Premature Chromosome Condensation (PCC): Tools in chromosome and cytogenetic research

Eisuke Gotoh

Department of Radiology, Jikei University of School of Medicine, 3-25-8, Nishi-Simbashi, Minato-ku, Tokyo, 116, Japan (EG)

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Eukaryote chromosomes usually condense during mitosis. Chromosome condensation is strictly regulated by cellular elaborated machinery but the details of the mechanism still remains to be elucidated. Occasionally, chromosomes condense at outside of mitosis under several circumstances (Gotoh et al., 1995; Gotoh and Durante, 2006). This phenomenon is known as premature chromosome condensation (PCC), prematurely mitosis (PM); the resulted condensed chromosomes are called prematurely condensed chromosomes (PCCs). About a half century ago, the first description of PCC phenomenon was reported by several artisans from 1967 to 1969 by observing the chromosomes in virus infected mammalian cell (Kato and Sandberg, 1967; Kato and Sandberg, 1968). They reported that the several kind of virus infected cells showed something strange "shattered" or "pulverized" appearance chromosomes. At that time, shattering of chromosomes was thought as that clastogenic activity of infected virus affected the cell nuclei, resulted in severe chopping of chromosomes of infected cells.

Thereafter, detailed analysis of premature chromosome condensation by fused with synchronized cells in mitotic cells using Sendai virus as fusogen (Johnson and Rao, 1970). They found that the "pulverized appearance" of chromosomes is selectively observed in s-phase nuclei. In addition, either G1 or G2 cells do not show "pulverized" chromosomes but showed condensed form of univalent or bivalent chromosomes, respectively. These findings suggested that the "pulverized appearance of chromosome" did not due to the actual breakage of chromosomes. They concluded, therefore, that the nuclei of cells fused in mitotic cells condensed prematurely by unknown material which rich accumulated in mitotic cells, and observed chromosome structures are equivalent to that of the chromosomes of individual cell cycle stage at the time of cell fusion.

They named this phenomenon as premature chromosome condensation (PCC). They also named the material which increased in mitotic cells and promotes chromosome condensation as mitosis promoting factor.
(MPF). At the same time a similar phenomenon to PCC is known to occur at second polar body stage during oocyte maturation. This phenomenon is known as Germinal Vesicle Breakdown (GVBD).

Cytoplasm factor which induces GVBD in oocyte maturation was identified and named as maturation promoting factor (MPF) (Masui and Markert, 1971; Masui, 1974). The MPF of somatic cells and oocyte were later identified as identical substances, so it is often called as mitosis/maturation promoting factor (MPF). MPF is now known as complex of p34cdc2/cyclinB. p34cdc2/cyclinB is regulated negatively by cdc25 and cdc25 is sensitive to type 1 and type 2A protein phosphatases such as okadaic acid and calyculin A.

After recognized of PCC phenomenon, PCC has been utilized in widely in cytogenetic fields. Because PCC has a unique advantage that allows the cell nuclei to be analyzed like as condensed chromosomes in G1, S and G2 cell cycle. The fusion-PCC protocol using fusogenic virus has, however, several drawbacks. The PCC efficiency is highly depend on virus activity and with a risk of infection during virus manipulation.

Thus, chemical induced fusion-PCC protocol was introduced by means of replacing fusogenic virus by cell fusion substance such as polyethylene glycol (Pantelias and Maillie, 1983). Thereafter, fusion-PCC method has been widely used in cytogenetic field including radiation biology or other fields (Cornforth and Bedford, 1983; Mullinger and Johnson, 1983; Hittelman, 1984; Hittelman et al., 1984; Hittelman and Pollard, 1984; Pantelias and Maillie, 1984).

Fusion-PCC using polyethylene glycol is very useful and utilized in wider cytogenetic fields, however the method is still problematic. The PCC efficiency depends on product lot which requires a preparation of substantial amount of mitotic arrested inducer cells. Therefore, the PCC utilization has been very limited in specialized institute. To overcome the drawbacks of fusion-mediated PCC, drug-induced PCC technique was introduced using protein phosphatase inhibitors such as okadaic acid or calyculin A (Gotoh et al., 1995).

This method is very simple and easier than fusion-mediated PCC, and even more easier than conventional chromosome preparation method using colcemid (Gotoh et al., 1995). Therefore, drug-induced PCC method has been used in many fields of chromosome research and cytogenetic study (Gotoh and Asakawa, 1996; Asakawa and Gotoh, 1997; Johnson et al., 1999; IAEA, 2001; Gotoh, 2007; IAEA, 2011).

Molecular mechanism of PCC

Mechanism of fusion-mediated PCC

The precise mechanism of chromosome condensation is still almost unclear. Therefore, the molecular mechanism of induction of PCC is still not completely elucidated either. However, accumulated evidence has revealed several molecules that might be playing key roles in PCC induction. Maturation/Mitosis Promoting Factor (MPF) is a key enzyme that induces PCC or GVBD in somatic cell or oocyte, respectively. MPF is now known as the complex of p34cdc2/cyclinB (Dunphy et al., 1988; Gautier et al., 1988; Maller et al., 1989) and is playing a central role in cell cycle regulation and cell growth control. Activation of p34cdc2/cyclinB complex (phosphorylated form) commences at mitotic stage and accumulated activated p34cdc2/cyclinB complex promotes chromosome condensation (Moreno et al., 1989). Therefore, following cell fusion by either viruses or PEG, the interphase nuclei is exposed to activated MPF which is supplied from the mitotic nuclei; consequently interphase cell nuclei condense prematurely condensed to mitotic-like chromosomes.

Further mechanism for chemical-mediated PCC

Cdc25 is a cell cycle check point proteins which regulates entry of mitosis together with p34cdc2/cyclinB (Ducommun et al., 1990; Moreno et al., 1990). Cdc25 is a tyrosine phosphatase which activates p34cdc2/cyclinB complex by dephosphorylation of tyrosine residue (Gould et al., 1990; Dunphy and Kamagai, 1991; Gautier et al., 1991; Kamagai and Dunphy, 1991; Strausfeld et al., 1991). The activity of cdc25 depends on the phosphorylation/dephosphorylation of itself. Activity of cdc25 is sensitive to PP1 and PP2A (type 1 and type 2A protein phosphatase) (Izumi et al., 1992; Kumagai and Dunphy, 1992; Kinoshita et al., 1993). Okadaic acid or calyculin A is specific inhibitors of PP1 and PP2A. These inhibitors may influence the activity of cdc25 and p34cdc2/cyclinB, finally promoting the premature entry in mitotic stage (Kumagai and Dunphy, 1992; Kinoshita et al., 1993). Activated MPF molecule (p34cdc2/cyclinB) may finally promote chromosome condensation prematurely. This is, presumably, a possible mechanism of drug-induced PCC induced by okadaic acid or calyculin A.

Recently, number of molecules which involved in the mitotic events have been identified such as SMC proteins, including condensin (chromosome condensation), cohesin (chromosome cohesion of replicated chromosomes) (Swedlow and Hirano, 2003), or aurora kinases in centromere function (Tanno et al., 2006; Meyer et al., 2010; Tanno et al., 2010).
These molecules cooperatively regulate the condensation/compaction and formation of eukaryote chromosomes, but the detail mechanism is still unknown and will be elucidated in the future.

**Protocol of drug induced premature chromosome condensation**

**Materials**

Calyculin A, a toxin extracted from marine sponge Discodermia Calyx, is a very strong inhibitor for protein serine/threonine phosphatase (molecular weight 1009.18, molecular formula: C_{50}H_{81}N_{4}O_{15}P, IC_{50} for PP1: 0.5 - 2 nM and IC_{50} for PP2A: 0.1 - 1 nM). Calyculin A is available from many chemical companies. Calyculin A dissolves in ethanol, methanol or DMSO but does not dissolve in water. Calyculin A is permeable in cell and cell nuclear. For stock solution, Calyculin A is dissolved in ethanol or DMSO as 1 mM concentration and stored at -20°C. Fifty nM of final concentration is usually used to induce PCC in much kind of cells. Okadaic acid is also a toxin from marine sponge Halichondria okadai, and is a protein phosphatase inhibitor (molecular weight: 805.2, molecular formula: C_{44}H_{68}O_{13}, IC_{50} for PP1: 10 - 60 nM, IC_{50} for PP2A: 0.1 - 1 nM). It is also used as an inducer of PCC but the PCC efficiency is usual lower than that of calyculin A, in particular for attached growing cells. Thus, using of calyculin A is much recommended. Stock preparation of okadaic acid is same as calyculin A. Figure 1 shows the chemical structure, chemical formula and molecular weight (WM) of okadaic acid and calyculin A. Other kinds of protein phosphatase inhibitors such as 35 methyl okadaic acid, cantharidin, cantharidic acid or endothal are also used as PCC inducers. Microcystin-LR is a very strong inhibitor for protein phosphatase too but not able to be used as PCC inducer (since microcystin-LR is not permeable in cells).

**Method**

Drug-induced premature chromosome condensation technique is very simple and has been described elsewhere. The procedure steps are almost same or even easier than that of conventional mitotic
chromosome preparation using colcemid for mitotic cell arrest, so the artisans who are mastering the chromosome preparation technique will be soon able to handle. So I do not intend to describe the details of protocols but simply summarize it (please see the following reviews for the details (Gotoh and Durante, 2006; Gotoh, 2009).

1. Cell culture and maintain exponentially growing condition is a key point to obtain good PCC index. The PCC index depends also on cell types for the experiment use. Usually 50 nM calyculin A for 30 minutes of incubation is enough to obtain substantial number of PCCs. Note that the optimal condition will be different for individual cell types. The researcher should fix the best combination of concentration and incubation time of calyculin A (in general, PCC index increases as either concentration or incubation time increase, but the shape of chromosome will be more condense).

2. Thirty-minutes prior to cell harvest, add 50 nM final concentration of calyculin A (1 mM stock solution in ethanol or DMSO) to the medium and another culturing.

3. At harvest time, cells usually rounded and loosely attached or even detached from the culture flask and suspended in the medium due to calyculin A effect. Gently pipetting the medium and harvest the cells with a medium into a centrifuge tube.

4. Spin down gently (250 g ~ 800 rpm, a couple of minutes), remove supernatant and re-suspend the cell pellet with 0.075 M KCl to swollen the cells at 37°C for 20 minutes.

5. Fix the cell with same volume of Carnoy’s fixative (methanol: acetic acid 1:3 vol: vol), then spin down again the same above condition. The cell pellet is again re-suspended with the same fixative. Repeat 3 times spin-down/re-suspend/fixation steps, and finally samples are suspended in smaller volume of fixative to make adequate concentration for chromosome preparation (chromosome suspension: if cells are too rich, spin down again chromosome suspension, and suspend again the pellet using more volume of fixative. In case of cells are too sparse, do the same steps but with less volume of fixative).

6. One drop (usually 10 - 15 µl; using yellow pipet tip of Pipetman) of chromosome suspension is placed on the cleaned glass slide, chromosome suspension spontaneously spreading and vaporizing and air-dried. Usually one slide glass will enough to score and analyze substantial number of chromosome spreads. Chromosome sample is then subject to conventional staining protocol such as Giemsa staining or FISH study if required.

**Application of PCC technique for various cytogenetic investigations**

PCC is a very interesting phenomenon not only of a biological point of view but also as useful tools for cytogenetic fields. Chromosomes are usually prepared from mitotic cells using colcemid block method. The obtained chromosomes are therefore mitotic chromosomes only because colcemid simply inhibits assembly of spindle body at mitotic phase, thus the cell’s arrest in mitosis with condensed chromosomes. In contrast, PCC forces the chromosomes to be condensed 'prematurely' not only in mitosis but also in interphase nuclei of any cell cycle (G1-, S-, G2-, M-phase) (Gotoh et al, 1995), hence interphase nuclei is allowed to be visualized as condensed chromosome form like as mitotic chromosomes. Due to this unique aspect, PCC has been used for analyze various nuclear events that proceed during interphase, such as chromosome breakage and repair after exposure of ionizing irradiation or mutagens either using fusion-mediated PCC (Hittelman and Rao, 1974; Hittelman and Rao, 1975; Cornforth and Bedford, 1983; Hittelman and Pollard, 1984; Sen and Hittelman, 1984; Maillie et al., 1986), or chemical-mediated PCC (Gotoh and Asakawa, 1996; Asakawa and Gotoh, 1997; Gotoh et al., 1999; Ito et al., 2002; Horstmann et al., 2004; El Achkar et al., 2005; Srebniak et al., 2005; Terzoudi et al., 2005).

One of most successful application of PCC technique is on radiation biodosimetry, in particular to estimate the irradiation dose after an exposure accident of large dose irradiation (Gotoh and Asakawa, 1996; IAEA, 2001; Gotoh and Tanno, 2005; Gotoh et al., 2005; IAEA, 2011; Gotoh, 2012). Estimation of the body irradiation exposure dose by means of cytogenetic approach by scoring chromosomal aberrations is widely used as a standard biodosimetry. Prepare and score chromosomes of peripheral blood lymphocytes, using the colcemid block protocol which is a simple and well established method (cytogenetic biodosimetry, see reviews by Lloyd 1984, International Atomic Energy Agency (IAEA), 1986). However, after human body is irradiated with large doses (i.e. greater than 10 Gy whole body), the remained cells in the peripheral blood arrest at G2 or G1 phase and do not undergo mitosis or even undergo mitotic cell death or apoptosis. As a consequence, it is usually impossible to obtain chromosome from severely damaged cells for cytogenetic analysis, which has limited the application of the conventional colcemid block method for cytogenetic biodosimetry to estimate radiation doses above 10 Gy. This dose limitation was first overcome using drug-induced PCC technique (Gotoh et al., 1995), and showed that the maximum estimable dose was 40 Gy of γ-rays (Gotoh and Asakawa, 1996). Figure 2 shows the Giemsa stained severely damaged human peripheral blood lymphocyte chromosomes, after 48 hours of exposure to 40 Gy of γ-irradiation in vitro. As clearly seen, all chromosomes are severely damaged and no intact chromosome remains.
Such chromosomes, extremely destroyed, have not ever been obtained from the cells exposed to high dose of irradiation. Since then, number of cytogenetic biodosimetry approaches for high exposure irradiation dose estimation (more than 10 Gy irradiation equivalent of $\gamma$-ray) have been applied and reported (Gotoh and Tanno, 2005; Lamadrid et al., 2007; Wang et al., 2009; Balakrishnan et al., 2010; Gotoh, 2012; Romero et al., 2013). IAEA has qualified PCC technique as one of standard protocol for biological dosimetry (IAEA, 2001; IAEA, 2011). Newer cytological approaches have been challenged utilizing with PCC technique. Classically, it was thought that chromosomes condense during mitotic phase. However, recently accumulated findings revealed that chromosome condensation is not an independent event proceeding during mitosis, but tightly coupled with DNA replication or chromosome repair process (Zink et al., 1998; Manders et al., 1999; Pflumm, 2002; Samaniego et al., 2002). But visualizing approaches for chromosome dynamics coupled with DNA replication was somehow limited, because it should be required to observe chromosomes in S-phase, but chromosomes are usually invisible in S-phase as they are de-condensed. Visualizing dynamics of chromosome condensation has been accomplished using drug-induced PCC technique (Gotoh, 2007), which showed that chromosome condensation is very tightly coupled with DNA replication. Involvement of condensin II in sister

Figure 2. Chromosomes obtained from 48 hours after 40 Gy of in vitro $\gamma$-irradiated peripheral blood lymphocytes. As clearly shown, all of chromosomes are severely destroyed by $\gamma$-irradiation, and there is no more remain intact chromosome.
chromatid separation during the S-phase has also elucidated by means of drug-induced PCC (Ono et al., 2013).

Molecules (proteins/enzymes: such as condensin and cohesin) involved in chromosome condensation mechanism have been recently clarified (Swedlow and Hirano, 2003), lamina, microtubules, histone H1 and topoisomerase II (Kubiak, 1991; Rattner and Wang, 1992; Ishida et al., 1994; Van Hooser et al., 1998; Vass et al., 2003), shugoshin and protein phosphatase 2A in chromosome cohesion (Kitajima et al., 2006; Tanno et al., 2010), aurora kinases in centromere function (Tanno et al., 2006; Meyer et al., 2010; Tanno et al., 2010).

However, precise mechanism is still mostly unclear. PCC is partly the similar but not completely the same phenomenon as normal chromosome condensation (Ghosh et al., 1992; Rattner and Wang, 1992; Guo et al., 1995), but elucidate the PCC phenomenon will provide a new insight for understanding the mechanism of eukaryote chromosome condensation (Vagnarelli, 2012).

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References


This article should be referenced as such: