Nuclear Bud Formation: A Novel Manifestation of Zidovudine Genotoxicity

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Key Words

Aneuploidy · AZT · FISH · Mouse painting probes · Nuclear buds · Zidovudine

Abstract

Normal diploid somatic mammalian cell division generates 2 daughter cells as a result of a strict and well-controlled mitotic process. However, some defects during the progression of that process could generate an unbalanced distribution of chromosomes, aneuploidy and eventually, a malignant phenotype. Previous observations using a transgenic mouse model with diminished DNA repair capacity revealed the presence of nuclear buds (NBs) induced in vitro by the nucleoside analog zidovudine (Retrovir®, 3'-azido-3'-deoxythymidine, AZT). Here we used bone marrow mesenchymal cells, taken from mice with the *Xpa*^{-/-} *Trp53*^{+/-} genotype, that were cultured and exposed to 0 and 100 μ M AZT for 24 hours. Fixed and denatured cells were processed by fluorescence in situ hybridization (FISH) with whole chromosome painting probes used to identify chromosomes in cells growing on glass chamber slides (2 probes/slide). A variety of sizes and shapes of NBs were observed. Some NBs had a large connection with the main nucleus ($>1/4$ of the NB diameter), others had a smaller connection ($<1/4$ of the NB diameter), some were circular and positioned close to the nucleus, while some resided in the cytoplasm separated from the nucleus or connected by a thin chromatin strand. We had hypothe-

sized that NBs would progress in the process of budding until separation occurred, but this was not proven by time-lapse photography studies performed for 20 hours. From 1,126 cells scored in the unexposed cultures, 10.39 % of cells carried NBs, while from 1,108 cells scored in the AZT-exposed cultures 29.16% of cells carried NBs ($p = 0.001$). In AZT-exposed cells there were a total of 322 NBs scored; 46.6% or 150 NBs contained positive signals for one or both probes used, while 53% or 172 NBs had no probe signal. In addition, FISH analysis showed no preferential localization of any chromosome within the NBs. Among the NBs that carried no probe signal, the presence of positive signals with inversion of DAPI imaging demonstrated centromeric content. It has been hypothesized that NBs occur as a result of expulsion of amplified DNA from the main nucleus; however, this data demonstrates that NBs may contain any chromosome, suggesting that NBs do not consist of just amplified DNA.

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Normal diploid somatic mammalian cell division generates 2 daughter cells as a result of a strict and well-controlled mitotic process. However, some defects during the progression of that process could generate an unbalanced distribution of chromosomes, aneuploidy and eventually, a malignant phenotype. In that regard, expansion of the nucleus into structures designated as nuclear buds (NBs) has attracted the attention of many researchers. NBs have

been defined as structures that are responsible for the expulsion of undesirable DNA content, such as amplified DNA [Shimizu et al., 1998, 2000]. Some reports indicate that NBs, called 'blebs', are precursors of micronuclei and contain specific chromosomes, like chromosomes 1, 4 and 12 in human liposarcomas [Pedeutour et al., 1994; Suijkerbuijk et al., 1994], or chromosomes 17 and 21 in folate deficiency-induced aneuploidy in human lymphocytes [Wang et al., 2004].

The nucleoside analog drug zidovudine (Retrovir®, 3'-azido-3'-deoxythymidine, AZT) is a DNA replication chain terminator able to induce genomic centrosome amplification and aneuploidy in a number of species [Borojerdi et al., 2009; Yu et al., 2009]. In addition, AZT has been shown to induce NBs in AZT-exposed cultured hamster cells [Borojerdi et al., 2009].

Here we expand those original observations and present experiments designed to identify the content of NBs induced in mesenchymal-derived cells grown from the bone marrow of mice null for the *Xpa* gene and haploinsufficient for *Trp53* [van Steeg et al., 2001; Nostrand et al., 2009]. Employing fluorescent in situ hybridization (FISH) techniques, individual chromosomes were identified by localization within NBs. Additionally, the process of formation of the NBs was monitored by time-lapse photography. These studies show that the content of the NBs is diverse, and the genesis of the NB remains to be understood.

Materials and Methods

Transgenic Mouse Model

Experiments have been performed utilizing C57 BL mice that are null for the nucleotide excision repair gene *Xpa* and heterozygous for the tumor suppressor gene *Trp53*. The nomenclature used in this manuscript to represent the genotype will be *Xpa*^{-/-} *Trp53*^{+/-}. Animals were maintained and treated at the NCI Animal Facility under conditions specified by the Association for the Assessment and Accreditation of Laboratory Animal Care, in accordance with humane principles for laboratory animal care. Procedures were approved by the NCI Animal Care and Use Committee. The selection of this transgenic model is based on prior observations demonstrating an enhanced response to the genotoxicity induced by the nucleoside analog AZT. Mice of this genotype are susceptible perhaps due to the fact that the animals lack the first step in nucleotide excision repair, and the lack of *Trp53* allows the cells to progress through the cell cycle regardless of the DNA damage.

Cell Culture

Bone marrow from *Xpa*^{-/-} *Trp53*^{+/-} mice was collected from the femur under sterile conditions employing a disposable LO-DOSE ½ cc U-100 Insulin Syringe with a permanently attached

28G × ½" needle (Becton Dickinson, Franklin Lakes, N.J.). The material was resuspended in RPMI media (ATCC, Manassas, Va.) with 10% fetal bovine serum (ATCC) and cells were plated on one-chamber polystyrene vessel glass slides (BD Falcon, Bedford, Mass.). Cells remained undisturbed for 48 h to allow attachment. After that period, media was removed, and replaced with fresh media. Mesenchymal-derived cells with a typical fibroblast appearance were attached by that time. When cultures were semi-confluent, the media was removed and replaced by media containing either 0 or 100 µM AZT (Sigma, St. Louis, Mo.). After 24 h cells were washed with PBS and fixed with ice-cold methanol at -20°C for 20 min.

Chromosome Identification by Fluorescent Painting Probes

A single whole mouse chromosome paint system (Applied Spectral Imaging, Vista, Calif.) was used to identify 2 pairs of chromosomes per slide by simultaneously using Spectrum Orange probes for one pair and Spectrum Green probes for the second pair. Slides were incubated for 2 min at room temperature in 2× SSC (0.3 M NaCl and 0.3 M sodium citrate) and then dehydrated sequentially in 70%, 80%, and 90% ethanol. Chromosomal DNA was denatured in 70% formamide, 2× SSC, for 2 min at 72°C followed by dehydration in ethanol washes of 70%, 90%, and 100%. FISH was carried out as described by Pinkel et al. [1986] and Lichter et al. [1988] with minor modifications. On each slide 10 µl of labeled DNA was applied. Labeled DNA was denatured at 78°C for 10 min and then preannealed at 37°C for 30 min. Slides were hybridized overnight at 37°C. Post-hybridization washes were performed at 45°C as follows: (1) 50% formamide, 2× SSC, 15 min, (2) 1× SSC, 10 min, and (3) 0.1× SSC, 10 min. Slides were counterstained and mounted with DAPI-Antifade 250 ng/µl (Vector, Burlingame, Calif.).

Microscopy and Chromosomal Content Analysis

FISH slides were imaged with a Zeiss Fluorescent Imager M1 upright microscope system (Carl Zeiss Inc, Thornwood, N.Y.) equipped with a Hamamatsu Digital Camera (Hamamatsu, Bridgewater, N.J.) and IPLab software (BioVision, Exton, Pa.). Approximately 100 cells per pair of painting probes were scored with the NB content being analyzed for the presence or absence of the specific pair of painting probes in cultured *Xpa*^{-/-} *Trp53*^{+/-} bone marrow mesenchymal derived cells either unexposed or exposed to 100 µM AZT for 24 h.

Time-Lapse Imaging

Mesenchymal fibroblasts obtained from *Xpa*^{-/-} *Trp53*^{+/-} mice were cultured in 35 mm glass bottom culture dishes (Mat-Tek Corporation, Ashland, Mass.) and grown until semi-confluence was reached. Cells were then treated with 0 and 100 µM AZT and stained with Hoechst 33342 FluoroPure grade (Invitrogen, Eugene, Oreg.). Cells were placed inside an environmental chamber (enclosing most of the microscope) set to 37°C. A small 6" × 4" × 1" transparent chamber was placed over the Mat-Tek dish in order to supply 5% CO₂ and humidity to the cells. All experiments were imaged for at least a minimum of 17 and maximum of 20 h with no more than 30 locations per dish. All images were collected using a wide-field Personal DeltaVision system (Applied Precision Inc., Issaquah, Wash.) mounted on an inverted Olympus IX71 microscope with an oil immersion PlanApo N 60×/1.42 objective lens. All images were acquired using a CoolSNAP ES2

camera with a frame rate of 2, 4 and 8 min per acquisition, 2×2 binning and a 512×512 pixels imaging field. Z-stacks were collected (3 images per stack) using excitation 360/40 (10%) and emission filter 457/50 for all image sets. Videos of the live cells were created using Applied Precision's SoftWoRx software package version 4.0.0.

Results

Incidence of NBs

A summary of the incidence of NBs in cultured *Xpa*^{-/-} *Trp53*^{+/-} bone-marrow mesenchymal cells exposed to 0 or 100 μ M AZT is presented in table 1. The total number of cells scored for 0 and 100 μ M AZT is shown, along with cells with and without NBs. Percentages of NB-containing cells are indicated in table 1 (bottom row). A Student's t test comparison of the NBs in 0 and 100 μ M AZT treatments showed statistical significance ($p = 0.001$).

FISH Analysis of Specific Chromosomal Content of NBs

A representative NB is shown in figure 1A. A total of approximately 100 cells was analyzed per FISH experiment. From those, the content of the NBs was scored to identify the presence of one or both of the mouse painting probes hybridized on that particular slide. A representative example is shown in figure 1B. Table 2 shows the number of NBs positive or negative for the painting probe hybridized on each slide. The first column shows the chromosome pair probed on each slide together with the labeling dyes: Spectrum Orange (red) and Spectrum Green (green). The second column represents the total number of NBs that contain positive signals for the probes used on that FISH experiment. The last column indicates the total number of NBs not containing positive signals for the specific pair of probes used in that hybridization. It is important to mention that the NBs classified as not containing FISH signal did frequently contain chromosomes that were not hybridized on that particular slide. From a total of 322 nuclei analyzed, 172 (53.42%) showed no positive signals, however DAPI staining revealed centromeric signals, suggesting chromosomal content inside the NBs (fig. 1G, K).

Interpretation of NB Images by Fluorescence Microscopy

Careful scoring of FISH cells by fluorescence microscopy suggested that NBs could be classified in a hypothetically-sequential progression based on size and mag-

Table 1. Incidence of NBs in cultured *Xpa*^{-/-} *Trp53*^{+/-} bone marrow mesenchymal cells exposed to 0 or 100 μ M AZT for 24 hours

	Unexposed	AZT Exposed
Total cells	1,126	1,108
Without NB	1,009	782
With NB	117	326
% cells with NB ^a	10.39	29.16

^a Student's t test revealed a significant difference between NBs present in the AZT group compared to the unexposed group. $p = 0.001$.

nitude of connection with the nucleus (compare fig. 1B, D, F). Some NBs had a large connection with the main nucleus ($>1/4$ of the NB diameter) (fig. 1D, F), others had a smaller connection ($<1/4$ of the NB diameter) (fig. 1A, arrowhead), some were circular and positioned close to the nucleus (fig. 1F, G), while some resided in the cytoplasm separated from the nucleus (fig. 1K, arrowhead) or connected by a thin chromatin strand (fig. 1A, arrowhead, also see online supplementary movie 1; for all online supplementary material, see www.karger.com/doi/10.1159/000298794).

Evaluation of NB Formation by Time-Lapse Photography (Frames 1 and 2)

We had hypothesized that cells could progress from NBs with a large connection to the separated NB, but this was not proven by time-lapse photography studies performed for 17–20 hours. Live-cell imaging techniques were performed with a time-lapse rate of 2, 4 and 8 minutes per acquisition. Obtaining photos every 2 minutes induced cell death in 6–8 hours due to phototoxicity by overexposure from the UV lamp used to reveal Hoechst images. Acquisitions every 8 minutes were not fast enough to follow the nuclei and process of NB formation. We found that acquisitions every 4 minutes worked well, and used this interval to follow nuclear changes and NB formation for 5 independent experiments each with 17–20 hours duration. The sequential progression of NBs from the initial stage of formation to the separated state could not be confirmed when 50 cells/experiment were observed over a time period of 17–20 hours, with a collection rate of 2 minutes per acquisition. A sudden expansion of the nuclear content to the final size of the NB was observed in the majority of the cases. Occasionally, the NBs were separated from the nucleus but frequently they remained at-

Fig. 1. NBs. **A** Frame obtained from time-lapse movie 1 showing inverted Hoechst image of nuclei of *Xpa*^{-/-} *Trp53*^{+/-} bone marrow mesenchymal derived cells exposed to 100 μ M AZT for 24 hours. A nucleus (arrow) exhibits an NB attached by a DNA strand (arrowhead). **B, D, F, H** *Xpa*^{-/-} *Trp53*^{+/-} mesenchymal-derived cells exposed to 100 μ M AZT hybridized with Spectrum Orange-labeled whole chromosome 1 and Spectrum Green-labeled whole chromosome 12 probes. **B** Nucleus bearing an NB in an initial state of formation containing part of chromosome 1 (arrow). **C** Inverted DAPI image of cell in (**B**) showing chromatin containing NB (arrow). **D** Nucleus with a larger NB showing the absence of both chromosomes 1 and 12 in the NB; **E** inverted DAPI image of cell in (**D**) showing NB-containing centromeres and chromatin

(arrow); **F** nucleus with NB containing labeled signal for both chromosomes 1 (red) and 12 (green); **G** inverted DAPI image of cell in (**F**) showing centromeres and chromatin containing NB (arrow). **H** Normal nucleus showing typical localization of both Spectrum Orange-labeled whole chromosome 1 and Spectrum Green-labeled whole chromosome 12 probes. **I** Inverted DAPI image of nucleus in (**H**). **J, K** Inverted DAPI images of *Xpa*^{-/-} *Trp53*^{+/-} bone marrow mesenchymal derived nuclei from cells exposed to 100 μ M AZT for 24 hours showing different types of NBs. **J** NB containing no centromere staining (arrow); **K** nuclei with attached NB containing centromere (arrow), and a detached NB containing multiple centromeric signals (arrowhead).

tached by a thin strand of DNA (fig. 1A arrow and online suppl. video 1). The attached remote NBs and the nuclei kept the same degree of chromatin condensation, with synchronized progression of the condensation in the 2 independent bodies. The size of the NB depended on the ploidy of the cells, as larger NBs were more frequently associated with larger nuclei. Additionally, with time-lapse

imaging it was recognized that polyploid cells are more resistant to cellular death, than their diploid counterpart (online suppl. video 2).

NB Content Is Variable

At times whole chromosomes appeared to be located within an NB although the NB was not always positive

for the painting probe (fig. 1D). Unstained (FISH negative) whole chromosomes can be identified by inverting the DAPI signal (fig. 1E, G, K, arrows). In many occasions NBs were positive for the painting probes, revealing the presence of specific chromosomes (fig. 1B, F) and in some cases the NBs were negative for any centromere or specific painting probe (fig. 1J, arrow). Figure 1B shows an NB containing part of chromosome 12 (arrow) and figure 1F shows both chromosomes in a large NB (arrow). Specific chromosome content observed in a normal nucleus is shown in figure 1H, where Spectrum Orange-labeled whole chromosome 1 and Spectrum Green-labeled whole chromosome 12 each exhibits 2 positive FISH signals.

Discussion

In previous studies the antiretroviral drug AZT has induced cell cycle arrest, micronuclei, sister chromatid exchanges, shortened telomeres, and alterations in gene expression [Olivero, 2007]. A novel form of DNA response to damage, described as budding of the nucleus, has been reported recently in CHO cells [Borojerdi et al., 2009]. Here we investigated the content of NBs, induced in cultured fibroblasts taken from transgenic mice with a DNA repair deficiency and a *Trp53* deficiency. Painting probes specific for mouse chromosomes were used to identify the presence/absence of whole chromosomes located within the NBs. According to Fenech's definition 'NBs are bodies of DNA attached to the nucleus by a narrow stalk of nucleoplasmic material, depending on the stage of the budding process' [Fenech, 2007]. Although the time involved in the complete maturation of a bud is uncertain, some reports indicate that gamma irradiation induced NBs about 3 hours after the initial treatment [Haaf et al., 1999]. Although not specific, some evidence points towards the preferential material being expelled from the nucleus as unwound chromatin [Xu et al., 1999]. Furthermore, it has been proposed that the content of NBs originates from interstitial or terminal fragments, or excess DNA extruded from the nucleus [Lindberg et al., 2007]. Here we provide evidence that nuclear budding is a process that starts early after cells have been exposed to AZT, as shown in the time-lapse experiments obtained during a 17–20 hour period. Some NBs were observed as early as 2 hours after treatment was initiated. Analysis of content using whole chromosome painting probes revealed that AZT induces NBs in ~30% of cells while 10% of untreated cells have NBs. Around 47% of the NBs were

Table 2. FISH analysis of specific chromosomal content of NBs in *Xpa*^{-/-} *Trp53*^{+/-} cultured bone marrow mesenchymal cells exposed to 100 μ M AZT for 24 hours

Chromosome probe ^a	Cells with NBs containing FISH signal	Cells with NBs containing no FISH signal
1 (red) + 12 (green)	11	16
2 (red) + 13 (green)	14	17
3 (red) + 14 (green)	9	12
4 (red) + 15 (green)	17	20
5 (red) + 16 (green)	17	19
6 (red) + 17 (green)	13	18
7 (red) + 18 (green)	12	15
8 (red) + 19 (green)	20	18
9 (red) + X (green)	5	6
10 (red) + Y (green)	19	21
11 (red)	13	10

^a A total of 322 NBs were scored for 11 FISH experiments, 150 of those (46.58%) were positive for FISH signals indicating the presence of either one or 2 chromosomes of the pair being analyzed, while 172 (53.42%) show no FISH signal for the pair being hybridized.

positive for chromosome painting probes and NB content is not chromosome specific. DAPI-stained inverted images showed the presence of centromeric signals evidencing localization of whole chromosomes contained inside NBs. Nevertheless, time-lapse photography revealed that NBs: are dynamic structures that can change shape in space and time; can contain centromeres or DNA material; can remain attached to the main nucleus by a strand of DNA of variable size; maintain synchrony with the nucleus at least in terms of chromatin condensation; and, vary in size with ploidy of the cell, rather than the extent of DNA damage (i.e. larger nuclei produce larger NBs).

Live-cell imaging techniques were used to provide insights into the nature and function of NBs. Time-lapse photography experiments showed that NBs could be detached in several pieces from the original nucleus before reaching apoptosis. All the observations done in this study obtained by both FISH analysis and time-lapsed photography help to characterize NBs and clarify the genesis and fate of these structures in the life of the cell. We demonstrated that no specific chromosome is preferred to form NBs but most NBs show chromatin content. It remains to be understood, however, if there is a real function associated with expulsion of amplified DNA.

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