypothesis. Karyotypes obtained from metaphase spreads showing this type of damage revealed that different chromosomes were damaged in different cells (11). The results so far are consistent with the hypothesis that inhibition of post replication repair at the sites of photolesions caused aberrations to appear in microirradiated chromosomes (16,11).

Other mitotic figures were unexpectedly found where most (type D) or even all chromosomes (type E) were damaged (Figs. 4,5). While some aberrations were expected to appear in chromatin outside the microirradiated area because of the small number of photolesions which might be introduced in this chromatin, there is no apparent reason why the microirradiated chromosomes or chromosome segments themselves should not show a much more pronounced effect. Using this criterion microirradiated chromosomes can be tentatively identified in some but not in all cases classified as type D and E.
Fig. 6 shows that the proportion of type D and type E increase with dose. The caffeine concentration and the stage of the cell cycle are other important parameters in determining whether intact (type A) or severely damaged mitotic figures (types C - E) are obtained (data not shown).
The number of photolesions produced in any interphase chromosome is not a sufficient criterion to predict the probability of the number of aberrations in mitosis originating at this chromosome. This conclusion is supported by the following comparison. At the lowest dose (1/125 sec) the concentration of photolesions in the microirradiated part of the nucleus is still rather high, the energy density within the focus being in the order of 750 ergs/mm^2. Unscheduled DNA synthesis at the microirradiation site of such nuclei could be detected, but there was no significant yield of uv-induced chromosome aberrations (Fig. 6b). At higher doses, however, chromosome disintegration was obtained even in unirradiated parts of microirradiated nuclei, although the data described in 3.1 suggest a much lower concentration of photolesions in these unirradiated parts at any dose, as compared with the concentration of photolesions in chromatin microirradiated with the lowest dose.

The effects of microirradiation of a small part of the nucleus were compared with those of a larger part (Fig. 6b-e). For any investigated uv-dose the percentages of types A - E were found similar in both types of experiments.
In further experiments microirradiation of a small part of the nucleus was compared with microirradiation of whole nuclei at the same dose (1/15 sec) (unpublished data). Under the experimental conditions of these latter experiments the percentages for type C and D were very low. The percentages

for type E were found to be 40 % and 37 %. These data indicate that demolition of all chromosomes may be achieved in sensitive cells with any distribution of a given number of photolesions.

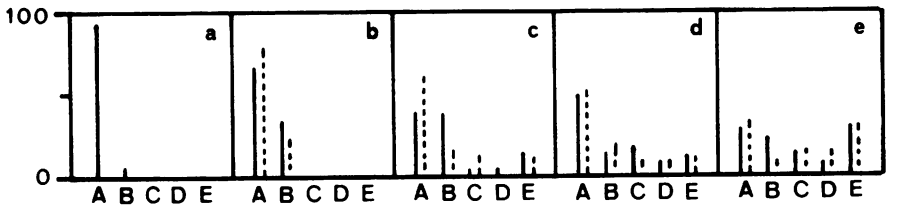

Fig. 6: Chromosome damage after laser-uv-microirradiation of nuclei with different doses and posttreatment with 2 mM caffeine. For experimental details and classification of mitotic figures see Fig. 1. Abscissa: type of damaged mitotic figure. Ordinate: proportion of each type (%)

| : data obtained with the uv-focus placed inside the cell nucleus (as in experiments of Fig. 1);

¦ : data obtained with the uv-focus placed above the cell, resulting in an irradiation field of approx. 4 µm diameter.

The first number gives the irradiation time, the second and third number give the number of analyzed mitotic figures using the irradiation modes with the focus inside (|) and above the cell (¦), respectively.
a) no irradiation, 142 mitotic figures, b) 1/125 sec, 3/18, c) 1/60 sec, 28/19, d) 1/30 sec, 52/65, e) 1/15 sec, 30/60.

4. Discussion

Mitotic figures showing disintegration of all chromosomes induced by microirradiation of a small part of the nucleus do not support the hypothesis I that photolesions in the chromatin are the only important sites at which uv-induced chromosome aberrations can originate.
To fit our results with the hypothesis, we have to assume that the probability of a single photolesion to create an aberration depends on the total number, but not on the distribution of other photolesions in the nucleus. If the total number of photolesions is high enough, then very few photolesions in a chromosome not detectable by the autoradiographic method described in 3.1., could be sufficient to trigger its complete demolition. In cases where all chromosomes are completely disintegrated (Fig. 5), the much higher concentration of photolesions in microirradiated chromosomes cannot be distinguished by cytological observation. This interpretation, however, does not satisfactorily explain why microirradiated chromosomes do not show a more severe disintegration than unirradiated ones in many mitotic figures of type E, where chromosome demolition is not complete (Fig. 4).

To interprete our results we now consider a second hypothesis (II):
A significant number of aberrations can occur at chromatin sites which do not contain photolesions. Both the sites of photolesions and these other sites contribute to the total yield of uv-induced chromosome aberrations. The proportion of aberrations obtained from each source in any cell may depend on some unknown properties of the cell and on the experimental conditions. At least one of the two assumptions leading to hypothesis I is not compatible with hypothesis II. Two new assumptions (a) and (b) may be formulated as alternatives.

(a) Besides photolesions in the chromatin other photochemical reactions are also important for the formation of uv-induced chromosome aberrations. In this case assumption (ii) may still be valid. There are several possibilities. 1. Mutagens may be produced in the microirradiated part of the karyoplasm and create chromatin lesions anywhere in the nucleus (1). 2. UV-damage of the nuclear envelope may lead to uncontrolled fluxes of important substances in both directions at the microirradiated site. Such an effect, as well as an uv-dependent formation of toxic substances may lead to severe changes in the internal milieu of the whole nucleus which may become more sensitive to the action of caffeine. It is known that caffeine at high concentrations ($\geq$ 10 mM) induces chromosome disintegration in unirradiated cells (17 and our unpublished data).

Alternatively, assumption (i) may be supposed to be valid. Then assumption (ii) has to be abandoned and assumption (b) may be formulated: A significant number of chromosome aberrations which depend on the formation of photolesions in the chromatin do not originate at the sites of the photolesions. Again several possibilities may be considered.
1. The genetic information at the irradiated part of a nucleus including genes important for the morphological integrity of all mitotic chromosomes may be blocked by transcription terminating photolesions in a dose dependent way (18). A codogenic strand of such genes free from transcription terminating lesions may be necessary shortly after semiconservative DNA replication of these genes and obtained by DNA repair processes, but caffeine might prevent such genes from transcription at the correct time by inhibition of post replication repair.
Many such genes would be required distributed throughout the nucleus to explain the frequent occurrence of type E at higher doses (Figs. 1e, 6e).
2. Increased DNA degradation after uv-irradiation has been reported for mammalian cell systems and several mutants in bacteria (19,20). These findings may justify a speculation whether generalized chromosomal demolition could be due to the uncontrolled action of an uv-induced nuclease. Recently, inhibition of poly(ADPR) synthase by caffeine has been reported (21). A possible role of such enzyme(s) in the control of nucleases is exemplified by the inhibition of a Ca^{++} Mg^{++} dependent endonuclease by ADP ribosylation (22).
3. Finally, the possible role of competition between sites of post replication repair and DNA replication points for some limiting enzyme(s) may be considered. Both sites provide discontinuities in newly synthesized strands of DNA (23,25) and are known to be affected by caffeine. The molecular mechanism may involve binding of caffeine to single stranded DNA

regions (24) which occur at both sites. The function of limiting enzyme(s) binding to these sites and involved both in an accurate performance of semiconservative DNA synthesis and in post replication repair may be impaired in the presence of caffeine.
Inhibition by direct binding of caffeine to such limiting enzyme(s) may also be considered (17). At a low concentration of caffeine the function of the enzyme(s) may still suffice to avoid chromosomal aberrations occuring in unirradiated cells. A certain number of DNA photolesions, however, with any distribution of the lesions in the nucleus, may provide so many additional sites of binding for limiting enzyme(s) that faulty DNA synthesis occurs at DNA replication points and post replication repair becomes incomplete or faulty at other sites of chromatin lesions which occur spontaneously during the cell cycle (23).
Different sites may provide different probabilities of causing a chromosome aberration, but the probability for any site depends on the number of all other sites, present in the nucleus at the same time. According to this model uv-induced chromosome aberrations originate at the sites provided by three different sources:photolesions in the chromatin, other chromatin lesions and DNA replication points. The contribution of each source to the total yield of uv-induced aberrations may vary with the experimental conditions. Our model does not exclude the possibility that in the absence of caffeine most aberrations would occur at the sites of photolesions (2). It gives an explanation why uv-light produces chromosome aberrations in a S-phase dependent mode (2,13). It would also explain the finding that the change from cells containing no or few aberrations to cells with multiple aberrations is rather abrupt in experiments where uv is combined with caffeine (16).
The model is compatible with the finding of Griggs and Bender (4) that photoreactivation can prevent most uv-induced chromosome aberrations. On the other hand the above considerations indicate that photoreactivation is not sufficient evidence for the model of Bender et al. (2).

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