Biphasic chromatin structure and FISH signals reflect intranuclear order

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Abstract. Background and aim: One of the two parental allelic genes may selectively be expressed, regulated by imprinting, X-inactivation or by other less known mechanisms. This study aims to reflect on such genetic mechanisms. Materials and methods: Slides from short term cultures or direct smears of blood, bone marrow and amniotic fluids were hybridized with FISH probes singly, combined or sequentially. Two to three hundred cells were examined from each preparation. Results and significance: A small number of cells (up to about 5%), more frequent in leukemia cases, showed the twin features: (1) nuclei with biphasic chromatin, one part decondensed and the other condensed; and (2) homologous FISH signals distributed equitably in those two regions. The biphasic chromatin structure with equitable distribution of the homologous FISH signals may correspond to the two sets of chromosomes, supporting observations on ploidywise intranuclear order. The decondensed chromatin may relate to enhanced transcriptions or advanced replications. Conclusions: Transcriptions of only one of the two parental genomes cause allelic exclusion. Genomes may switch with alternating monoallelic expression of biallelic genes as an efficient genetic mechanism. If genomes fail to switch, allelic exclusion may lead to malignancy. Similarly, a genome-wide monoallelic replication may tilt the balance of heterozygosity resulting in aneusomy, initiating early events in malignant transformation and in predicting cancer mortality.

Keywords: Chromatin, intranuclear order, epigenetics and malignancy

1. Introduction

In routine smear preparations nuclei of mononuclear and polymorphonuclear cells of blood and bone marrow exhibit a special feature that may be called biphasic chromatin condensation. These cells can be recognized after staining with Giemsa or DAPI and can be observed in FISH preparations stained with chromosome-specific probes. Homologous FISH signals are distributed equally in the two nuclear regions with condensed and decondensed chromatin structures.

Ploidywise distribution of chromosomes, i.e., segregation of parental sets of chromosomes, were reported by us based on DNA ploidy measurement with image cytometry and analysis of FISH data from murine and human cells [4,5] and also by Nagele et al. [22,23] by examining chromosome-specific FISH signals on metaphase plates and interphase nuclei. Segregation of the parental sets of chromosomes was previously reported in various cell types of different organisms – plants and animals [1,9,10,26,27]. Sanchez et al. [29] and Sanchez and Wangh [30] examined the relationship between chromosome location and nuclear morphology in special human neutrophils by FISH analysis of chromosomes 2, 18, X and Y and reported that they do not segregate randomly. Although the number of neutrophils studied were limited, these observations support the earlier findings of Hsu et al. [14] and Manuelidis [18], i.e., that the chromocentres cluster symmetrically or non-randomly in different mouse cells and in different cells of human central nervous system. An elaborate study by Koss [15] revealed that the two human sex chromosomes were distributed, one each, in the proximal and distal half of the polarised ellipsoid nucleus of bronchial epithelial cells. Genomewise separation of chromosomes has been elegantly demonstrated in early embryos [19,20] and also observed in blood and bone marrow cells of...
children with hematological disease applying sequential stainings with GTG and FISH protocols and SKY for 24-colour karyotyping [7]. The aim of this work was to study the association between differential chromatin condensation and spatial distribution of the two parental sets of chromosomes.

2. Materials and methods

Twenty specimens of blood and bone marrow were received from cases of Chronic and Acute Myeloid Leukaemia (CML, AML), Aplastic Anaemia (SAA), Myelodysplastic Syndrome (MDS), Chronic and Acute Lymphoblastic Leukaemia (CLL, ALL), Non-Hodgkin Lymphoma (NHL), etc. at diagnosis and follow up of therapy in the Tumor Cytogenetic Unit, Munich University and IMPATH/Genzyme, Phoenix (Arizona). Preparations of cells from blood and bone marrow screenings, lymphoid cell lines and from amniotic fluid (AF) and other sources of the perinatal diagnostic laboratory Dr. Waldenmeyer, Munich (FRG), a total of 12 specimens, served as control material.

Preparations were made using both native smears and cytogenetic techniques. Bone marrow and peripheral blood smears were fixed with ethanol-acetic acid (3:1) in a Coplin jar for 20 min. Alternatively, the cells were washed and fixed in suspension and then air-dried on slides. For cytogenetic preparations, routine short-term cultures were used with and without cytokines (GM-CSF) in RPMI 1640 medium supplemented with 20% fetal bovine serum (Seromed, Berlin). Following colchicine and hypotonic treatments the cells were harvested and fixed in suspension with ethanol-acetic acid before being spread and air-dried on slides.

Fluorescent in situ hybridisation (FISH) was done using centromeric and locus-specific DNA probes, directly labeled with the fluorophores spectrum orange, green and aqua (Abbott-Vysis, Downers Grove, IL, USA). The following probes were used: probe pairs CEP 3/7, 7/8, 7/9, 8/9, 9/11, X/Y, LSI 9q/22q, 11q/14q, 12p/21q and 13p/21q; tri-colour probe X/Y/18; and bi-colour fusion probes ALK at HSA 2p23, BCL6 at HSA 3q27, 5p5q (EGR), MLL, IGH at HSA 11q, IGH at HSA 14q32 and MALT1 at HSA 18q (Table 1). Specimens were stained sequentially with Giemsa and FISH protocol using more than one combination of probes or applying panels covering 5 to 11 chromosomes on parallel preparations. The probe and target DNA were melted simultaneously at 76°C for 2 min and then allowed to hybridise at 42°C for about 1 h and subsequently at 37°C for 4 h to overnight. After washing, the cells were

Table 1

| Probe | Location | DNA clones or sequences |
|-------|----------|------------------------|
| CEP 3 | 3p11–q11 | D3Z1                   |
| CEP 7 | 7p11–q11 | D7Z1                   |
| CEP 8 | 8p11–q11 | D8Z2                   |
| CEP 9 | 9p11–q11 | D9Z1                   |
| CEP 11| 11p11–q11| D11Z1                  |
| LSI 5p5q| 5p15.2/5q31| DSS23, DSS721/EGR1 |
| LSI 9q/22q| 9q34/22q11.2| ABL/BCR |
| LSI 11q/14q| 11q13/14q32| CCND1/IGH |
| LSI 12p/21q| 12p13/21q22| TEL/AML1 |
| LSI 13p/21q| 13q14/21q22| RB1/D21S259, S341, S342 |
| LSI 15q/17q| 15q22/17q21| PML/RARA      |
| ALK 2p23|                | ALK, flanking the gene |
| BCL6 3q27|                | BCL6, flanking the gene |
| MLL 11q|                | MLL, covering the gene |
| IGH 14q32|                | IGH, partly flanking and covering the gene |
| MALT1 18q|                | MALT1, flanking the gene |
| X/Y Xp11–q11/Yq12|    | DXZ1/DYZ12 |
| X/Y/18 Xp11–q11/18p11–q11|    | D18Z1 |
counter-stained by mounting with a buffered medium containing 4′,6-diamidino-2-phenylindole (DAPI) for microscopic examination [7,12].

Microscopy and photomicrography were done using Zeiss Axioskop® with triple band pass and other suitable filters for epifluorescence. Fuji colour diapositive films were used for photographic documentations. Alternatively, an Olympus microscope with digital camera served us for convenient capturing of cells with FISH signals. Two to three hundred cells were examined as regards biphasic chromatin structure and the FISH signal distribution.

3. Results and discussions

3.1. Biphasic nuclei with FISH signals of chromosomal homologues

Biphasic nuclei (i.e., nuclei with two areas, one with condensed and one with non-condensed chromatin, Fig. 1a) were observed in every third or fourth specimen scrutinized. In smears, observation of biphasic nuclei was generally less frequent than after short-term culture, although the highest frequency so far, 4.3%, was observed in a blood smear (Table 2). Their presence, in simple smear and other direct preparations, indicates that such biphasic nuclei are not artifacts caused by the special cytogenetic treatments designed to induce swelling and spreading of the nuclear contents to obtain good metaphase spreads. Such special treatments may, however, augment the sporadic biphasic feature. Although comparatively infrequent this feature seems to be important for nuclear architecture with respect to genome organisation in structure and function. FISH staining with various probes of sex and autosomal chromosomes singly, combined or sequential, demonstrated that, members of chromosome pairs were distributed equally in the two areas of a biphasic nucleus (Figs 1b–f, 2 and 3). So far we have observed biphasic nuclei with corresponding distribution of the chromosome-specific signals for the following probes: paired probes CEP 3/7, 7/8, 7/9, 9/11, X/Y, LSI 9q/22q, 11q/14q, 12p/21q and 13p/21q; one tri-colour probe X/Y/18; and bi-colour fusion probes ALK at HSA 2p23, BCL6 at HSA 3q27, MLL at HSA 11q, IGH at HSA 14q32 and MALT1 at HSA 18q. Some of these probes were applied sequentially or as panels covering 5 to 11 chromosomes on parallel preparations of the same specimen. These observations strongly indicate that each phases of a biphasic nucleus represents one of the two sets of parental chromosomes. However, only a small fraction of cells was observed with this biphasic chromatin structure. Therefore, either this occurrence has a small time window or it is limited to a tissue-specific stem cell population. The latter possibility was also discussed by Pathak et al. [25].

The frequency of these cells were small, but we have ample examples of important biological entities which are rare, e.g., stem cells in bone marrow are less than 1%

![Fig. 1. Biphasic nuclei with its condensed and non-condensed chromatin areas apparently represent the two genomes of a diploid nucleus. Photomicrographs of a biphasic nucleus from bone marrow with DAPI filter (a) and triple band pass showing signals specific to chromosomes HSA 7 red and HSA 9 green (b). Distribution of signals of HSA 3 red and HSA 7 green in a biphasic nucleus from blood (c). In the non-condensed area homologous FISH signals are often either bipartite (b), or extended (d, e and f), indicating advanced replication or enhanced transcription.](image-url)
Table 2
The frequency of biphasic nuclei, where two parental genomes display different chromatin compaction and equitably distributed FISH signals, obtained from control and patient specimens

| Specimen | Tissue | Age | Sex | Diagnosis       | No cells with biphasic nucleus | Percent nuclei with open part | X in open part | Y in open part | X+Y in open part | X or Y marginal or unclear |
|----------|--------|-----|-----|-----------------|--------------------------------|-----------------------------|---------------|--------------|-----------------|-----------------------------|
| Control 01 | AF     | F   | Age | 1/200           | 0.5                            | –                           | –             | –            | –               | –                           |
| Control 02 | AF     | F   | Age | 1/200           | 0.5                            | –                           | –             | –            | –               | –                           |
| Control 03 | LCL    | M   |     | 1/200           | 0.5                            | 1                           | –             | –            | –               | –                           |
| Control 04 | LCL    | M   |     | 0/300           | 0.0                            | –                           | –             | –            | –               | –                           |
| Control 05 | LCL    | M   |     | 0/300           | 0.0                            | –                           | –             | –            | –               | –                           |
| Control 06 | LCL    | M   |     | 0/300           | 0.0                            | –                           | –             | –            | –               | –                           |
| Control 07 | PB     | F   | Normal | 1/300           | 0.3                            | –                           | –             | –            | –               | –                           |
| Control 08 | PB     | F   | Normal | 1/300           | 0.3                            | –                           | –             | –            | –               | –                           |
| Control 09 | PB     | M   | Normal | 0/300           | 0.0                            | –                           | –             | –            | –               | –                           |
| Control 10 | PB     | M   | Normal | 0/300           | 0.0                            | –                           | –             | –            | –               | –                           |
| Control 11 | BM     | F   | Normal | 0/300           | 0.0                            | –                           | –             | –            | –               | –                           |
| Control 12 | BM     | M   | Normal | 0/300           | 0.0                            | –                           | –             | –            | –               | –                           |
| Patient 01 | PB/BMT | 15  | F   | Apl anemia      | 13/300                         | 4.3                         | 8             | 3            | 2               | –                           |
| Patient 02 | PB     | 15  | M   | CML            | 1/200                         | 0.5                         | –             | –            | –               | –                           |
| Patient 04 | PB     | 48  | F   | Thromcytosis   | 2/200                         | 1.0                         | –             | –            | –               | –                           |
| Patient 05 | PB     | 74  | F   | ?MDS          | 2/300                         | 0.7                         | –             | –            | –               | –                           |
| Patient 07 | PB/BMT | 50  | F   | ALL            | 5/200                         | 2.5                         | 13            | 5            | 2               | 1                           |
| Patient 11 | PB     | 65  | F   | ?MDS         | 2/300                         | 0.7                         | –             | –            | –               | –                           |
| Patient 12 | PB     | 49  | F   | NHL           | 6/200                         | 3.0                         | –             | –            | –               | –                           |
| Patient 15 | PB     | 57  | F   | ?CML      | 3/200                         | 1.5                         | –             | –            | –               | –                           |
| Patient 03 | BM     | 48  | F   | Lymphoma     | 4/300                         | 1.3                         | –             | –            | –               | –                           |
| Patient 06 | BM     | 54  | M   | APL           | 3/300                         | 1.0                         | –             | –            | –               | –                           |
| Patient 08 | BM     | 50  | F   | Leukopenia   | 5/300                         | 1.7                         | –             | –            | –               | –                           |
| Patient 09 | BM     | 31  | M   | AML           | 3/300                         | 1.0                         | –             | –            | –               | –                           |
| Patient 10 | BM     | 92  | F   | ?MDS./MPD   | 1/200                         | 0.5                         | –             | –            | –               | –                           |
| Patient 13 | BM     | 77  | F   | ET            | 3/200                         | 1.5                         | –             | –            | –               | –                           |
| Patient 14 | BM     | 20  | F   | Thromcytosis | 3/300                         | 1.0                         | –             | –            | –               | –                           |
| Patient 16 | BM     | 44  | F   | ?CML      | 1/200                         | 0.5                         | –             | –            | –               | –                           |
| Patient 17 | BM     | 32  | F   | CML           | 4/200                         | 2.0                         | –             | –            | –               | –                           |
| Patient 18 | BM     | 75  | F   | CML           | 0/300                         | 0.0                         | –             | –            | –               | –                           |
| Patient 19 | BM     | 84  | M   | Hodgkin     | 6/200                         | 3.0                         | –             | –            | –               | –                           |
| Patient 20 | BM     | 66  | M   | ?NHL        | 6/300                         | 2.0                         | –             | –            | –               | –                           |

AF: amnion fluid, BM: bone marrow, LCL: lymphoid cell line, PB: peripheral blood, BMT: bone marrow transplantation, and ?: diagnosis not confirmed by cytogenetics or FISH.
Fig. 2. A biphasic nucleus stained sequentially with dual colour FISH probe ALK in panel (a) and a probe combination of abl (red) and bcr (green) in panel (b), representing chromosomes HSA 2, 9 and 22. Signals are equitably distributed in the two chromatin areas. Signals in the non-condensed area are elongated. (Accompanying nucleus of a polymorphonuclear cell (PMN) serve as a reference for size, morphology, etc.)

in 10,000. Moreover uneven ploidies, like tri-, penta-, hexa- (also duplicated triploidies), heptaploidy etc, observed in cancer possibly begin as biphasic nuclei giving rise to triploidy.

3.2. Structural and functional organisation of the nucleus

Earlier we reported that up to 18% of the human and murine granulocytic nuclei observed displayed bilateral symmetry (i.e., symmetric order of nuclear lobes), which often exhibited bilateral distribution of the chromosome-specific FISH signals [4,5,7]. While identifying the chromosomes of rat liver cells after partial hepatectomy using a banding pattern obtained by 8-oxychinolin, Gläss [9,10] observed that, in many metaphase preparations, the two haploid sets were lying side by side. This kind of genomic segregation could be more easily recognized in polyploid cells. In a monograph on polyploidy by Pera [26] a hexaploid metaphase from a vole (Microtus agrestis) displays six distinct groups, each representing one set of chromosomes. Both Gläss and Pera [9,10,26,27] also reported on the existence of a number of haploid nuclei in their specimens, as if the chromosomes of each of the parental genomes could segregate as a coherent unit.

We may therefore presume that this phenomenon of biphasic chromatin condensation may indicate a ploidywise distribution of chromosomes. This was observed in interphase cells of individuals of all age groups (prenatal, juvenile, adult and old age) in both sexes ([7] and our unpublished data). It may thus be concluded that the parental genome segregation reflects a general structural principle of nuclear organisation. As such it is likely to have functional implications. The FISH signals in the noncondensed chromatin are often bipartite or stretched while those in...
the condensed phase are often dense (Figs 1b–f, 2 and 3). In normal interphase nuclei the chromosome-specific FISH signals of each homologous pair may also display differences in size and form [15], which often are dismissed as signs of chromosomal polymorphisms, which may not always be true. It is not uncommon to find one of the homologues of FISH signals showing the morphology of S or G2 phase, while the other retains the G1 morphology. Gläss [9,10], reporting on differences of the chromosomal homologues, observed that in regenerating liver of male rats the maternal metaphase chromosomes were less condensed. Chromatin decondensation is generally associated with transcription and replication activities [2,17,34,35]. Decondensation of chromatin of this order is also seen in cultures of blood cells when small lymphocytes undergo blast transformation on mitogenic stimulations accompanied by transcription and replication. But in this case the two haploid sets decondense, as usual, synchronously.

3.3. Quantitative observations

Though low in frequency, biphasic nuclei were observed often and in a large number of clinical specimens. A quantitative sample presentation in Table 2, of control and patient specimens, has shown that this biphasic chromatin distribution tends to occur more often and at a higher frequency in various cases of leukemia and lymphoma than in control specimen. Moreover parallel preparations from the same specimen hybridised to different probes yielded very similar values of biphasic nuclei with equitable distribution of the homologous signals. At the same time different kinds of cells are observed to have biphasic nuclei.

3.4. Involvement of mononuclear and polymorphonuclear cells

This kind of biphasic chromatin condensation has been observed in both mononuclear and polymorphonuclear (PMN) cells of blood and bone marrow (Figs 1–3). PMN synthesize several gene products and may also undergo mitoses upon cytokine stimulation in vivo and in vitro. Biphasic nuclei with equitable distribution of homologous FISH signals seen in amniotic fluid cells are probably precursors of blood or brain cells.

3.5. Epigenetic regulations

The reported observations in diverse systems like plants [1], amphibians [32], mammals [9,10,26,27] and cancer patients [13] indicate that chromosomes are probably handled or addressed to function ploidywise, i.e., genomewise. Regulation of such function would very probably be associated with microtubule mechanisms of centrioles [11] and may, moreover, involve inter-chromosomal tether-like structures described by Nagele [24]. Each of the two centrioles of a diploid cell develops microtubules controlling possibly one of the two sets of chromosomes [7]. Electron microscopy indicated that the number of centrioles of a cell corresponds to the number of sets of chromosomes [16]. The differential compaction of the two parental genomes may indicate that one of them has acquired priority to operate. This kind of epigenetic regulation may switch between maternal and paternal genomes during differentiation of cells as is also known to occur during development in context of allelic exclusion [31]. Data from our two female patients with transplantation of male bone marrow cells may be seen as evidence for switching of this chromatin condition between the two genomes, because in the same specimen some cells had chromosome X in the non-condensed chromatin while others had chromosome Y (Table 2). It may therefore be assumed that involvement of the genomes in a biphasic nucleus does not show restrictions of parental bias.

In a number of human and mammalian genes generally only one allele is expressed in a cell – in some maternal in others paternal. The relevant genes of such cells exhibit functional hemizygosity. Extension of this allelic exclusion to one entire parental genome may, therefore, give rise to a biphasic nucleus.

3.6. Asynchrony of the two sets of chromosomes and implications for malignancy

The differential behavior of chromatin compaction of the two parental genomes is also evident in the asynchrony of their cell cycle progression. Thus, one may find only one of the two centromeres of a homologous chromosome pair to be bipartite, i.e., at G2 or beyond (Fig. 1b). An extreme form of a biphasic nucleus displaying a harlequin-like condition may be designated as inter-metaphase nucleus, where one haploid set attained metaphase while the other lagged behind and remained at interphase (Fig. 4). To date, we have observed three harlequin nuclei. It may be hy-
Fig. 4. A special form of biphasic nucleus with one set of chromosomes at metaphase while the other set remained lagging at interphase. Such “harlequin” nuclei were described earlier as partial Prematurely Condensed Chromosomes (PCC). Note the red–green signal of the fusion probe MLL on the homologues of chromosome HSA 11 (panel (a)). Panel (b) shows the inverted DAPI image of the “harlequin” nucleus revealing its half meta- and half interphase status.

pothesized that these cells may well be associated with neoplastic transformation through loss of heterozygosity. Presumably they may result in triploidy or break apart into two daughter nuclei, e.g., one haploid (hemizygous) and one diploid (with regular heterozygosity or with uniparental disomy for all chromosomes). The hemizygous haploid cell may in its turn duplicate its total DNA resulting in uniparental diploidy, which amounts to a complete loss of heterozygosity. In each of these cases nuclei would suffer gross imbalance of heterozygosity. Each of them – triploid, uniparentally diploid or haploid – are known in cancerous conditions [3,8,13]. In many specimens from cancer patients we have observed uneven ploidy conditions, like tri, penta, hexa (duplicated triploidy) or heptaploidy, which might have developed by asynchrony of the two sets of parental chromosomes.

Recently aneuploidy has been shown to be an early event in malignant transformation and to predict cancer mortality [33]. Pathak et al. [25] hypothesized that organ specific stem cells on acquiring aneuploidy initiate malignancy. The origin and impact of aneuploidy has also been recently elucidated in context of cancer in a special issue of Cellular Oncology [21,28]. Aneusomy would also grossly disturb the balance of heterozygosity.

In conclusion the observed biphasic compaction of chromatin of a nucleus may be an important aspect of general genomic organisation with respect to both structure and function and may relate to loss of balance of heterozygosity and to neoplastic development via aneuploidy or aneusomy.

Acknowledgements

This work was generously supported by research grants from the Sturm-Stiftung of Munich City and the Sander-Stiftung, Munich (Grant No. 97.080.1 Molekular Zytogenetik).

The authors are thankful to Ms. Debra Stegeman, Site Director, Ms. Carol Berger, Laboratory Supervisor and Dr. Sultan Karamanov, Molecular Geneticist, Mr. Norman Ottleben, System Administrator of Genzyme Genetics, Phoenix, AZ, for constant encouragement and qualified assistance. Thanks are also due to Ms. Deborah A. Dugan, Senior Patent Counsel, Genzyme Corporation, Cambridge, MA for reviewing the manuscript.

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