Mitosis-promoting Factor Activity of Inducer Mitotic Cells May Affect Radiation Yield of Interphase Chromosome Breaks in the Premature Chromosome Condensation Assay

Xinbo Cheng, Gabriel E. Pantelias, Ryuichi Okayasu, Nge Cheong, and George Iliakis

Abstract

We measured mitosis-promoting factor (MPF) activity in two cell lines, CHO and HeLa, which were used as mitosis inducers in the assay of premature chromosome condensation to study the yield and the repair kinetics of radiation damage in interphase chromosomes of diverse cell lines. We found a 2.5-fold higher MPF activity in HeLa as compared to CHO mitotic cells, when used as inducers of premature chromosome condensation. Consequently, we observed two more interphase chromosome breaks in irradiated, non-stimulated human lymphocytes as compared to CHO mitotic cells. A 2-fold increase in the yield of interphase chromosome breaks with HeLa mitotics was also observed in G1 cells from a plateau-phase CHO culture. Thus MPF activity may be a contributing factor of the process that transforms radiation-induced DNA damage to chromosome breaks, and subsequently to other types of mitotic chromosomal aberrations. We speculate that the level and control of the cell cycle of MPF activity may influence the radiosensitivity of cells to killing. The results strongly suggest that a direct comparison between the yields of interphase chromosome breaks measured in different laboratories may not be possible unless similar inducer cells with similar MPF activity are used.

Introduction

Fusion of a mitotic with an interphase cell by treatment with either Sendai virus or PEG4 induces mitotic events in the interphase nucleus leading to a nuclear membrane breakdown and premature condensation of interphase chromatin (1). An activity present in the mitotic cell, termed MPF, is implicated for the events observed in the interphase nucleus (2). Purified MPF consists of two polypeptide chains identified as the homologue of the gene product of cd2 in the fission yeast Schizosaccharomyces pombe, p34cdc2, and a cyclin of the B family, p34cdc2 is a serine threonine kinase and cyclin B acts as a regulatory subunit in the MPF complex (3–5).

PCC is used extensively not only for studies related to cell cycle control, but also as a means of visualizing and scoring damage induced by radiation and other clastogenic agents in mitotic chromosomes (6–8). It allows measurement of the yield and the repair kinetics of chromosome damage in G1 or G2 cells immediately after irradiation. The approach bridges in this way results obtained at the DNA and the cell level, and is helpful in the elucidation of the mechanism that leads to the formation of chromosome aberrations as visualized at metaphase (9).

Since the process of premature chromosome condensation is predominantly the result of MPF acting on interphase chromatin, we examined the contribution of MPF activity to chromosome breakage. The experimental approach chosen is based on a preliminary observation that mitotic cells from different origins display different levels of MPF activity. Here, we show that higher MPF activity in the inducer mitotic cell used for PCC is associated with higher yields of chromosome breaks in the irradiated interphase cell. This finding implicates a cellular activity with a tight regulation throughout the cell cycle in the transformation of radiation-induced damage to lethal chromosomal aberrations.

Materials and Methods

Cell Culture and Irradiation. Experiments were carried out with normal human lymphocytes and CHO cell strains, 10B1 and 10B2. Human lymphocytes were obtained from blood freshly drawn from normal subjects into heparin syringes by venipuncture. The lymphocytes were separated by ficoll-paque and were incubated in McCoy’s medium 5A supplemented with 10% fetal calf serum after washing once in the medium (10). Experiments were initiated immediately after preparation. CHO cells were grown as monolayers in McCoy’s medium 5A supplemented with 10% fetal calf serum and antibiotics. All experiments were performed with plateau-phase cells obtained by growing 1 X 10^6 cells in 60-mm dishes (3 ml medium) for 4–5 days without medium change (11). Human lymphocytes rest in G1 and plateau-phase CHO cultures have approximately 95% of cells in G1 (G1-like stage), 2% in S and 3% in G2 + M phases.

Irradiations were carried out with a Siemens therapeutic X-ray machine operated at 250 kVp, 15 mA, with a 2-mm aluminum filter (effective photon energy about 70 keV), at a dose rate of 2.0 Gy/min. Dosimetry was performed with a Victoreen dosimeter. Cells were irradiated on ice, in suspension.

p34cdc2 Kinase Assay. A synthetic peptide with the sequence ADAQHATP-PKKKKVVEDPKDF derived from simian virus 40 large T-antigen, a known substrate of p34cdc2 kinase (12), was used to measure MPF activity (GIBCO-BRL). In a mitotic cell the majority of this activity is taken to reflect the activity of MPF. The peptide was modified by altering the serine residues at positions 3 and 6 (120 and 123 in the protein) with alanine residues, leaving thus a single phosphorylation site and reducing the probability of phosphorylation by other protein kinases. The peptide is not phosphorylated by protein kinase C, cyclic AMP-dependent protein kinase, casein kinases 1 or II, glycogen synthase kinase, phosphorylase b kinase, or calmodulin kinase II (13). The kinase that phosphorylates the peptide can be depleted by using antibodies to p34cdc2 or by immobilized p13, a p34cdc2-binding protein (13). All these findings suggest a high specificity of the peptide as a substrate for p34cdc2 kinase. The peptide is equivalent to histone H1 as a substrate for the p34cdc2 kinase, and has a Km value of 74 mM (13).

To prepare cellular extracts, 3 to 5 million mitotic HeLa or CHO cells (mitotic index about 98%, kept frozen as described previously (11)) were washed twice in Hanks’ balanced salt solution and lysed in a buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 0.1% Triton X-100, 150 mM NaCl, 15 mM MgCl2, 50 mM β-glycerol phosphate, 1 mM EDTA, 100 mM PEG, 4 M urea, and hexokinase and glucose 6-phosphate (13). All these steps were carried out at 4°C.
dithiothreitol, 20 mM ethyleneglycol bis(β-aminooxyethyl)N,N',N'-tetraacetic acid, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml aprotinin. They were homogenized in a glass Dounce homogenizer with the use of a type B pestle (30 strokes), and centrifuged at 16,000 × g for 15 min. The supernatant was transferred to a new tube and was either immediately used, or stored frozen at −85°C. The protein concentration of the cell lysates was determined by using a colorimetric protein assay kit (Bio-Rad).

Kinase activity was measured in a 30-µl reaction mix containing: 365 µM peptide substrate, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM ethyleneglycol bis(β-aminooxyethyl)N,N',N'-tetraacetic acid, 100 µM ATP, 3.33 µCi of [γ-32P]ATP (6000 Ci/mmol: NEN), and appropriate amounts of cell extract as required by the experimental protocol. The reaction was allowed to take place at 30°C for 20 min, and was stopped by the addition of 15 µl of 30% trichloroacetic acid. Protein was allowed to precipitate for 15 min on ice, and was pelleted by a 15-min centrifugation at 4°C. A 10-µl aliquot of the supernatant was then spotted onto a phosphocellulose disk (GIBCO-BRL) that binds the peptide substrate. Phosphocellulose disks were washed twice for 10 min in 100 µM phosphoric acid, and twice for 5 min in deionized water before counting in a scintillation counter.

**Premature Chromosome Condensation.** Induction of damage induced at the chromosome level in human lymphocytes was measured by a procedure developed for PEG-mediated cell fusion and PCC induction (10). Briefly, 10⁶ mitotic cells (CHO or HeLa, depending on the experimental protocol) were mixed with an equal number of interphase cells, centrifuged, and resuspended in 0.1 ml of 50% ice-cold PEG solution (M, 1450; Sigma, prepared in phosphate-buffered saline, 50% (w/v)). The pellet was held in this solution for 1 min and subsequently 2 ml of cold phosphate-buffered saline were slowly added. The cell suspension was centrifuged, the pellet resuspended in 0.7 ml of McCoy's growth medium 5A containing colcemid at 10⁻⁶ M, and cells were incubated at 37°C for 60 min; in that time, cell fusion and induction of PCC was completed.

Damage induced at the chromosome level in plateau-phase CHO cells, and in some experiments in human lymphocytes fused with HeLa mitotics, was measured by Sendai virus-mediated fusion and induction of PCC (1, 7, 8). Briefly, 10⁶ mitotic cells were resuspended in 0.1 ml of 50% ice-cold PEG solution, centrifuged, and resuspended in 0.1 ml of 50% ice-cold PEG solution (M, 1450; Sigma, prepared in phosphate-buffered saline, 50% (w/v)). The pellet was held in this solution for 1 min and subsequently 2 ml of cold phosphate-buffered saline were slowly added. The cell suspension was centrifuged, the pellet resuspended in 0.7 ml of McCoy's growth medium 5A containing colcemid at 10⁻⁶ M, and cells were incubated at 37°C for 60 min; in that time, cell fusion and induction of PCC was completed.

After chromosome condensation, cells were treated in hypotonic (0.075 M) KCl solution and fixed in 10 ml methanol:acetic acid (3:1). Cells were washed once more in fixative, dropped on precleaned wet slides, air dried, and stained with 2% Giemsa (Sigma). Analysis for PCC fragments was performed by means of light microscopy. Routinely, 25–30 cells were scored for excess chromosome fragments. The results obtained are presented as mean ± SE of three independent experiments. We have previously shown that the choice of the fusogen (PEG versus Sendai virus) does not affect the yield of interphase chromosome breaks in the PCC assay.5 The experiments carried out with human lymphocytes as part of the study presented here confirm this observation.

**Results**

Fig. 1 shows phosphorylation of peptide substrate by crude extracts from HeLa or CHO mitotic cells. Plotted in the figure is the pmol of phosphorylated substrate per reaction as a function of the amount of crude protein added. There were 10.95 nmol of peptide substrate present and 2.42 × 10⁶ dpm corresponded to the phosphorylation of 1 nmol of peptide substrate in each reaction. Fitting of the results to a straight line gave a slope of 2.10 ± 0.14 pmol/µg and 5.29 ± 0.35 pmol/µg for CHO and HeLa cells, respectively. From these values we calculated that the phosphorylation activity is 2.52 times higher in HeLa than in CHO extracts. There were (2.9 ± 0.5) × 10⁻⁴ µg protein per HeLa cell, and 2.2 ± 0.4 × 10⁻⁴ µg protein per CHO cell. HeLa mitotics had 1.7 times the volume of CHO mitotics based on measurements carried out with an electronic counter.

Due to the presence of inhibitory activities in the extract, the phosphorylation of the peptide substrate was substantially higher in HeLa than in CHO cells, per µg of crude protein, for all points examined. From the ratio of the slopes of the lines shown in Fig. 1, we calculated a 2.5-fold higher phosphorylation activity in HeLa as compared to CHO mitotic cells. Based on the specificity of the peptide substrate used for p34cdc2 kinase (see “Materials and Methods”), we assume that the difference in the phosphorylating activity between HeLa and CHO cells reflects predominantly a difference in MPF activity.

We investigated the ability of the same batch of mitotic HeLa and CHO cells to induce premature chromosome condensation in human lymphocytes and to express radiation damage in interphase chromosomes. Characteristic micrographs of the results obtained in these experiments are shown in Fig. 2. We scored, as expected, 46 prematurely condensed chromosomes in human lymphocytes fused with HeLa mitotics (Fig. 2A). Exposure to 2 Gy X-rays led to chromosome fragmentation and increased, in the cell shown in Fig. 2B, the number of chromosome pieces to 62, which is equivalent to 16 breaks per cell. When we fused CHO mitotics with human lymphocytes, we also observed 46 prematurely condensed chromosomes in non-irradiated cells (Fig. 2C). However in cells exposed to 2 Gy X-rays the number of chromosome pieces scored was 53, which corresponds to only 7 breaks per cell (Fig. 2D). Thus, HeLa mitotics uncovered over twice

---

MPF ACTIVITY OF INDUCER MITOTIC CELLS

Fig. 2. A, syncytium of a lymphocyte with a mitotic HeLa cell showing premature condensation of the lymphocyte chromatin into 46 distinct chromosomes. B, same as A but with a lymphocyte cell exposed to 2 Gy X-rays; 62 chromosome pieces can be seen. C, syncytium of a lymphocyte with a mitotic CHO cell. Here again 46 distinct prematurely condensed chromosome can be seen. D, same as in C but with a lymphocyte cell exposed to 2 Gy X-rays.

as many interphase chromosome breaks than CHO mitotics in irradiated human lymphocytes.

To study this phenomenon in greater detail, we exposed nonstimulated human lymphocytes to a range of radiation doses and scored the number of interphase chromosome breaks by fusing the same initial cell suspension with either HeLa or CHO mitotics. Fig. 3 summarizes the results obtained in three such experiments. The formation of excess interphase chromosome breaks increased linearly with dose after fusion either with HeLa or CHO mitotic cells. However, at all doses examined, we scored approximately twice as many interphase chromosome breaks in lymphocytes fused with HeLa mitotics than in lymphocytes fused with CHO mitotics. Comparison of the slopes of the best fit lines shown in Fig. 3 shows that, on the average, there were 1.96 times more interphase chromosome breaks after fusion with HeLa than with CHO mitotics.

We wished to examine the effect of inducer mitotic cells on the formation of interphase chromosome breaks after exposure to ionizing radiation in a different cell system to exclude that our observations reflect a peculiarity of the human lymphocytes, e.g., differences in chromatin structure that may affect MPF action. Therefore, we fused plateau-phase CHO cells exposed to various doses of X-rays with either HeLa or CHO mitotics, and measured the yield of interphase chromosome breaks. Fig. 4 summarizes the results obtained in three such experiments. Similar to the observations with human lymphocytes, cells fused with HeLa mitotics had almost twice as many interphase chromosome breaks than cells fused with CHO mitotics. Comparison of the slopes of the best fit curves shown in Fig. 4 gives a 2.05-fold higher induction of interphase chromosome breaks after fusion with HeLa than after fusion with CHO mitotic cells.

Discussion

The results presented in the previous section indicate that MPF may be a contributing factor in the process that transforms radiation-in-
Reduced DNA damage to chromosome breaks. Among the lesions induced by ionizing radiation the DNA dsb is commonly considered as the critical lesion for chromosome fragmentation visualized by PCC (9). Our results suggest that the efficiency of transformation of a DNA dsb to an interphase chromosome break depends upon the type of mitotic inducer cell used, and may be higher when MPF activity is high. This observation offers yet another way to explain in part the difference between the yields of DNA dsb and interphase chromosome breaks measured in irradiated G1 cells. We have reported that in CHO cells irradiated in G1 there are 21 DNA dsb but only 2 interphase chromosome breaks per cell per Gy. We have presented evidence that this difference is partly caused by repair taking place during the period allowed for chromosome condensation, and that it can be prevented by mutations affecting radiosensitivity or by agents inhibiting DNA repair (14-16). The present set of data adds MPF as an additional parameter in the process.

The results presented pertain at first for syncytia formed between mitotic and interphase cells whereby mitotic events are induced in the interphase nuclei. However, similar events may also take place in the absence of fusion and PCC induction in interphase cells irradiated at a stage of the cycle localized timewise shortly before mitosis, e.g., late in G2. It is possible that depending upon the level of MPF activity reached at mitosis the efficiency of transformation of dsb to interphase chromosome breaks will vary, varying thus the rate of production of lethal chromosome aberrations. Such phenomena are expected to mainly affect the radiosensitivity to killing of cells irradiated in the late part of the cell cycle; however, an effect on cells irradiated at earlier stages of the cell cycle cannot be excluded entirely. That MPF activity may affect cell radiosensitivity to killing is also indirectly suggested by the observation that two oncogenes, ras and mos, implicated in the regulation of MPF activity (17-19) modulate, when activated, cell radiosensitivity in model cell systems (20-23). Higher MPF activity may cause a rapid condensation of interphase chromatin, and as a result, an increased fragmentation for a given radiation dose.

In agreement with this suggestion, we presented evidence that chromatin condensation is a prerequisite for interphase chromosome breakage (24).

Differences in MPF activity, or in the regulation of chromatin condensation under the influence of MPF, may also underlie the observation that the frequencies of chromatid breaks and gaps in metaphase cells after G2-phase irradiation are higher in tumor cells and in fibroblasts from individuals with genetic conditions predisposing to cancer than in fibroblasts from normal individuals (25, 26). An upregulation of MPF activity in tumor cells will selectively lead to more chromosome breaks in cells irradiated 1-2 h before metaphase when MPF peaks and chromatin condensation begins. Such a model would account for the high incidence of chromosome aberrations in tumor cells irradiated toward the end of the cycle, without requiring increased radiosensitivity throughout the cell cycle (tumor cells are generally equally or less radiosensitive as compared to normal cells). This model is testable and if validated may provide means of measuring a parameter with predictive potential (i.e., chromosome fragmentation as outlined in Refs 25 and 26) indirectly via MPF activity measurements.

The finding that different types of mitotic inducer cells give widely different yields of interphase chromosome breaks has ramifications for the use of the PCC assay for measurements of radiation-induced damage in interphase chromatin. Yields obtained with different types of inducer mitotic cells may not be directly compared with each other.
Indeed, wide variations have been reported in the yield of interphase chromosome breaks in human cells irradiated in G1 and fused either with CHO or HeLa mitotics (8, 27, 28). It is likely that differences in inducer mitotic cells used is at least one of the causes for the differences in the results obtained.

If MPF activity is a contributing factor in the chromosome fragmentation observed by means of PCC in irradiated interphase cells (G1 or G2), parameters presently neglected when performing the assay will need to be considered. In addition to the total MPF activity present in the mitotic cell, one should also consider the volume of the interphase cell and the total volume of the syncytium generated. Further parameters will include the presence in the interphase cell of activities neutralizing MPF, as well as alterations in chromatin that will affect its action as substrate for MPF. Careful evaluation of the contribution of all these parameters in chromosome breakage as measured by means of PCC will help not only in the standardization of the assay, but also in the elucidation of the mechanism of formation of chromosome aberrations.

Acknowledgments

The authors are indebted to Nancy Mott for secretarial help and for editing the manuscript.

References

Mitosis-promoting Factor Activity of Inducer Mitotic Cells May Affect Radiation Yield of Interphase Chromosome Breaks in the Premature Chromosome Condensation Assay

Xinbo Cheng, Gabriel E. Pantelias, Ryuichi Okayasu, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/23/5592

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.