Endopolyploidy in irradiated p53-deficient tumour cell lines: Persistence of cell division activity in giant cells expressing Aurora-B kinase

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Abstract

Recent findings including computerised live imaging suggest that polyploidy cells transiently emerging after severe genotoxic stress (and named ‘endopolyploid cells’) may have a role in tumour regrowth after anti-cancer treatment. Until now, mostly the factors enabling metaphase were studied in them. Here we investigate the mitotic activities and the role of Aurora-B, in view of potential depolyplloidisation of these cells, because Aurora-B kinase is responsible for coordination and completion of mitosis. We observed that endopolyploid giant cells are formed via different means in irradiated p53 tumours, by: (1) division/fusion of daughter cells creating early multi-nucleated cells; (2) asynchronous division/fusion of sub-nuclei of these multi-nucleated cells; (3) a series of polyplloidising mitoses reverting replicative interphase from aborted metaphase and forming giant cells with a single nucleus; (4) micronucleation of arrested metaphases enclosing genome fragments; or (5) incomplete division in the multi-polar mitoses forming late multi-nucleated giant cells. We also observed that these activities can release para-diploid cells, although infrequently. While apoptosis typically occurs after a substantial delay in these cells, we also found that ~2% of the endopolyploid cells evade apoptosis and senescence arrest and continue some form of mitotic activity. We describe here that catalytically active Aurora-B kinase is expressed in the nuclei of many endopolyploid cells in interphase, as well as being present at the centromeres, mitotic spindle and cleavage furrow during their attempted mitoses. The totally micronucleated giant cells (containing sub-genomic fragments in multiple micronuclei) represented only the minor fraction which failed to undergo mitosis, and Aurora-B was absent from it. These observations suggest that most endopolyploid tumour cells are not productively inert and that Aurora-B may contribute to the establishment of resistant tumours post-irradiation.

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Keywords: Mitotic catastrophe; Tumours; Polyploidy; Aurora-B kinase

1. Introduction

The application of genotoxic insults including irradiation is an established method of treatment of malignant tumours. Tumours lacking functional p53 are defective in many cell cycle checkpoints and often respond to genotoxic stress by undergoing mitotic catastrophe (MC). Although MC is defined as “cell death occurring during or shortly after a failed mitosis” (Kroemer et al., 2005; Galluzzi et al., 2007), p53-deficient tumours undergoing MC are resistant to genotoxic treatments. As a result of mitotic failure, cells alternatively reset interphase becoming tetraploid (Castedo et al., 2004a,b). Therefore, MC has also been defined as mitotic events that produce tetraploid progeny cells in the first post-damage...
observations were made and reviewed by Rajaraman et al. (2006). Association of genotoxic resistance with the induced endopolyploidy was found in rodent and human tumours (Baroja et al., 1998; Come et al., 1999). Our earlier observations revealed that transient endopolyploid p53-Burkitt lymphoma cells were able to facilitate DNA repair and release para-diploid mitotic progeny post-irradiation (Erenpreisa et al., 2000; Illidge et al., 2000; Ivanov et al., 2003). These observations led us to hypothesise that transient endopolyploid cells, which are capable of depolyploidisation, may in fact constitute an alternative survival pathway (Erenpreisa and Cragg, 2001, 2007). Similarly, de la Hoz and Baroja (1993) and Baroja et al. (1996, 1998) reported that rodent tumour cells of high ploidy are capable of proliferating, despite certain peculiarities in their cell cycle. Using computerised live imaging, Ianzini and Mackey (2002) have demonstrated that a small proportion of endopolyploid cells formed in vitro post-mitotic catastrophe successfully undergo polyploidy reduction and form viable clones. Prieur-Carrillo et al. (2003) found that ~2% of human bladder carcinoma giant cells formed after irradiation potentially clonogenic 2N progeny. Stewenius et al. (2005) showed that events of mitotic catastrophe in colorectal cancer are compatible with survival, and underlined the role of anaphase bridge formation in clonogenic growth. Furthermore, the striking live-imaging studies of Chu et al. (2004) on CDKN1A-deficient cells (CDKN1A is up-regulated by the tumour suppressor p53 controlling G1/S checkpoint) have clearly shown the viability of the endopolyploid cells produced by multiple mitotic catastrophe events. These authors concluded that MC is not directly responsible for individual cell death. Similar observations were made and reviewed by Rajaraman et al. (2006).

These intriguing reports underscore the importance to study further the division potential of endopolyploid cells in p53-deficient tumours. Although the presence of high ploidy cells in malignant tumours has long been documented (Baroja et al., 1998), their biological significance is not well understood, with much controversy persisting over their proliferative potential. However, if a result of genotoxic treatment, genetically unstable giant cells can give rise even to a few selected clones, these might be genetically changed, promoting resistant regrowth and further tumour progression. Therefore detailed study of the mechanisms of the reproductive/apoptotic behaviour of giant cells is important.

We have investigated the reproductive activities of endopolyploid cells post-irradiation in p53 defective human cell lines through the involvement of Aurora-B kinase, the essential regulator of mitosis (Carmena and Earnshaw, 2003; Vannarelli and Earnshaw, 2004). Aurora-B belongs to the group of mitosis regulators called “chromosome passengers”. Within this group, Aurora-B kinase provides for fidelity and procession of mitosis by coordinating chromosome alignment onto metaphase spindle with anaphase and cytokomy (Ditchfield et al., 2003). The easily recognisable immunocytochemical markers of its presence are the attachment of Aurora-B to centromeres in metaphase plate, to microtubules of the central mitotic spindle during anaphase B and participation in the formation of the mid-body in ana-telophase. The mid-body is marked by the two bands of Aurora-B and two lateral bands of tubulin. In immunofluorescent staining for the two proteins, these two-coloured bands and a central split in the mature mid-body (the place of the centrolin ring) assign the whole structure its unique appearance. While the main events of mitosis occur within 1 h, the mid-body, which is responsible for cytokomy completion, persists in the cytoplasmic bridge between daughter cells for 2–4 hours longer (Gromley et al., 2005). Thus the mid-body represents a characteristic marker of the process of mitosis. Our data reveal that catalytically active Aurora-B kinase is intimately associated with the formation, division, and extended survival of endopolyploid cells resulting from MC in functionally p53-deficient tumour cell lines.

2. Methods

2.1. Cell lines

Namalwa Burkitt’s lymphoma cells (ATCC) were grown as suspension cultures in RPMI 1640 medium, 10% foetal calf serum (Gibco or Sigma) at 37 °C in a 5% CO2 in air humidified incubator. HeLa S3 cells (ATCC) were grown either in suspension culture or as adherent clone 3. Suspension HeLa culture was grown under constant rotation in Joklik’s MEM media containing 10% heat-inactivated calf serum (Hyclone) and antibiotics. Suspension cultures were maintained in logarithmic phase of growth for at least 24 h prior to irradiation. Namalwa cells were further cultivated by replenishing culture medium every 48–72 h, and HeLa S3 every 24 h.

HeLa adherent clone 3 cells were grown as monolayer in F-10 medium (Hyclone) containing 10% heat-inactivated foetal calf serum (Sigma or Hyclone) and antibiotics (100× penicillin–streptomycin, Sigma P4333) in a 37 °C incubator supplied with 5% CO2 in air, either on 13 mm polylysine-coated coverglasses in 24× wells, for immunocytochemistry and DNA cytometry or in a T-25 tissue culture flasks for live-imaging.

To determine the cells in S-phase, BrdU was added at 10 μg/ml to the cell culture for 60 min prior to cell fixation on slides with methanol. DNA denaturation was performed by 2 N HCl, 37 °C for 20 min. After washes in PBS, the primary and secondary antibodies were applied (Table 1). In some experiments, proteasome inhibitors (Sigma) Mg-132 (5 μg/ml), inhibitor of calpain (25 μg/ml), and lacticocystin (10 μg/ml) were added for 2 h prior to cell harvest. Autofluorescence vacuoles were detected by monodansyleadaverin (MDC) and by Sa-β-galactosidase. For MDC (Sigma) staining the cultures were incubated with 0.05 mM MDC at 37 °C for 60 min followed by fixation in 4% paraformaldehyde, washing twice in PBS. The slides were mounted into Perm Mount and immediately scored in the DAPI channel. For Sa-β-galactosidase detection, the instruction of theSigma kit (Code CS0030) was followed (staining was extended overnight).
2.2. Irradiation

Irradiation for live imaging at the University of Iowa was delivered at room temperature using a PANTAK Bipolar 2HF320 irradiator (200 kV, 0.35 Cu/1.5 Al filter). Dosimetry was performed using a Victoreen electroscope at a dose rate of 1.27 Gy/min. In vitro cell culture irradiation was applied using a Gullmay D3 225 X-ray source at a dose rate of 0.77 Gy/min. A single dose of 10 Gy was delivered in all experiments.

2.3. LSDCAS imaging analysis

Large-Scale Digital Cell Analysis System (LSDCAS), a live imaging and analysis system that includes quantitative cell analysis software, is amenable to determine the kinetics of various cellular mechanisms on a cell-by-cell basis (Ianzini and Mackey, 2002; Davis et al., 2007). For live imaging, HeLa adherent clone 3 cells were maintained for irradiation by the scheme described above. Two days prior to irradiation, 1 × 10⁵ cells were plated as a cell suspension and placed into the CO₂ incubator. After irradiation, the cells were re-incubated for 2 h, at which time an equal volume of fresh, complete and warmed media was added prior to the flask being positioned on to the LSDCAS stage for imaging acquisition for 72 h. One hundred random fields were manually chosen. Division-related events were categorised as follows: normal, normal followed by cleavage regression (sister cells fusion), and unrelated cell fusion (non-sister cell fusion). The number of cells alive at the end of the experiment was also counted.

2.4. Immunofluorescent (IF) staining

Suspension cells were pelleted, resuspended in FCS and cyto spun on to polylysine-coated slides. Cultures on cover glasses were rinsed before fixation in PBS and FCS. Cells were allowed to dry for 1 min (drying was not done when turbulins were being detected) and were fixed at −20 °C in methanol followed by 5–10 short rinses in cold acetone or methanol/acetone (1:1) at −20 °C. A 1 min rinse in 70% methanol and several in PBS followed, after which blocking in 1% bovine serum albumin in PBS (for some stains adding 0.01–0.05% Tween-20) was performed for 15 min. Application of the primary antibody and further manipulations were done in the usual routine manner (antibodies listed in Table 1). Post-staining was performed with propidium iodide (5 µg/ml), DAPI (1 µg/ml) or 1 µg/ml of amino actinomycin (7-AAD). Cells were finally embedded in Prolong Gold (Invitrogen).

2.5. DNA image cytometry

For DNA in situ cytometry, cells were fixed in ethanol/aceton (1:1, v/v) for 30 min at room temperature and stained with by modified Feulgen reaction using toluidine blue. Images were taken with a Leitz Ergolux L03-10 microscope equipped with a calibrated Sony DXC 390P colour video camera. DNA content was measured as the integral optical density in the green channel or in the red channel with interference filter 289 nm, using Image Pro Plus 4.1 software (Media Cybernetics; REO 2001, Riga, Latvia). The stoichiometry of DNA staining was verified using the values obtained for metaphases compared to anaphases and telophases (ratio 2.0); arbitrary diploid (2C) DNA values were averaged from measuring 50 anaphases in non-treated tumour cells. The device error was estimated at 0.5%. The variation coefficient for DNA content was also assessed in normal human lymphocytes where it was determined as being in the range 2–5%, while for HeLa mitotic cells it reached 20%.

2.6. Fluorescent in situ hybridisation (FISH)

HeLa cells were harvested, treated with 75 mM KCl at room temperature for 10 min and fixed with five changes of methanol/glacial acetic acid (3:1). The suspension was dropped onto slides and allowed to dry. Satellite probes for chromosomes 10 and X were used in the kits provided by Molecular Cytogenetics (Q-BIOgene) by the applied instruction. These chromosomes were chosen as containing three normal copies and not participating in clonal markers (Macville et al., 1999). The number of labels per individual nuclei was counted. The results were grouped as normal (three labels) and abnormal (all other counts) and compared in pairs by Fisher’s Exact Test (http://www.matforsk.no/ola/fisher.htm).

2.7. Fluorescent, bright field, and confocal microscopy

For these images, a Leica confocal laser microscope DM 600 and a Leitz Ergolux L03-10 microscope were used.
3. Results

3.1. Irradiation causes anaphase bridging and bi-nucleation

Irradiation (10 Gy dose) induces a G2 arrest that persists for 8–10 h in HeLa cells. Cells then enter mitosis displaying elevated mitotic indices of 10–11% at 24 h and 15%, at 48 h. About 25% of metaphases in HeLa become arrested and many of them restitute interphase as micronucleated (many small nuclear vesicles enclosing separate chromosomes or their groups) or mono-nucleated polyploidy cells. However, the majority of irradiated HeLa cells proceed through anaphase. About 70% of all HeLa cells in the first mitosis at 19–24 h and 96% of cells in the second mitosis at 42–48 h display anaphase bridges due to dicentric chromosomes (checked by CREST-immunoserum staining for centromere proteins), compared to 2–5% in controls. As a result, 40% of cells become bi-nucleate on day 1 post-irradiation. Live-imaging confirmed that mitotic cleavage furrow regression between nuclei-bridged daughter cells was responsible for the initiation of bi-nuclearity in these cells, as 82.2% of daughter cells fused (n = 107), of which 96.7% were still alive at the end of the 3-day filming session (Fig. 1 and Table 2). The nuclei of the bridged post-mitotic daughter cells often display irregular contours and/or contain in addition a few micronuclei; however, as judged by DNA image cytometry, segregation was mostly equal (Fig. 2a—c,e,f). FISH studies using pericentromeric probes for chromosomes X and 10, which do not participate in HeLa clonal markers, showed that a small proportion of nuclei (5%) aneusomic by these chromosomes was present in the control population, which increased to 15% after the first cell cycles in irradiated samples (Table 3). A second smaller wave of bridged anaphases appeared 3 days post-irradiation, with increased bi-nucleation following 24 h later.

Irradiated Namalwa cells arrest in G2 for 24 h, start aberrant mitoses from day 2, which are mostly arrested in metaphase, giving an increased mitotic index on days 3–4 (Ivanov et al., 2003). Contrary to HeLa cells, which mostly pass anaphase and form bi- and multi-nucleated giant cells (MNGC), the later contain three or more nuclei. The majority of Namalwa cells restitute the arrested metaphases as mononucleated giant cells (MONGC).

Table 2

<table>
<thead>
<tr>
<th>Results of computed live imaging</th>
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<tbody>
<tr>
<td>Analysed cells that divide normally in 72 h</td>
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<tr>
<td>Analysed cells that divide normally and then fuse</td>
</tr>
<tr>
<td>Cells that divide, then fuse and live to the end of the movie</td>
</tr>
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<td>Non-sister cell fusions in 72 h</td>
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Fig. 1. A series of images of irradiated HeLa cells in LINDCAS system starting live-imaging 12 h post-irradiation. Four cells (A–D) divide remaining connected by nuclear bridges and then each daughter pair regresses a cleavage furrow forming a bi-nucleate cell.
3.2. Aurora-B localises normally in cells during the first division events post-irradiation

During mitosis in untreated tumour cells, Aurora-B kinase localises sequentially to the centromeres, spindle mid-zone and equatorial cortex, and the mid-body (Fig. 3a and c). Despite the clear presence of bridged chromosomes in irradiated cells (Fig. 2a and b), Aurora-B kinase localised normally and the microtubules forming the mitotic spindle, the anaphase mid-zone, and the mid-body, were organised correctly (Fig. 3b and c). However, some of these cells showed additional diffuse staining in the cytoplasm (Fig. 3b).

3.3. Polyploidy doubles with a periodicity of one generation time

After the second round of mitosis (day 2) the frequency of bi-nucleate cells (4C) fell in HeLa cells from 40% to 20%. However, the incidence of MNGC increased from 10% to ~70%, with most multi-nucleated cells containing eight chromosome

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Table 3

<table>
<thead>
<tr>
<th>Samples</th>
<th>Chr #X Control</th>
<th>Chr #X 48 h</th>
<th>Chr #10 Control</th>
<th>Chr #10 48 h</th>
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<tr>
<td>Number of normal nuclei (3 labels)</td>
<td>577</td>
<td>450</td>
<td>637</td>
<td>667</td>
</tr>
<tr>
<td>Number of abnormal nuclei</td>
<td>28</td>
<td>69</td>
<td>35</td>
<td>101</td>
</tr>
<tr>
<td>% Abnormal (aneusomic) nuclei</td>
<td>4.85</td>
<td>15</td>
<td>5.5</td>
<td>15</td>
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<tr>
<td>Fisher Exact Test, p-value</td>
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<td>$p &lt; 0.001$</td>
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</tbody>
</table>
sets. Polyploidy increased each day post-damage. By day 4, HeLa cells typically displayed DNA contents of 8C, 16C and 32C corresponding to completion of four polyploidising cycles-phases over the 4-day period (Fig. 4). The proportion of polyploid cells reached 50–60%. Consistent with our previously published results, the same polyploidy limit (32C, occasionally 64C) was exhibited in irradiated Namalwa cells, which also increased in ploidy by a daily doubling (Erenpreisa et al., 2000).

3.4. Asynchronous mitosis occurs in multi-nucleated cells

At 2–4 days post-irradiation, ~30% of HeLa cells were paired with long DNA bridges (Fig. 5a–g) surrounded by the nuclear envelope and thin layer of cytoplasm, as judged by staining for lamin B and tubulin (Fig. 5f). Frequently the two daughter cells comprising the DNA-bridged pair exhibited a loss in synchrony during their second or third division, such that one daughter nucleus/cell was in mitosis, while the other was in interphase (Fig. 2c,e,f). About 5% of MNGC exhibited asynchronous division of only one of sub-nucleus (Fig. 5a,d,e). The same phenomenon, although at a smaller scale and at later terms, was found in Namalwa (Fig. 5h). These divisions had normal or nearly normal Aurora-B positive mid-bodies, suggesting that elements of mitosis were retained during this event (Fig. 5a,c,g,h). Similar quantities of DNA appeared to be segregating by this asynchronous division, as judged by voxel intensity of the DAPI signal (see Fig. 5d and e). These divisions had normal or nearly normal Aurora-B positive mid-bodies, suggesting that elements of mitosis were retained during this event (Fig. 5a–c,g,h). Similar quantities of DNA appeared to be segregating by this asynchronous division, as judged by voxel intensity of the DAPI signal (see Fig. 5d and e). These secondary divisions were also often DNA- and nuclear envelope-bridged (Fig. 5d and f). These apparently symmetric “daughter nuclei” on either side of the bridge suggest that early bi- and multi-nucleated cells have mostly originated from successful previous mitosis followed by fusion of daughter nuclei or cells. This process continued later on a smaller scale, where both asynchronous bipolar mitosis and cytoplasmic mid-bodies were found in multi-nucleate cells (Fig. 5h,i). Synchronous divisions of two sub-nuclei in bi-nucleate cells were rarely observed (Fig. 2d). Totally micronucleate cells, which represented a minority, were Aurora-B-negative (Fig. 6a, arrow); they never entered mitosis and often died (by necrosis). However, some of them, as seen from inclusion of BrdU and increasing size, grew indefinitely solely by endoreduplication (data not shown).
In summary, the majority of early multi-nucleated HeLa cells (days 1–3) resulted from a series of bipolar mitosis/sister fusion events, with a ~10-fold smaller amount occurring by non-sister fusion of stressed cells, and a minor population by micronucleation of the failed mitosis. The majority of endopolyploid cells (~70%) from irradiated Namalwa were mononucleate.

3.5. Aurora-B kinase is present in MONC and MNGC nuclei but is targeted for degradation

Control cells undergoing mitosis stain positively for Aurora-B. Aurora-B is also present in the nuclei of G2 cells and rare giant cells, albeit far less abundantly than in mitotic cells (Fig. 3a). About 60% of giant cells on days 3–4 post-irradiation display enhanced nuclear positivity for Aurora-B. However, Aurora-B-negative MONG and MNGC cells lacking signs of degradation are also present in the population (Fig. 6b). On day 5 the positive giant cells comprise about 40%, on day 7 only 10%. MONGC sometimes have a very strong karyoplasmic reaction for Aurora-B (Fig. 6a). Expression of Aurora-B in the interphase nuclei of giant cells was much more prevalent after treatment with the proteasome inhibitors, lactocystin, MG132 or inhibitor of calpain (2 h), suggesting that Aurora-B is normally targeted for proteasome-mediated degradation when present in the nucleus (Fig. 6d and e).
We also noted that Aurora-B was absent from annexin V-positive apoptotic cells (Fig. 6f), but again its expression could be rescued by the application of proteasome inhibitors (not shown).

Since Aurora-B is generally considered a mitotic kinase, and its activity is stimulated primarily by its mitotic partner protein, inner centromere protein INCENP (Adams et al., 2001), we studied the co-localisation of Aurora-B with centromere proteins by CREST immunoserum (Fig. 7a–c). In metaphase plates of control cells Aurora-B co-localises with individual centromeres (Fig. 7a). After irradiation, the majority of Aurora-B-rich nuclei of giant cells contain one large central nucleolus and clustered centromeres. Aurora-B-positive nuclear foci in giant interphase cells rarely co-localise fully with individual centromeres (Fig. 7b), but rather are found as larger patches in centromere clusters, mostly in the perinucleolar heterochromatin, in chromocentres, and at the nuclear envelope in some cells (Fig. 7c,d). To test whether Aurora-B present in giant interphase nuclei was active, we used the immunoprobe for phospho-H3ser10, a specific substrate of Aurora-B kinase. Some giant cell nuclei contained speckles of phosphorylated histone H3, mostly around their nucleoli (Fig. 7e). However, giant aberrant metaphases were extensively positive for phosphorylated histone H3, indicating a high level of Aurora-B kinase activity (Fig. 7f).

Fig. 6. Distribution of Aurora-B kinase in and among irradiated cells. (a) Strongly positive MONGC; however, always negative micronucleated HeLa cells (arrow). (b) A proportion of cells with Aurora-B-positive nuclei in Namalwa. (c) A positive MNGC of HeLa (green for Aurora, counterstained with red for α-tubulin). (d) Thin dotted arrays of Aurora-B (green) in the chromatid of the nucleus restituting from the arrested polyploid metaphase: fixed 2 h after application of Mg-132. (e) A rare finding: clear Aurora-B dots over minimally condensed preprophasic chromosomes; red for Aurora-B, green a false colour for overexposed DAPI. (f) Annexin-positive dying cells (red) are negative for Aurora-B kinase (Namalwa cells). Bars = 20 μm.
3.6. Multi-polar mitoses and formation of late multi-nucleated cells

A small proportion of HeLa cells undergo tripolar mitoses in the first days post-irradiation, which show Aurora-B kinase-positive mid-body with three spindle twigs (Fig. 8a). These cells often complete division into three daughters (not shown). However, the majority of multi-polar mitoses in giant cells which they attempt before day 5 become arrested in metaphase showing clumped, poorly condensed, often partially polytenised chromosomes (Fig. 8b). In metaphase arrested cells, Aurora-B-kinase is often found both on the chromosomes and in the cytoplasm. Massive apoptosis observed around days 5–6 closely follows this period in both cell lines, as determined by cell morphology and caspase 3 activity (not shown), involving ~30% of giant HeLa cells and up to 80–90% of giant Namalwa cells, as reported previously (Illidge et al., 2000; Ivanov et al., 2003). In the survivors, occasionally from day 5–6 and then more frequently from day 10 post-irradiation, MNGC and MOGC underwent true endomitosis (as initially defined by Geitler (1937)). In these cells, individual chromosomes were condensed, but the nuclear envelope and nucleoli remained intact. Despite the absence of nuclear envelope breakdown, Aurora-B strongly accumulated at these endomitotic chromosomes (Fig. 8c). At the same late period, Aurora-B was concentrated on metaphase centromeres of well condensed chromosomes in a number of multi-polar mitoses (Fig. 8d) and on the central spindles.
of multi-polar (usually tri-polar) anaphase cells (Fig. 8e). Hence, after a series of mitoses aborted in metaphase at the earlier post-irradiation period, these cells attained the capacity to pass a spindle checkpoint and were found segregating their DNA and attempting to cleave cytoplasm into a number of discrete progeny. In most cases, full cytokinesis did not occur and mid-bodies did not form. However, in rarer cells, daughter sub-cells were released tearing (disjoining?) the chromosome bridges, as previously shown (Erenpreisa et al., 2005). The non-segregated anaphases reverted in a circular or semi-circular arrangement of sub-nuclei at the periphery of giant cytoplasm (Fig. 8f). DNA image cytometry revealed circularly arranged post-mitotic sub-nuclei (Fig. 2h–j), ranging from 1C to 4C (Fig. 2h), often in odd numbers. However, ~60% of these sub-nuclei were para-2C. At later times (1–2 weeks post-irradiation) when regrowth of para-diploid mitotic fraction was renewed, post-mitotic giant cells underwent replicative senescence, as showed by positive staining for

Fig. 8. Multi-polar mitoses in irradiated samples. (a) Clear central mid-body. Aurora-B (red) and α-tubulin (green) in a tri-twigged spindle. However, telophase nuclei (DAPI) possess very irregular contours. (b) Arrested multi-polar metaphase: α-tubulin revealing poles is red, Aurora-B (green) is poorly attached to chromosomes (blue), which are not sufficiently condensed and seem polytenised. (c) True endomitosis with condensed chromosomes (DAPI) and Aurora-B richly bound to them. Aurora-B (green), α-tubulin (red). (d, e) The cells were fixed 2 h after application of the calpain inhibitor. (d) A tripolar metaphase, chromosomes are well condensed and Aurora-B (red) is richly attached to them; α-tubulin of the poles is green. (e) Three spindles are seen decorated in their central part with the tandemly arranged Aurora-B-positive grains (green; tubulins were not stained), while DAPI reveals several anaphase figures. (f) Radial arrangement of the equal-sized Aurora-B-immunopositive (red) nuclei in the large adherent HeLa cell with active microtubular skeleton (green). Inset: the nuclei in DAPI channel. (a) 2, (b) 5, (c–e) 6, and (f) 8 days post-irradiation. Bars = 20 μm.
monodansylcadaverine and Sa-β-galactosidase, absence of BrdU inclusion, and Aurora-B negativity (not shown). However, ∼10% of late giant cells were Aurora-B positive (Fig. 8f) and also labelled for BrdU and cyclin B (not shown), indicating that they remained in an active cell cycle during at least 2 weeks of observation. These data demonstrate that active Aurora-B may contribute to the long-lasting reproductive capacity of endopolyploid cells.

The reproducible schedule of main reproductive activities of the two cell lines after genotoxic insult is summarised in Table 4. Although there are some differences, the main timing of events is similar in all of them.

4. Discussion

In developmental systems, endopolyploidy is usually a terminal point of cell differentiation (Nagl, 1978). In Drosophila metamorphosis salivary glands, the giant polyteneic cells cycle only between G1 and S phase, with the down-regulated activity of mitotic cyclin kinase complex (Edgar and Orr-Weaver, 2001). However, in contrast to the absence of mitotic activity Drosophila, we have demonstrated that p53-deficient giant tumour cells formed by MC undergo ongoing mitosis. Firstly, we observed that endopolyploid giant cells are formed in irradiated p53 tumours as a result of aberrant mitotic activities in several ways, by: (1) division/fusion of daughter cells creating early MNGC; (2) asynchronous division/fusion of sub-nuclei of these multi-nucleated cells; (3) a series of polyploidising mitoses resetting replicative interphase from arrested metaphase and forming MOGC; (4) micronucleation of arrested metaphases; or (5) incomplete division in the multi-polar mitoses forming late MNGC. We also observed that these activities are able to albeit infrequently release para-diploid cells. Some of those descendants are likely to initiate a further round of MC. All these events are displayed after genotoxic stress in a reproducible sequence extended for 1–2 weeks (summarised in Table 4).

In brief, in the first period there are aberrant mitoses, mostly bridged, followed in the second phase (day 3–5) by the production of increasingly large polyploid cells, mostly due to metaphase arrest of polyploidising mitoses. This is followed in the third phase (day 5–6) by segregation by multi-polar anaphases, and in the fourth, from day 7, regrowth of paradiploid clones commences. Within our tumour model the events that occur between days 5 and 6 are a critical period, when the cells shift from the formation of increasingly large polyploid cells to the breakdown of these large polyploid cells and the start of an apoptotic crisis. Therefore it is worth noting that, preceding this trigger, endopolyploid Namalwa cells are undergoing the delayed wave of recombination repair of DNA double-strand breaks on days 4–5 which protects them from apoptosis (Ivanov et al., 2003). Only 10–20% of giant cells evade apoptosis (Illidge et al., 2000; Ivanov et al., 2003), and as we have seen in this study, ∼10% of those further overcome senescence arrest and continue cyclical mitotic activity. Hence the fraction of potential long-living survivors originating from endopolyploid cells may be 1–2% in this tumour, an estimation that corresponds with the counts obtained by live-imaging analysis in other systems (Prieur-Carrillo et al., 2003). We also found the same reproductive activities involving giant cells in the untreated tumour cell cultures during prolonged cell cultivation (10–20 days), although occurring slowly and to a much smaller proportion and extent.

Secondly, we have shown that 4 of the 5 mechanisms producing endopolyploid cells were marked by the presence of Aurora-B kinase, which localises at centromeres, the anaphase mid-zone, and the mid-body. Moreover, it was present in the interphase nuclei of giant cells, where immunoprobing for phosphor-H3 revealed that it was catalytically active.

Table 4

<table>
<thead>
<tr>
<th>Days after irradiation</th>
<th>HeLa</th>
<th>Namalwa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bridged mitoses/union of sister nuclei/cells</td>
<td>Arrest in G2</td>
</tr>
<tr>
<td>2</td>
<td>Bi-nucleation, bipolar mitoses of their sub-nuclei forming MNGC (about 70%); metaphase arrests of mono-nucleated cells</td>
<td>Aberrant mitoses, many of them are bridged</td>
</tr>
<tr>
<td>3</td>
<td>Bi-polar mitoses of sub-nuclei in bi-and MNGC</td>
<td>Metaphase arrests, emergence of MONGC (about 80%) and MNGC (about 20%)</td>
</tr>
<tr>
<td>4–5</td>
<td>A new, smaller wave of bi-nucleation a</td>
<td>Metaphase arrests of polyploidy mitoses, increase of polyploidy to 32C; bi-polar mitoses of sub-nuclei in MNGC</td>
</tr>
<tr>
<td>5–6</td>
<td>Major cessation of further polyploidisation, apoptotic crisis, true endomitosis, bi- and multi-polar cell divisions processing anaphase in surviving cells</td>
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</tr>
<tr>
<td>7–9</td>
<td>Beginning of clonogenic regrowth of small para-diploid cells; cessation of proliferative activities and appearance of senescence markers in the majority of giant cells; continuation of mitotic attempts in the minor fraction of giant cells</td>
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</tr>
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</table>

a The second and possibly next waves of polyploidisation were noticed also in later terms.
(supposedly in the G2 state). This activity was particularly high in true endomitotic and giant mitotic cells. During de-polyplodisation stage, aurora-B shifted from centromeres to central spindles in cells starting multi-polar anaphases and occasionally was found as a mid-body in tripolar mitoses undergoing cytotomy. Similarly, association of overexpression of aurora-B with increased proliferative potential of megakaryocytes has been shown in transgenic mice (Zhang et al., 2004) and in polyploid cell formation through aborted mitoses in vascular smooth vessel cells (Nagata et al., 2005), suggesting common mechanisms.

Thus, in line with some previous reports (Baroja et al., 1998; Chu et al., 2004; Stewenius et al., 2005), we herein report that endopolyploid p53-deficient tumour cells are derived by active, yet aberrant mitotic events, and also that the process of ploidy reduction can occur by mitotic mechanisms, although mostly modified. The only exception to this observation are entirely micronucleated cells which lose the ability to enter mitosis (and apoptosis as well) and which in our models represented a minor fraction. There is some evidence and arguments in literature that chromosome bridges in colon cancer do not prevent clonogenic growth, while multi-polar mitoses likely may produce genomically less perspective cells (Stewenius et al., 2005; Gisselson, 2005). However, the interference of recombination and true endomitosis found by us between these events may account for more crucial effects on the genomes than only their simple segregation and needs further studies on different models.

The literature reports that deregulation of Aurora-B kinase, both by overexpression or knock-down, leads to polyploidy (Nguyen and Ravid, 2006). This paradox becomes more understandable in view of the present results showing the necessity of both mitosis and its failure or reverse for the formation of endopolyploid cells.

Overexpression of Aurora-B kinase is characteristic of many tumours correlating with genetic instability, endopolyploidy and aggressive behaviour (Adams et al., 2001; Giet et al., 2005). These characteristics are particularly prevalent in tumours lacking functional p53 or its target (Ditchfield et al., 2003; Hamada et al., 2003). Deregulated Aurora-kinase B is capable of transforming cells in vitro. Moreover, the in vivo anti-tumour activity of the inhibitors of aurora-kinases has been reported and the question of their use as anti-cancer targets is currently under investigation (Warner et al., 2003; Wilkinson et al., 2007).

5. Conclusions

We have shown that p53-deficient endopolyploid tumour cells are formed mostly as a result of abortive mitoses and that part of these cells retain a long-lasting reproductive potential, crucially supported by the activity of Aurora-B-kinase. These data suggest that endopolyploid cells and Aurora-B kinase may contribute to the formation of genotoxically resistant growth.

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Authors’ contributions

J.E. designed experiments and carried them out cytologically and immunocytochemically in Riga, Iowa, Brighton, and Manchester, and drafted the paper; S.P.W. participated in experimental design, in establishing of some immunocytochemical methods, in microscopy, in the article drafting and editing; E.A.K. maintained in Iowa the HeLa cell culture, helped in the gamma-irradiation, worked at the LSDCAS imaging acquisition, analysed the LSDCAS images, and carried out DNA cytometry; F.I. and M.A.M. conceived the LSDCAS studies, participated in its design and coordination, and drafted the part of the paper related to the LSDCAS studies. F.I. also performed the gamma-irradiation at Uf; A.P.A. contributed in the cytological analysis of HeLa irradiated and control samples; P.J.D. designed and wrote the event analysis software for LSDCAS and helped in the analysis of the LSDCAS images; G.P. carried out the irradiation experiments on HeLa in Riga and participated in the analysis of their results; A.I. carried out with J.E. the experiments on Namalwa in Manchester and participated in analysis of the results; T.M.I. participated in experimental design, analysis of results, drafting and editing of the manuscript.

References

Andreasen PR, Lacroix FB, Lohez OD, Margolis RL. Neither p21WAF1 nor 14-3-3 sigma prevents G2 progression to mitotic catastrophe in human colo carcinoma cells after DNA damage, but p21WAF1 induces stable G1 arrest in resulting tetraploid cells. Cancer Res 2001;61:7660–8. 

Chu K, Teele N, Dewey MW, Albright N, Dewey WC. Computerized video time lapse study of cell cycle delay and arrest, mitotic catastrophe, apoptosis and clonogenic survival in irradiated14-3-3s and CDKN1A (p21) knockout cell lines. Rad Res. 2004;162:270–86.


