Atomic force microscopy of human metaphase chromosomes after differential staining of sister chromatids*

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Summary. Human metaphase chromosomes, in which 5-bromo-deoxyuridine (BrdU) had been incorporated into the DNA, were treated with the fluorescent plus Giemsa (FPG) method. Use of this method distinctly stained one of the paired sister chromatids with the Giemsa solution due to the difference in content of BrdU in the two chromatids. These chromosomes with their differential staining of sister chromatids were observed by atomic force microscopy (AFM). In the air-dried specimens, one of the paired chromatids that was stained strongly with Giemsa solution was about two times higher than the counterpart that was stained faintly with Giemsa solution. In the critical point dried chromosomes, the height of the Giemsa positive chromatid roughly matched that of the Giemsa negative counterpart. These findings imply that the arrangement of the Giemsa negative chromatid after FPG staining is fragile and easily collapses due to the surface tension of water during air-drying. At higher magnifications, the surface structure differed between Giemsa positive and negative chromatids; the Giemsa positive chromatid (i.e., unifilarly BrdU-incorporated chromatid) was composed of fibrous structures while the Giemsa negative chromatid (i.e., bifilarly BrdU-incorporated chromatid) exhibited a fine granular appearance. These structural

changes in the sister chromatids are thought to arise from the ultraviolet irradiation and heating of the chromosomes during FPG staining.

The differential staining of sister chromatids in the metaphase chromosome was first described by Taylor (1958). He incorporated tritium-labeled thymidine into the DNA of plant cells and observed the metaphase chromosomes after second duplication of DNA by an autoradiographic method. This method allowed the labeling of one of a pair of sister chromatids of each chromosome. Thereafter, several methods using non-isotope techniques were also introduced for the differential staining of the sister chromatids. Latt (1973) first noticed that the fluorescence of Hoechst 33258 was quenched in the 5-bromo-deoxyuridine (BrdU) incorporated portions of chromosomes, and succeeded in the differentiating BrdU incorporated chromatids in the metaphase chromosomes. A combination of Hoechst 33258 with a Giemsa solution was also introduced by Perry and Wolff (1974); using this fluorescent plus Giemsa (FPG) method, the bifilarly BrdU-incorporated chromatid was stained weakly with a Giemsa solution while the unifilarly incorporated chromatid was stained strongly with this solution. Because the FPG method is simple and effective for obtaining images of differentially stained chromatids with better resolution than those by previous methods, many researchers have applied this method for the visualization of sister chromatid exchanges in somatic cells, with some modifications also introduced by several investigators (Goto *et al.*, 1978; González-Gil and Navarrete, 1982). The mechanism of the sister chromatid differential staining in relation to the high order structure of chromosomes was also investigated by the combined use of light microscopy and scanning electron microscopy (SEM) (Takayama and

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Taniguchi, 1986; Taniguchi and Takayama, 1987; Jack *et al.*, 1989), though the details remain to be fully elucidated.

We have been studying the high-order structure of the metaphase chromosomes by atomic force microscopy (AFM). This microscope—invented by Binnig *et al.* (1986)—uses a sharp probing tip while scanning the sample surface and monitoring the interaction force between the tip and samples. Since this microscope has advantages in obtaining topographical images of non-conductive samples both in air and in liquid without any metal coating, it has been applied to studies of various biological samples (see reviews of Ushiki *et al.*, 1996 and Ushiki, 2003).

In this study, we employed AFM to observe those BrdU-incorporated chromosomes which were stained with the FPG method to differentiate bifilarly and unifilarly BrdU incorporated chromatids. The present study will show the structural difference between the sister chromatids after FPG staining and discuss this in relation to the mechanism of differential staining.

Materials and Methods

Chromosome preparation and differential staining of sister chromatids

Human lymphocytes were collected from fresh heparinized peripheral blood of healthy donors. Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for isolating lymphocytes, a process described in detail in our previous papers (Kimura *et al.*, 2002). The isolated lymphocytes were cultured in a karyotyping medium (PB-MAX, Gibco, Invitrogen Co, Carlsbad, USA) including BrdU (10 $\mu\text{g/ml}$) (Wako, Osaka) for 72 h, during which time two rounds of mitosis occurred. Thus, the metaphase chromosomes in the second mitosis in the presence of BrdU come to possess paired chromatids with different BrdU contents: one chromatid incorporates BrdU into both strands of the DNA helix (i.e. bifilarly BrdU-incorporated chromatid), while the other incorporates BrdU into only one strand of the DNA helix (i.e. unifilarly BrdU-incorporated chromatid) (Kimura *et al.*, 2002). One hour before harvest, the lymphocytes were treated with 50 ng/ml colcemid (Demecolcine, Wako). After the pretreatment with colcemid, the cell suspension was exposed to 0.075 M KCl as hypotonic treatment for 30 min and fixed with methanol-acetic acid (3:1). Spreads of metaphase chromosomes were made by dropping the cell suspension onto glass slides, followed by drying in air.

Differential staining of sister chromatids was performed by a fluorescent plus Giemsa (FPG) method based on the technique by Goto *et al.* (1978). Firstly, specimens were

stained with Hoechst 33258 (10 $\mu\text{g/ml}$) (Gibco) in Dulbecco's phosphate buffered saline (PBS) for 10 min, and then washed several times with PBS. Subsequently, the specimens were put in a staining vat containing PBS heated at 50°C and exposed to ultraviolet light under two lamp stands—each having a 15 W black light (FL15BLB-A, Toshiba, Tokyo)—for 120 min. The two lights were arranged parallelly and located about 8 cm above the staining vat. During exposure to light, the heated PBS was changed every 30 min. Finally, specimens were stained with 2% Giemsa solution (Merk, Darmstadt, Germany) in PBS (pH 6.8) for 10 min and washed with distilled water several times. These stained chromosomes were mounted in the Giemsa solution, observed and photographed with a light microscope.

After light microscopic observations, the specimens were washed with distilled water. Some of these were simply air-dried and observed by atomic force microscopy (AFM). The rest of the specimens were fixed with 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4), treated with 1% tannic acid solution for 5 min, and immersed in a 1% OsO₄ solution for 5 min. They were then dehydrated in a graded ethanol series, transferred to isoamyl acetate, dried in a critical point drier using liquid CO₂, and observed by AFM.

Atomic force microscopy

Specimens were first observed with a Nanopics AFM (Seiko Instruments Inc., Chiba). This microscope features a NPX 100 microscope unit controlled by a Nanopics 1000 controller (Seiko Instruments Inc.) and has the advantage of obtaining wide-ranging images (800 $\mu\text{m} \times 800 \mu\text{m}$ in x y scan range and 10 μm in z range) for analyzing the quality and condition of the specimens. The microscope was operated in a dynamic force mode with a self-sensing cantilever (force constant of 30 N/m). The images were collected as constant force images (i.e., height mode images). After observation by Nanopics AFM, the chromosomes of interest were also observed at higher resolutions with an SPA-400 scanning probe microscope controlled by an SPI 4000 probe station (Seiko Instruments Inc.). Cantilevers used in this microscope were rectangular and had a force constant of 42 N/m and a resonance frequency of 300 kHz (SI-DF40P, Seiko Instruments Inc.). All images were collected simultaneously as constant force images and variable deflection images in a dynamic force mode in air at room temperature.

Fig. 1. Light micrograph of human metaphase chromosomes after sister chromatid differential staining with the FPG method. Sister chromatid exchanges are also observed in some chromosomes.

Fig. 2. AFM image of the metaphase chromosomes shown in Figure 1. These chromosomes were air-dried after the FPG staining. The height of Giemsa positive chromatid is higher than that of Giemsa negative chromatid. Bar = 20 μm

Results

In specimens stained with our FPG method, chromosome spread with differentially stained chromatids was clearly observed by light microscopy. The staining intensity differed between chromatids in each BrdU-incorporated chromosome, in that one of the paired sister chromatids was

distinctly stained with the Giemsa solution. Sister chromatid exchanges (SCEs) were also confirmed in several portions of metaphase chromosomes (Fig. 1).

AFM of air-dried chromosomes after FPG staining

In AFM images of air-dried chromosomes after FPG staining, one of the paired chromatids in each chromosome was

Fig. 3. AFM images (**a**, **c**) and the line profile (**b**) of one of the chromosomes in Figure 2. **a:** The height of Giemsa positive chromatid is higher than that of Giemsa negative chromatid. Bar=2 μ m. **b:** The line profile shows the height of the chromosome indicated by a white line in **a**. X and Y in the profile correspond to the position labeled with X and Y in **a**. **c:** Closer view of a part of **a**. Giemsa positive portions consist of the aggregation of fibrous structures about 40–50 nm. In contrast, no obvious fibers can be observed in Giemsa negative portions. Bar=200 nm

clearly differentiated from its counterpart chromatid by the obvious difference in height present between the two (Fig. 2). Comparison of AFM images with light micrographs of the same chromosomes showed that the height of the Giemsa positive chromatid was higher than that of the Giemsa negative chromatid (Fig. 3a). The Giemsa positive chromatid being roughly 120–200 nm in height, which was almost two times that of the Giemsa negative chromatid when the line profile was examined (Fig. 3b). At high

magnification, Giemsa positive portions were observed as aggregations of fibrous structures about 40–50 nm (Fig. 3c). The structure of the Giemsa negative portions was not clearly determined, although the finer fibrous structures appeared to form a meshwork. Although the portion of sister chromatid exchanges was also observed at high magnification, it was impossible to obtain clear views of the features of the portion in relation to the high-order structure of the chromosomes.

AFM of critical point dried chromosomes after FPG staining

In the critical point dried chromosomes after FPG staining, no obvious height difference in a paired chromatids was recognized, even though the differential staining of sister chromatids was confirmed by light microscopy (Fig. 4a, b). The height of both Giemsa positive and negative chromatid was about 200 nm, which is 1.5 times greater than the height of the Giemsa positive chromatids in air dried chromosomes (Fig. 5a, b). At high magnification, the surface structure was different between Giemsa positive and negative chromatids (Fig. 5c). The surface of the Giemsa positive chromatid showed a fibrous appearance with strongly twisted fibers about 40–50 nm. In contrast, the surface of the Giemsa negative chromatid was composed of fine granules whose size was estimated at about 30–40 nm.

Discussion

AFM studies of chromosomes have seen various attempts made since Degrooth and Putman (1992) first applied the AFM to such research (Tamayo and Miles, 2000; Hoshi and Ushiki, 2001; Ushiki *et al.*, 2002). The present study has first shown the AFM images of BrdU-incorporated chromosomes stained with a FPG method. Perry and Wolff (1974), who developed the FPG method, noted that the chromatid containing BrdU in both strands of DNA (bifilarly BrdU-incorporated chromatid) stained faintly with Giemsa solution while the chromatid containing BrdU in only one strand of DNA (unifilarly BrdU-incorporated chromatid) stained distinctly. Previous investigators have proposed that the loss of Giemsa staining in bifilarly BrdU-incorporated chromatid is caused by the alternation of protein, the change of interaction between protein and BrdU-substituted DNA, and DNA loss during staining (Speit, 1984; Speit and Haupt, 1985; Jack *et al.*, 1989).

In the present study, we have demonstrated that the bifilarly BrdU-incorporated chromatid can be also differentiated from the unifilarly incorporated chromatid due to the difference in height between the two chromatids when the air-dried chromosomes after FPG staining are observed by AFM. Because the height of the Giemsa negative chromatid was roughly half of the Giemsa positive chromatid, the bifilarly incorporated chromatid is considered to be flattened in comparison with the unifilarly incorporated chromatid. It is noteworthy that no height difference was present between the paired chromatids in the critical point dried chromosomes after FPG staining. This finding indicates that the Giemsa negative chromatid is fragile and easily collapses due to the surface tension during air-drying.

Fig. 4. Light micrograph (a) and AFM image (b) of chromosomes after differential staining with the FPG method. For AFM studies, chromosomes were critical point dried after the differential staining. Bar=20 μ m

Fig. 5. Closer view of chromosomes shown in Figure 4. **a:** Bar=2 μm . **b:** The line profile for the height of Giemsa positive and negative chromatids in the portion indicated by a white line of **a**. X and Y in the profile correspond to X and Y in **a**. **c:** Closer view of Giemsa positive and negative chromatids in **a**. Bar=500 nm

On the other hand, in our unpublished data obtained by AFM, the chromosomes with Giemsa staining were substantially taller than non-stained chromosomes. Thus, it is also probable that Giemsa staining reinforces the structure of the unifilarly incorporated chromatid against the surface tension during air-drying.

The present study has also revealed the fine structure of BrdU-incorporated chromosomes by AFM. We have shown that the Giemsa positive chromatid (i.e. unifilarly incorporated chromatid) is composed of strongly twisted fibers about 40–50 nm while the Giemsa negative chromatid (i.e. bifilarly incorporated chromatid) shows a fine granules appearance. Similar findings were reported by Jack *et al.* (1989), whose SEM studies using chromosomes of Chinese hamster ovary (CHO) cells confirmed that loosely packed fibers could be observed in unifilarly incorporated chro-

matid while the surface became flattened in the faintly stained chromatid. On the other hand, we previously investigated the structure of BrdU-incorporated chromosomes immunostained with an anti-BrdU antibody by AFM, and showed that no structural difference was apparent between the unifilarly and bifilarly-incorporated chromatids (Kimura *et al.*, 2002). This implies that the change in structure of the two chromatids was caused during application of the FPG method. The combination of ultraviolet irradiation and heating is probably crucial for the change in structure of the two chromatids, as proposed by Jack *et al.* (1989). An AFM observation of chromosomes during each step of the FPG method could serve to clarify the mechanism of the structural change of the unifilarly and bifilarly incorporated chromatids.

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