Ultrastructural Analysis of Radiation Induced Chromosome Breaks and Rearrangements

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Light microscopy studies on chromosome breaks and rearrangements have provided some information about organization of the chromatin fiber within the chromosome. It has been reported that spontaneous, radiation-induced chromosome breaks as well as structural rearrangements show a non-random distribution between chromosomes and chromosome arms (Bauchinger and Götz 1979, Moore 1979, Cermeño and Lacadena (1985). Also cytogenetic data on tumor cells show rearrangements that exhibit a non-random involvement of some chromosomes and hot break-points (Yunis 1983, Changanti and Koduru 1987) along chromatids. Furthermore, chromosome fragile sites were described as regions expressed as chromatid or isochromatid breaks or gaps under certain cell culture conditions or following treatment with particular chemicals (Sutherland and Hecht 1985). Distribution of break-points in constitutional chromosome rearrangements in tumor cells has a possible relationship with the chromosome position of fragile sites, which would contribute to their genesis (Yunis 1986). In fact, fragile sites behave as targets for some mutagens and carcinogens, including ionizing radiation (Yunis *et al.* 1987).

Other chromosomal phenomenon where breaks are involved are Sister-Chromatid Exchanges (SCE). It consists of the reciprocal and symmetrical exchange between the two sister chromatids, which is produced during replication-modulation of DNA (Taylor 1957). Their frequency increases when cells are exposed to mutagens and carcinogens, being the most sensitive cytological method to detect potential clastogenic agents (Schvartzman 1987). A non-random distribution of SCE within chromosomes has been reported (Kato 1979, Dimitrov 1986) and it has been recently proposed that fragile sites are hot spots not only for chromosomal lesions but also for SCE formation (Glover and Stein 1987).

In order to explore the probable relationship between the characteristics of chromosome breaks-rearrangements with the several levels of chromatin fiber organization of the metaphase chromosome, here we present an ultrastructural approach on gamma-irradiated Chinese DON cells. Chromosome breaks and rearrangements were examined by transmission electron microscopy employing a variety of whole mounting or thin sectioning procedures. Furthermore, the angle of the border of breakage with respect to the chromosome axis was measured in whole-mounted chromosomes where DNA-breaks were performed by a photoinduction technique (Fernández *et al.* in press).

Material and methods

Condensation-inhibition of G-bands and cell irradiation

Chinese Hamster DON cells were exponentially growing in the dark at 37°C in RPMI medium supplemented with 15% fetal calf serum, 100 UI/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Treatment by BrdU (20 µg/ml) and Hoechst 33258 (40 µg/ml) was performed for 12 hr, and 300 cGy of gamma-radiation (from a ⁶⁰Co source, Theratron 780,

Canada) were administered 2 hr before harvesting. Cells were arrested at metaphase for 3 hr with 0.5 $\mu\text{g}/\text{ml}$ colchicine and mitosis were preferentially detached from the monolayer by being gently shaken off the culture flasks. After harvesting, mitosis were hypotonically treated for 10 min at 37°C with 0.075 M KCl.

Isolation and whole-mounting

Mitotic cells hypotonically treated were centrifuged at 1200 rpm for 5 min and the pellet covered with 2 ml of acid isolation buffer (1 % citric acid, 1 % Triton X-100, 6 mM MgCl_2) and left at room temperature for 30 min. The cells were gently syringed 7–10 times through a No. 22-gauge needle and the chromosome isolation monitored by phase microscopy. The chromosome suspension was again centrifuged for 5 min at 2000 rpm through a cushion of isolation acid buffer onto Formvar-coated EM grids, which were immediately immersed into 50 % ethanol for 10 min. After two 5-min steps in 75 % and 100 % ethanol, the grids were immersed into two 5 min baths of 100 % amyl acetate and air-dried. Electron microscopic examination and photographic recording were performed with a Zeiss 109 Turbo EM operated at 50 kV.

Embedding and sectioning

Chromosomes were embedded and thin sectioned according the procedure previously described (Goyanes and Schwartzman 1983). In brief, irradiated Chinese Hamster DON cells (without BrdU-Hoechst 33258 treatment) growing in monolayers, were treated for 10 min with hypotonic solution (0.075 M KCl) at 37°C in the flask, fixed with methanol: acetic acid (3:1), and washed with acid isolation buffer for 30 min. Care was taken to prevent detachment of the monolayer. Cells were scraped off the flasks with a rubber policeman, centrifuged at 1200 rpm for 10 min, and the resulting pellet covered with 8 % formaldehyde in Hanks for 16 hr at 4°C. The cell pellet was embedded in Epon, and 0.1 μm thick sections were cut with an LKB ultra-microtome. Sections were placed on 200 mesh copper grids and stained with uranyl acetate and lead citrate (Reynolds 1963).

Photoinduction of DNA break-down in one of the sister chromatids

Chinese Hamster DON cells exponentially growing were treated with BrdU (16.6 $\mu\text{g}/\text{ml}$) for 8 hr. Then the culture medium was removed and the cells grewed in a fresh medium without BrdU and with thymidine (10 $\mu\text{g}/\text{ml}$) for 12 hr. Colchicine (0.5 $\mu\text{g}/\text{ml}$) and Hoechst 33258 (13.2 $\mu\text{g}/\text{ml}$) were added for the last 2 hr and 10 min, respectively. Afterwards, cultures were exposed to a 280–313 nm UV-radiation for 15 min. Finally, chromosomes were isolated and processed by whole-mounting electron microscopy technique, as described previously.

Results and discussion

Scarce information has been reported where chromosome rearrangements are related with the levels of hierarchy of the chromatin fiber organization at metaphase (Mouriquand *et al.* 1974, Mouriquand *et al.* 1975, Bahr 1978, Mace *et al.* 1978, Yu 1979). At this respect here we attempt several approaches of DNA-chromatin damage and their relationship with the ultrastructure of metaphase chromosomes employing preservative technique of whole-mounting EM. Also, a first quantitative approach on the break border is reported.

Position of break-points was examined by EM in rearranged chromosomes irradiated at G-2 phase of the cell cycle. In order to locate accurately the break-points along the chromatid, a high resolution R-banding pattern was induced by means of condensation-inhibition of G-bands throughout a BrdU-Hoechst 33258 treatment along S and G-2 phases (Goyanes and Méndez 1981). When examined, chromosomes showed well-preserved three-dimensional

organization, with chromatin fibers organized in sequences of condensed and relaxed looping structures (R-bands) alternating with tracts of tightly packed longitudinally running fibers (G-bands) (Fig. 1). Condensation-inhibition was more pronounced in some G-bands than in others. This *in vitro* BrdU-Hoechst 33258 treatment inverts the normal pattern of packaging of the chromatin fiber within the chromosome (Bahr *et al.* 1973, Yunis and Bahr 1979, Goyanes and Méndez 1981) thus resulting in chromomere like R-bands intercalated with interchromomeric G-bands.

Nine from twelve rearranged chromosomes showed their breaks located at the point of transition between G- and R-bands (Figs. 1a, and b). The chromosome average diameter of break-points and rearrangements was 71.65% of the wide condensed R-bands, a result similar

Fig. 1. a, b). Electron micrographs of chromatid-type rearrangements in Chinese hamster chromosomes gamma-irradiated at G-2. Whole-mounted chromosomes show a high resolution R-banding pattern induced by condensation-inhibition of G-bands. Because sister chromatids retain strict pairing, position of break-points can be mapped. Transition between chromomeric and interchromomeric chromatin structures represents the preferential points of breakage and rearrangements. Bar: 0.5 μm .

to the average diameter of narrow G-bands (69.79%). At this respect, light-microscopy analysis performed by Dutrillaux *et al.* (1977) showed the preferential involvement of the R-G interbands when complementary banding techniques were used for the location of the break-points. Also, Holmquist *et al.* (1982) argued that the junctions of R- and G-bands might be specially sensitive to breakage because of their different timing of replication (Comings 1978).

Furthermore, it has been proposed that fragile sites represent regions where DNA sequences are, or can be induced to be, late-replicating during S or G-2 phases of the cell cycle, resulting in incomplete chromatin condensation. Rearrange and recombinogenicity at fragile sites would be consequence of the replication junctions that flank late-replicating DNA (Laird *et al.* 1987).

Chromatin fiber damage was also examined in thin sectioned chromosomes. Three chromosomes presented their broken terminal end organized as a coil (Siebert and Loidl 1984) (Fig.

2). Measurements pointed that this coil was constituted by two 23 nm diameter chromatin fibers coiling together, showing 38.09 nm average width. This results support the hypothesis that coils should be excentrically located to constitute the chromatid, because coils diameter was 43.70% of the mean chromatid diameter.

Another most exciting data can be deduced of characteristics of the border of breakage of the chromatid. This border was examined in whole-mounted chromosomes where DNA-breaks were photoinduced. Photoinduction technique is based on the ability of UV-radiation

to induce extensive single breaks in BrdU substituted DNA strand after a brief Hoechst 33258 treatment (Matsukuma and Utakoji 1984). Chromosomes which incorporated BrdU in part of one of their chromatids showed loss or dispersion of chromatin fibers at the places where DNA-breaks were photoinduced.

As ionizing radiation, photoinduced damage mainly expresses as DNA affectation. Then, the "border of damage" between affected and non-affected chromatin defines the course of DNA molecule within the chromatid (Fernández *et al.* in press). This border is visualized like a sharp break in

Fig. 2. Electron micrograph of an ultrathin sectioned chromosome showing a broken chromatid. Terminal end is organized as a coil of 38.09 nm average width (arrow). Bar: 0.5 μ m.

Fig. 3. Electron micrographs of whole-mounted chromosomes where breaks were photoinduced in BrdU-substituted DNA. Affected chromatin appears lost or widely dispersed. The border of damage (arrow) defines the course of DNA molecule within the chromatid. a) Early condensed chromosome. b) and c). High condensed chromosomes. The angle of the border of damage with respect to the chromatid axis tends to be more perpendicular as condensation is progressing. Bar: 0.5 μ m.

hardly affected samples, where damaged chromatin was widely dispersed or lost (Figs. 3a, b, c).

To establish the angle of the border of breakage with respect to the chromatid axis, 23 breaks were measured in 16 whole-mounted chromosomes with photoinduced DNA-breaks. Fourteen breaks from 11 early condensed chromosomes presented an average angle of 69.43° , while 9 break-borders from 5 high condensed chromosomes showed 78.22° (Fig. 3). Then, as Goyanes and Schwartzman (1983) pointed about the "border of exchange" in SCE, the tilting

degree of the breakage-border with respect to the chromatid axis is dependent on the chromosome condensation level, tending to be more perpendicular as condensation is progressing, possibly by a coiling packing mechanism.

Our results suggest that DNA break-down at any of the interphase stages of the cell kinetic cycle deeply influences the higher order structure of the mitotic chromosome. Also, the border of breakage, besides representing the ending tract of the chromatin fiber, constitutes a hot point for chromosome linkage with other chromatid fragments. The fact that broken ends are unstable and sticky is clearly viewed in chromosomes where part of chromatin was removed by photoinduced DNA-breaks (Fernández *et al.* in press).

The interphase between G- and R-bands, that is to say between chromomeric and interchromomeric chromatin structures, represents the change between a coiled to a longitudinal pattern of organization. These interphases show to be critical and fragile spots places for breakage and rearrangements.

The eukariotic chromosome can be considered as a long chromatin fiber that generates a three-dimensional volume in mitosis as a consequence of a programmed folding in space and time (Goyanes 1985). Chromosomal differentiated regions would be constituted by segments of specific DNA sequences and or characteristic non-histone associated proteins, with their own transcriptional, replication and condensation behaviour. Transition points between these stretches would represent the preferential points of chromosomal breakage-rearrangements.

Abstract

Chinese Hamster chromosomes R-banded *in vitro* were gamma-irradiated and chromatid breaks and rearrangements examined by electron microscopy employing whole-mounting technique. Breaks were preferentially located at the point of transition between G- and R-bands where the chromosome showed an average diameter 71.65% of the wide condensed R-bands. This result was similar to the average diameter of narrow G-bands. Three chromosomes which were thin sectioned presented their broken terminal end organized as a coil constituted by two 23 nm wide chromatin fibers coiling together. Coils diameter was 43.70% of the mean chromatid diameter. The border of damage-breakage was analyzed in whole-mounted chromosomes where breaks were photoinduced in BrdU-substituted DNA. Measurements of the angle of the sharp border of damage with respect to the chromatid axis showed a tendency to be more perpendicular as condensation progressed. These results clearly correlate with the several levels of chromatin fiber organization of the metaphase chromosome.

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