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Letter to the Editor

A cellular machine generating apoptosis-prone aneuploid cells

Dear Editor,

HeLa cells manipulated to express the human immunodeficiency virus (HIV-1)-encoded envelope glycoprotein complex (Env) can fuse with HeLa cells expressing the Env receptor CD4 as well as a chemokine coreceptor (e.g. CXCR4), thus forming syncytia.[1,2] We have used such Env-elicited syncytia to dissect a lethal p53-dependent signal transduction cascade[3–5] relevant to AIDS,[6–8] as well as 'mitotic catastrophe', an apoptosis-like cell death that occurs during the metaphase, after fusion of nonsynchronized cells and inactivation of the cell cycle checkpoint kinase Chk2.[9,10] One of the intrinsic advantages of a model of cell death affecting giant multinuclear cells is the ease with which the subcellular localization of apoptosis-regulatory proteins can be studied.[11]

In a coculture of Cell Tracker® Green-labeled HeLa Env cells and Cell Tracker® Red-labeled HeLa CD4 cells, syncytia can be easily detected as double-strained cells,[12,13] the vast majority of which have a $\geqslant 4n$ DNA content. However, a fraction ($\sim 10\%$) of such *bona fide* syncytia have an $\sim 2n$ DNA content, as determined by simultaneous staining with Hoechst
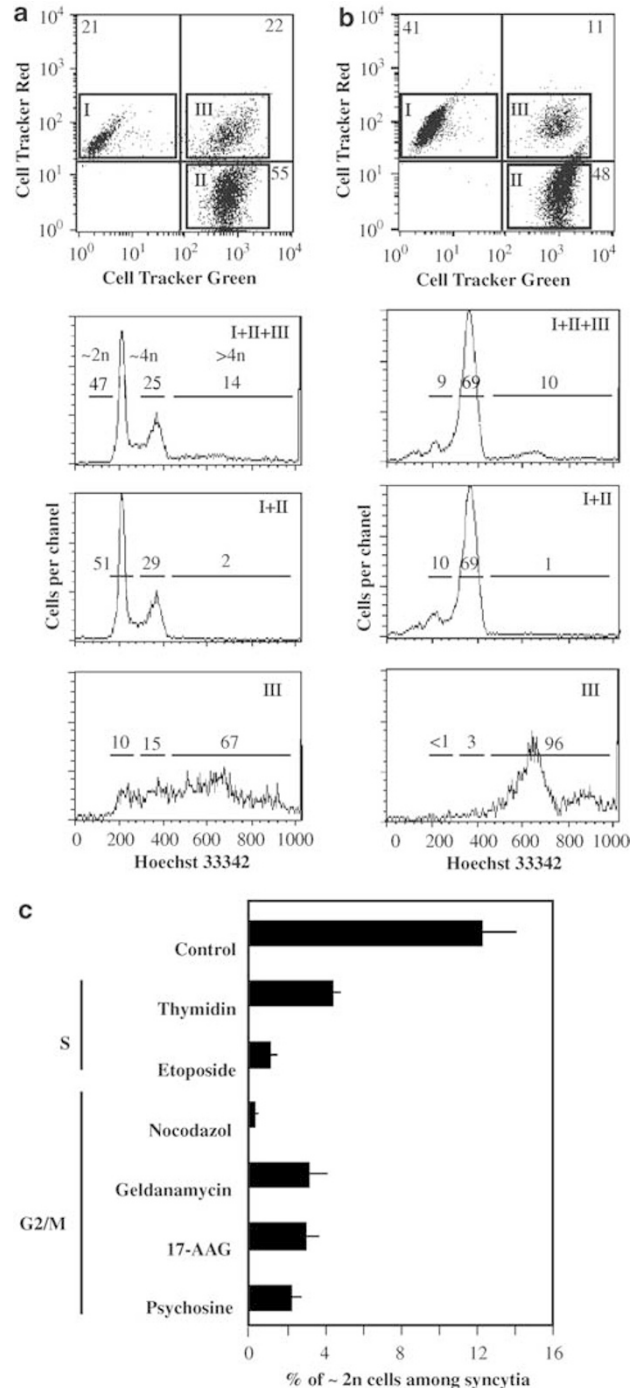

Figure 1 Detection of heterokarya arising from the fusion of HeLa CD4 and HeLa Env. (**a**) Fluorescent-activated cell sorter (FACS) profile of the fusion events and their DNA content. Subconfluent HeLa Env^LAI (NIH AIDS Research program) and HeLa CD4 cells[14] were stained for 45 min with 5-chloromethyl-fluorescein diacetate (Cell Tracker® Green CMFDA, 10 μM, 37°C; Molecular Probes), 5-and-6-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (Cell Tracker® Red CMTMR, 10 μM, 37°C; Molecular Probes), respectively, washed three times, trypsinized and cocultured for 36 h at a density of 1–1.5 × 10³ cells/mm² as described.[1,2] Then, cells were detached from the support by trypsinization, stained with Hoechst 33342 (2 μM, 30 min, 37°C) and analyzed in a FACS Vantage (Becton Dickinson), yielding a net separation of unfused HeLa CD4 cells (in window I), HeLa Env cells (in window II) and double-stained syncytia (in window III), based on the Cyto Tracker® staining profiles. The Hoechst 33342-detectable DNA content was determined for the entire population (windows I + II + III), single cells (I + II) and syncytia (III). 2n refers to the normal DNA content of interphase HeLa cells. Numbers refer to the percentages of cells with an $\sim 2n$, $\sim 4n$ and $> 4n$ DNA content. (**b**) Effect of cell cycle blockade on the DNA content of syncytia. Syncytia were generated and analyzed exactly as in (**a**), with the difference that nocodazole (500 nM, Sigma) was added during the coculture of HeLa Env and HeLa CD4 cells. Note the disappearance of syncytia with an $\sim 2n$ DNA content as compared to (**a**). (**c**) Effect of different cell cycle blockers on the abundance of syncytia with an $\sim 2n$ DNA content. Heterokarya produced as in (**a**), which is without addition of drugs during the coculture period (control), or syncytia produced in the presence of S phase blockers (1 mM thymidine, Sigma; 5 nM etoposide, Sigma)[15] or G2/M blockers (nocodazol as in (**b**), 500 nM geldanamycin; 10 μM 17-(allylamino-)-17-demthoxygeldanamycin[17AAG]; 25 μM psychosin, all from Calbiochem)[16] were analyzed for their DNA content and the percentage of syncytia with a DNA content of $\sim 2n$ was blotted ($n = 3$, means $\pm$ S.E.M.)

33342 (Figure 1a) and a normal cell size, as determined by analyses of the forward and side scatters in the cytofluorometer (see below). In the past, we have neglected this population of cells due to their normal size and mononuclear morphology, which is indistinguishable from that of normal cells (see below). Importantly, when the cell cycle of HeLa Env or HeLa CD4 cocultures was blocked by the microtubule poison nocodazol at the G2/M border ($4n$), most syncytia had an octaploid ($\sim 8n$) DNA content, and the percentage of syncytia with an $\sim 2n$ DNA content dropped to $<1\%$ (Figure 1b). A similar reduction in the frequency of $\sim 2n$ syncytia was observed in response to a variety of cell cycle
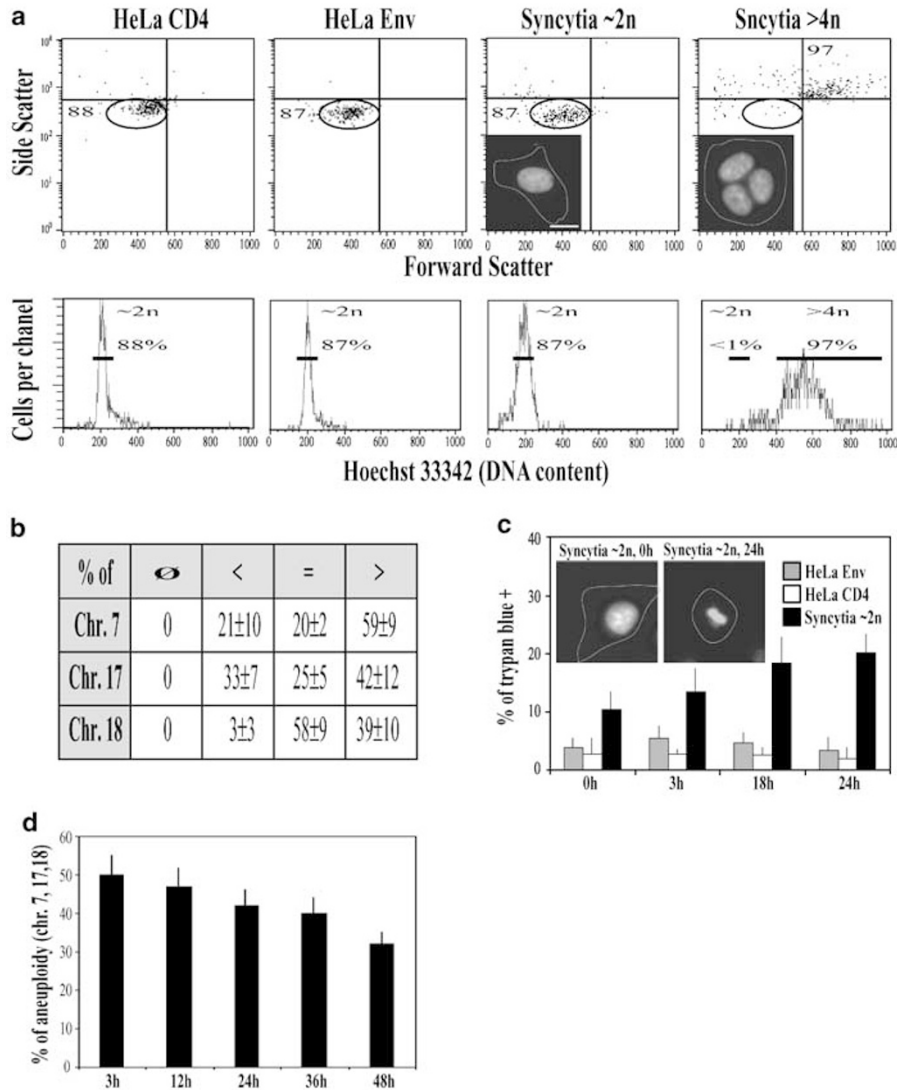

Figure 2 Characterization of aneuploid heterokarya. (**a**) FACS purification of heterokarya with a DNA content of $\sim 2n$. Syncytia were subjected to FACS purification (see Figure 1 for details and for the placement of gates) while simultaneously gating on window I plus $\sim 2n$ (HeLa CD4 cells), window II plus $\sim 2n$ (HeLa Env cells), window III (syncytia) plus $\sim 2n$ or window III plus $>4n$. Immediately after sorting, each of the populations was reanalyzed to confirm the homogeneity of the DNA content and the forward and side scatter characteristics. Inserts demonstrate the nuclear morphology of $\sim 2n$ syncytia (which possess one single nucleus) and that of $>4n$ nuclei (which have several nuclei), as detectable by Hoechst 33342 staining and fluorescence microscopy. The line follows the cellular contour and the bar indicates 10 μm. (**b**) Numeric chromosomal instability in syncytia with an $\sim 2n$ content. After FACS purification (as in **a**), syncytia with a content of $\sim 2n$ DNA were allowed to readhere on polylysine-coated glass slides (O Kindler Gmbh) for 3 h. Fluorescent *in situ* hybridization (FISH) was performed with a mixture of three commercial probes (Abbott/Vysis) that detect the centromeric region of chromosomes 7 (labeled with rhodamine), 17 (labeled with FITC) and 18 (labeled with Aqua). The percentage of cells ($X \pm$ S.D., $n = 3$) exhibiting complete loss (Ø), a reduced ($<$), normal ($=$) or increased ($>$) number of chromosomes was determined by fluorescence microscopic inspection of interphase nuclei in adherent cells, among the aneuploid population, which is $50 \pm 6\%$ of the total amount of cells, by the criterion of this tri-color FISH. Note that HeLa cells usually possess one extra copy of Chromosomes 17 and 18 and that this karyotype was considered as normal. (**c**) Mortality of aneuploid syncytia. FACS-purified single cells and syncytia with an $\sim 2n$ DNA content were cultured for the indicated period and then stained with the vital dye trypan blue to determine their mortality. Inserts show representative examples of Hoechst 33342-stained cells to illustrate that $\sim 2n$ syncytia undergo apoptotic chromatin condensation upon culture. (**d**) Percentage of aneuploid cells persisting in culture. Syncytia with an $\sim 2n$ DNA content were cultured for the indicated period and the frequency of cells exhibiting a loss or a gain of chromosomes was determined as in (**b**), by FISH of chromosomes 7, 17 and 18. Note that a minimum of 3 h of culture was required to allow cells to adhere. Purified syncytia were cultured in the presence of 500 nM of the Env-targeted peptide C34, thus avoiding *de novo* fusion events

blockers that arrest in the S or in the G2/M phases (Figure 1c). This indicates that ∼2n syncytia must result from the division of larger heterokarya.

Syncytia arising from the Env/CD4 interaction are known to undergo a first phase of cytoplasmic fusion (cytogamy), followed by nuclear fusion (karyogamy) after a latency period of several hours.[17] We wondered whether the division of syncytia would occur at the two-nucleus stage (in which case the two daughter cells would be genetically identical to their parental cells) or rather after mixture of the two genomes by karyogamy. In this latter case, multipolar cell division (as demonstrated in Castedo et al.[10]) would result in asymmetric cell divisions with consequent aneuploidy. To address this question, we FACS-purified syncytia (that is Cell Tracker® Red + Green cells) with an ∼2n DNA content, a normal size and one single nucleus, syncytia with a >4n DNA content and several nuclei, as well as nonfused cells with ∼2n DNA content which served as controls (Figure 2a), led them adhere to glass slides for a minimum of 3 h, and subjected them to multifluorescence in situ hybridization (FISH) for the detection of a random set of chromosomes (chromosomes 7, 17 and 18). While >95% of single HeLa Env or HeLa CD4 cells demonstrated an HeLa-specific, unaltered karyotype, up to 50% of the HeLa Env/CD4 syncytia with an ∼2n DNA content demonstrated either a loss or a gain of the three chromosomes investigated (Figure 2b), suggesting that an even higher proportion of cells have undergone numeric aberrations. Of note, we did not observe a single case of nullisomy (loss of all copies of a chromosome), presumably because such a genetic event would be acutely lethal and incompatible with readherence of the cells.

On theoretical grounds, a sudden change in chromosome numbers should provoke undesirable gene dosage effects, and loss of chromosomes should unravel genetic defects resulting from monoallelic deletions or mutations. Thus, we comparatively studied the survival kinetics of FACS-sorted HeLa Env cells, HeLa CD4 cells and ∼2n syncytia (Figure 2c). An increasing proportion of ∼2n syncytia spontaneously underwent cell death in standard culture conditions, while HeLa Env cells and HeLa CD4 cells survived although they had undergone the same staining procedure and the same shear stress during FACS purification. Cell death was detectable by incorporation of the vital dye trypan blue and was accompanied by apoptotic chromatin condensation (insert in Figure 2c). The mortality of ∼2n syncytia was roughly equivalent to that of large >4n syncytia (not shown). If aneuploidy was the factor that determined the spontaneous mortality of ∼2n syncytia, then one would expect that the percentage of aneuploid cells would decline among the population of surviving cells. In accord with this hypothesis, multi-FISH revealed a constant decrease in the fraction of cells exhibiting a gain or loss of chromosomes 7, 17 and 18 (Figure 2d).

In conclusion, we have generated a cellular machine for the rapid generation of a population of cells highly enriched for aneuploid heterokarya, a significant portion of which are apoptosis-prone. This is the formal demonstration of the concept that a numeric chromosomal instability (aneuploidy with monosomies, trisomies or higher-order polysomies) can trigger apoptosis as such.[18] Moreover, this experimental system should allow us to address the question as to whether an inhibition of the apoptotic machinery can increase the frequency of aneuploid cells, at the population level, and/or change the level of aneuploidization that is compatible with survival, at a cell-per-cell basis.

Acknowledgements

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