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Telomere dysfunction and chromosome structure modulate the contribution of individual chromosomes in abnormal nuclear morphologies

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ABSTRACT

The cytokinesis-block micronucleus assay has emerged as a biomarker of chromosome damage relevant to cancer. Although it was initially developed to measure micronuclei, it is also useful for measuring nucleoplasmic bridges and nuclear buds. Abnormal nuclear morphologies are frequently observed in malignant tissues and short-term tumour cell cultures. Changes in chromosome structure and number resulting from chromosome instability are important factors in oncogenesis. Telomeres have become key players in the initiation of chromosome instability related to carcinogenesis by means of breakage–fusion–bridge cycles. To better understand the connection between telomere dysfunction and the appearance of abnormal nuclear morphologies, we have characterised the presence of micronuclei, nucleoplasmic bridges and nuclear buds in human mammary primary epithelial cells. These cells can proliferate beyond the Hayflick limit by spontaneously losing expression of the $p16^{INK4a}$ protein. Progressive telomere shortening leads to the loss of the capping function, and the appearance of end-to-end chromosome fusions that can enter into breakage–fusion–bridge cycles generating massive chromosomal instability. In human mammary epithelial cells, different types of abnormal nuclear morphologies were observed, however only nucleoplasmatic bridges and buds increased significantly with population doublings. Fluorescent in situ hybridisation using centromeric and painting specific probes for chromosomes with eroded telomeres has revealed that these chromosomes are preferentially included in the different types of abnormal nuclear morphologies observed, thus reflecting their common origin. Accordingly, real-time imaging of cell divisions enabled us to determine that anaphase bridge resolution was mainly through chromatin breakage and the formation of symmetric buds in daughter nuclei. Few micronuclei emerged in this cell system thus validating the scoring of nucleoplasmic bridges and nuclear buds for measuring chromosome instability in telomere-dysfunction cell environments.

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1. Introduction

Over the last 30 years, biomarker-based approaches have been employed in environmental carcinogenesis research in the hope of refining exposure assessment and providing tools for the early detection of disease-related changes [1]. Cytogenetic biomarkers such as chromosomal aberrations and micronuclei (MN) are the most frequent endpoints in human population studies. They are widely used for screening exposure to mutagens and carcinogens and are early predictors of cancer risk [2]. Recently, other abnormal nuclear structures such as nucleoplasmic bridges (NPBs) and nuclear buds (Bs) have attracted the interest of researchers as biomarkers of genomic instability.

Micronuclei are formed from chromosomes and chromosome fragments that lag behind during anaphase and are left outside the daughter nuclei in telophase. Moreover, real-time *in vitro* studies in carcinoma cell lines have demonstrated that micronuclei can also be originated by complex chromosomal rearrangements which encounter mechanical difficulties during cell division [3]. Nuclear buds are morphologically similar to micronuclei with the exception that they are joined to the cell nucleus by a thin nucleoplasmic connection. Studies of human tumour cells have indicated that these structures contain amplified DNA sequences that are selectively concentrated at the cell nucleus periphery, which may later be extruded from the cell nucleus to form a micronucleus [4]. In contrast to a nuclear DNA extrusion origin, other authors suggest that the generation of buds may also be explained by the breakage of anaphase bridges [5]. In this case, these nuclear structures would in fact be regressing broken chromatin rather than protruding bodies as their name indicates. Anaphase bridges were first observed by Barbara McClintock after DNA damage in maize chromosomes caused by X-rays [6]. She determined that

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radiation-induced broken chromosomes can misrepair their ends leading to the formation of dicentric chromosomes that can be attached to both spindle poles. During division the two centromeres are pulled towards opposite poles creating an anaphase bridge. This bridge may then break, resulting in new chromosome free ends that can fuse again with other broken extremities, perpetuating a breakage–fusion–bridge (BFB) cycle. Alternatively, anaphase bridges can originate from extensive telomere shortening, when telomere uncapping leads to chromosome end-to-end fusion [7,8]. Telomeres, the specialised structures at chromosome ends, are essential for maintaining the stability of eukaryotic genomes by protecting natural ends from recombination and enabling cells to distinguish them from random DNA breaks.

Telomere integrity appears to be a critical element in the evolution of cancer. Numerous studies, mostly based on mouse models, clearly involve dysfunctional telomeres in the early phases of cancer development. A dysfunctional telomere-driven chromosomal instability model has been proposed to explain the origin of epithelial cancers [9]. This model postulates that the telomere–telomere fusions originated by non-balanced age dependent telomere erosion in proliferating epithelial cells together with frequent cell cycle checkpoint inactivation, could generate the major chromosomal instability that is responsible for initiating epithelial carcinogenesis in humans [10,11]. Anaphase bridges have been closely related to chromosomal instability in human tumour samples [12–14] and with tumourigenesis in mice [10,15]. It is therefore of relevant interest to ascertain whether genomic instability mediated by telomere dysfunctionality can be associated with the induction of micronuclei, buds and nucleoplasmatic bridges. We have assayed the usefulness of these biomarkers in human mammary epithelial cells (HMECs) derived from cosmetic breast reductions of healthy women. This cell system has proved to be a valuable tool in determining the initial pathways of carcinogenesis in human breast cancer [16]. It has been shown that HMECs spontaneously lose expression of the $p16^{INK4a}$ protein allowing *in vitro* proliferation of these cells beyond the Hayflick limit [17]. The continuous erosion of telomeric sequences in these cells ultimately leads to massive chromosomal instability generating the types of chromosomal abnormalities seen in the earliest stages of breast cancer [18–20].

Our studies of HMEC demonstrate that chromosome instability is manifested by an increasing formation of abnormal nuclear morphologies (ANMs) throughout the cell culture. In these cells NPB scoring has been a valuable tool, better than micronucleus scoring, for measuring ongoing instability. The use of DNA probes for selected chromosomes as well as real-time analysis of bridge resolution has provided information about the origin and fate of ANMs. In HMECs, chromosomes with eroded telomeres were mainly included in NPBs, MN and Bs. This observation, together with the internal location of MN and Bs in binucleated cells relative to the cell nucleus, provides evidence of a common origin for all ANMs. This hypothesis has been confirmed by the observation that both buds, predominantly, and micronuclei, to a lesser extent, arise after NPB breakage. All these results validate NPBs and Bs as biomarkers of chromosomal damage in a telomere-dysfunction cell environment.

2. Materials and methods

2.1. Cells and culture conditions

219-7 HMECs (BioWhittaker; Walkersville, MD) were derived from normal breast tissue. Cells were cultured in serum-free MEGM medium (BioWhittaker) and supplemented with epidermal growth factor, insulin, hydrocortisone, gentamicin/amphotericin-B and bovine pituitary extract. The cells were counted, plated at 2×10^5 cells per 75 cm^2 flasks and grown at 37 °C in a 5% CO_2 atmosphere. The number of accumulated population doublings (PDs) per passage was determined using the equation PD = initial PD + log (no. viable cells harvested/no. viable cells plated)/log2. The finite life span of post-selection HMECs when cultured in MEGM medium is about 22 passages, equivalent to approximately 65–75 PDs.

2.2. Cytokinesis-block micronucleus assay and metaphase preparations

Exponentially growing binucleated HMECs were obtained after adding cytochalasin B (2 mg/ml stock solution in dimethyl sulfoxide; Sigma; St. Louis, MO) at a final concentration of 6 μg/ml before harvesting the culture so as to block cytokinesis [21]. The tubes were centrifuged at 220 × *g* for 8 min, followed by hypotonic shock and three rounds of methanol/acetic fixation. Metaphase spreads were obtained after treatment with colcemid 0.02 μg/ml for 8 h, followed by hypotonic shock and methanol/acetic fixation. Cell suspensions were dropped onto clean slides, which were stored at −20 °C. Before hybridisation, the slides were mounted in antifade solution containing 4,6-diamidino-2-phenylindole (DAPI; Sigma) at a final concentration of 0.125 μg/ml. This enables good morphology chromosomes to be karyotyped before the denaturation step. After capturing metaphases and recording coordinates, metaphase karyotyping was performed by reverse DAPI staining, which results in a reproducible G band-like pattern that enables accurate identification of each pair of homologous chromosomes.

2.3. Fluorescence in situ hybridisation

2.3.1. PNA-FISH

Telomere and centromere PNA-FISH were performed on metaphase chromosome slides using a Cy3-$(CCCTAA)_3$ PNA-probe for telomeres and a FITC-AAACACTCTTTTTGTAGA probe for centromeres (PE Biosystems; Foster City, CA) [22]. Specific chromosome arms carrying critically short telomeres were identified rather than measuring mean telomeric reduction. Signal-free chromosome ends on the homologous chromosome arms in each analysed metaphase were recorded as a whole because the corresponding homologues, namely maternal and paternal chromosomes of each pair, could not be formally distinguished by G-banding.

2.3.2. Centromeric-FISH and chromosome painting

FISH of binucleated HMECs was performed using a mixture of three commercial probes (Abbott; Abbot Park, IL) that detect the centromeric region of chromosome 1 (CEP 1 Satellite II/III; *Spectrum Orange*) and 4 (CEP4; *Spectrum Aqua*) and the locus BCR region located at 22q11.2 close to the centromere of chromosome 22 (LSI22; *Spectrum Green*). The resulting signals were red, light blue and green for chromosome 1, 4 and 22, respectively. Chromosome painting probes (Abbott; Abbot Park, IL) for chromosome 1 (*Spectrum Orange*) and chromosome 22 (*Spectrum Green*) were also applied to slides. Before hybridisation, the slides were pre-treated with pepsin (0.1 mg/ml, Sigma) in 10 mM HCl and postfixed in 37% formaldehyde in PBS/1 M $MgCl_2$. The probes were denatured at 75 °C for 5 min, and then applied to slides in which the DNA had been denatured at 75 °C for 5 min in 70% formamide/2×SSC and dehydrated in cold ethanol series. Hybridisation was performed overnight at 37 °C in a moist chamber. The slides were washed for 1 min in 0.4×SCC that was previously warmed at 75 °C and in 2×SCC/0.05% Tween 20 for 5 min at room temperature. Finally, slides were dehydrated and mounted in antifade solution containing DAPI. Fluorescence signals were visualised under an Olympus BX microscope equipped with epifluorescent optics specific for each fluorochrome. Images were captured and analysed using Cytovision software (Applied Imaging, Inc., CA).

2.4. Scoring criteria

A binucleated cell was scored when the distance between the two daughter nuclei was equal to or lower than the radius of one nucleus. Moreover, the main nuclei could touch each other, but cells with overlapping nuclei were not considered. Finally, both daughter nuclei must be approximately equal in size, staining pattern and staining intensity. The analysis of abnormal nuclear morphologies was performed on DAPI counterstained binucleated cells using the criteria described by Fenech [23]. A nucleoplasmic bridge was considered to be the narrow/wide chromatin segment connecting two cell nuclei, micronuclei were morphologically identical to, but smaller than the cell nucleus, and round and oval protrusions of the nuclear membrane, connected to the cell nucleus, were classified as buds [5].

2.5. Transient transfection procedures and time-lapse microscopy

The day before transfection, HMECs were plated into a 35-mm glass bottom dish (MatTek, Ashland, MA) at a density of 7300 cells/cm^2. Transfection procedures using pEGFP-N1 plasmid encoding H2B-GFP sequence (Addgene Inc., Cambridge, MA) were performed using Fugene HD (Roche Diagnostics S.L., Indianapolis, IN) according to the manufacturer's instructions. Expression of the H2B protein fused with GFP was analysed under an inverted fluorescence microscope 24–48 h after transfection, while live cell imaging was performed in a Leica TCS SP5 confocal microscope. Cells were visualised with a HCX PL APO CS 40.0×1.25 OIL UV objective using the 488 nm line from an argon laser. Mitotic cells were imaged at 3× zoom using the software Leica LAS AF Lite (Leica Microsystems, Inc., Germany) for 2–4 h, at intervals of 3 min. Acquisition settings were established at 10% laser power through a pinhole of 5 AU, a line average of 2, and a scan speed of 400 Hz to avoid excessive cellular damage. Throughout the entire process, cells were kept at 37 °C and 5% CO_2.

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