

Contagious apoptosis facilitated by the HIV-1 envelope: fusion-induced cell-to-cell transmission of a lethal signal

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Summary

Cells expressing the human immunodeficiency virus (HIV-1) envelope glycoprotein complex (Env) can fuse with CD4⁺ cells. When the apoptotic pathway is initiated in Env⁺ cells ('donor cells'), co-culture with a healthy CD4⁺ fusion partner ('acceptor cells') results in apoptosis of the syncytium and thus is 'contagious'. The cell-to-cell transmission of the lethal signal was only observed when the nuclei from donor cells exhibited pre-apoptotic chromatin condensation (PACC), correlating with comet assay-detectable DNA strand breaks, which precede caspase activation, as well as the loss of the mitochondrial transmembrane potential. Transmission of the lethal signal resulted into mitochondrial alterations, and caspase-dependent nuclear pyknosis with chromatinolysis affecting

both the donor and the acceptor nuclei. In the presence of caspase inhibitors, all nuclei of the syncytium formed by fusion of the pre-apoptotic and the healthy cell manifested PACC, exhibited DNA lesions and lost transcriptional activity. Transmission of the lethal signal did not require donor cells to contain a nucleus or mitochondrial DNA, yet was inhibited when two mitochondrion-stabilizing proteins, Bcl-2 or vMIA, were overexpressed. Contagious apoptosis could be induced in primary human T cells, as well as in vivo, in T cells exposed to dying Env-expressing cells. Altogether, these data point to a novel mechanism through which HIV-1 can induce bystander killing.

Key words: Mitochondria, Bcl-2, HIV-1, DNA double strand breaks

Introduction

Viral infection can trigger apoptosis, in particular at late stages of the viral life cycle when viral spreading and/or subversion of the host's immune system can serve the purpose of the infectious pathogen. Accordingly, the human immunodeficiency virus (HIV-1) genome codes for a variety of different pro-apoptotic proteins (Badley et al., 2000; Fauci, 1996; Gougeon, 2003). To reveal the apoptogenic effect of some clinically important HIV-1-encoded proteins such as Vpr, it is necessary to take advantage of so called pseudotyped viruses, that is, genetically modified HIV-1 strains in which the endogenous envelope glycoprotein complex (Env) gene has been replaced by non-apoptogenic Env proteins from other viruses (Lum et al., 2003; Yao et al., 1998). This underscores the notion that Env is, at least in vitro, the principal apoptosis-inducing protein encoded by HIV-1 (Cicala et al., 2000; Laurent Crawford et al., 1993; Lifson et al., 1986; Sodroski et al., 1986).

Undoubtedly, Env can induce cell death through a cornucopia of different mechanisms. The Env glycoprotein precursor protein (gp160) undergoes proteolytic maturation to gp41 (membrane inserted) and gp120 (membrane inserted or shed from the cell surface). Soluble gp120 can stimulate pro-apoptotic signal via an action on chemokine receptors (CXCR4 for lymphotropic Env variants, CCR5 for monocytotropic Env variants) (Cicala et

al., 2000; Roggero et al., 2001; Twu et al., 2002), the p38 mitogen-activated protein kinase pathway (Kaul and Lipton, 1999), pertussis toxin sensitive G proteins (Twu et al., 2002) and/or a rapid cytosolic Ca²⁺ increase (Haughey and Mattson, 2002). The membrane-bound gp120/gp41 complex expressed on the surface of HIV-1-infected cells can induce apoptosis via interaction with uninfected cells expressing the receptor (CD4) and the chemokine co-receptor. Although this interaction can signal for apoptosis via a transient cell-to-cell contact involving a hemifusion-like event (Blanco et al., 2003), in most instances, this interaction induces cellular fusion (cytogamy) (Lifson et al., 1986; Sodroski et al., 1986; Sylwester et al., 1997) followed by nuclear fusion (karyogamy) within the syncytium (Ferri et al., 2000b). This nuclear fusion is the expression of an abortive entry into the mitotic prophase stimulated by the transient activation of the cyclin B-dependent kinase-1 (Cdk1) (Castedo et al., 2002b), accompanied by the permeabilization of the nuclear envelope, the nuclear translocation of mammalian target of rapamycin (mTOR), the mTOR-mediated phosphorylation of p53 on serine 15 (p53S15P) (Castedo et al., 2001), the p53-mediated transcription of pro-apoptotic proteins including Puma and Bax (Castedo et al., 2001; Perfettini et al., 2004), and finally, the Puma-triggered Bax/Bak-mediated mitochondrial membrane permeabilization (MMP) (Ferri et al., 2000a). This last step is accompanied by the activation of Bak (and Bax) on the surface

of mitochondria, associated with a conformational change leading to the exposure of the N terminus of Bak, which becomes immunodetectable (Perfettini et al., 2004). This leads to the release of cytochrome *c* from the mitochondrial intermembrane space, and cytochrome *c* then activates the apoptosome caspase activation complex. Activated, proteolytically mature caspase-3 can then trigger the biochemical cascade culminating in DNA fragmentation and nuclear apoptosis, including advanced chromatin condensation (pyknosis) and formation of nuclear apoptotic bodies (karyorrhexis) (Budijardjo et al., 1999; Roumier et al., 2003; Wang, 2002).

The above mentioned scenario of Env-elicited syncytial apoptosis describes a slow process in which an abortive advancement in cell cycle as well as the sequential activation of at least two transcription factors (NF κ B and p53) are obligate steps on the path to death. Thus, apoptosis affects approximately half of the syncytia generated by fusion of Env- and CD4-transfected HeLa cells after 2 days (Castedo et al., 2001; Castedo et al., 2002b; Ferri et al., 2000a; Perfettini et al., 2004). However, this phenomenon could be significantly accelerated (half life ~6 hours post cytogamy) when one of the two fusion partners was primed for apoptosis using a variety of different inducers, and in this case, apoptosis may be considered as a contagious phenomenon, as shown in the present work. We provide an extensive characterization of this novel form of apoptosis propagated through cell fusion. We demonstrate that contagious apoptosis obeys mechanistic rules that are completely different from 'classical' fusion-induced apoptosis, and we suggest that it may constitute a novel mechanism through which Env-expressing (that is HIV-1-infected) cells destroy CD4⁺ T cells.

Materials and Methods

Cell lines, culture conditions and transfection

HeLa cells stably transfected with the *Env* gene of HIV-1 LAI (HeLa Env) and HeLa cells transfected with CD4 (HeLa CD4) were cultured alone or together (1:1 ratio), at a density of 1.2×10^5 cells/ml (Ferri et al., 2000a). Cells were cultured in DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 10 mM Hepes and 100 U/ml penicillin/streptomycin at 37°C under 5% CO₂. HeLa Env cells were subjected to short-term exposure (3 hours) to staurosporine (STS, 3 μ M; Sigma), actinomycin D (ActD, 5 μ M) and the HIV-1-derived apoptogenic peptide Vpr 52-96 (3 μ M; kindly provided by Prof. B. Roques, University of Paris IV, France) (Jacotot et al., 2001). HeLa Env cells were then washed five times and co-cultured overnight with HeLa CD4 cells. *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.fmk, used at 100 μ M), as well as other inhibitors were added only once, at the beginning of the co-culture [roscovitine 10 μ M; purvalanol 3 μ M; cyclic pifithrin- α 10 μ M (Calbiochem), LY294002 10 μ M, (Calbiochem) rapamycin 1 μ M (Calbiochem), PDTC 50 μ M and the HIV-1 C34 peptide 50 nM (NIH AIDS Research)] (Castedo et al., 2002b). Polyethylene glycol (PEG)-mediated fusion was performed as described previously (Castedo et al., 2001). Transient transfection with 1 μ g of pcDNA3.1 control vector (Neo), with human Bcl-2 (Bcl-2), the cytomegalovirus UL37 exon 1 gene (vMIA; provided by V. Goldmacher) (Goldmacher et al., 1999) and the viral caspase inhibitor p35 from Baculovirus (kindly provided by S. J. Martin) was performed using Lipofectamine 2000TM (1 μ l; Invitrogen) 24 hours before the apoptogenic treatment and co-culture. HeLa Env cells deficient in mitochondrial DNA (ρ^0 cells) were generated by exposure for 8 weeks to ethidium bromide (0.5 mg/ml; Sigma) and maintained in supplemented DMEM with glucose (4.5 mg/ml) and uridine (50 μ g/ml). Jurkat, CEM and U937 cell lines

were cultured in RPMI supplemented with 10% FCS, 1 mM sodium pyruvate and penicillin/streptomycin (100 U/ml). Co-cultures with HeLa Env cells were performed at a ration of 2:1. Peripheral blood lymphocytes (PBL) isolated from healthy donors were maintained in a specific medium (AIM-V; Gibco) and co-cultured overnight with HeLa Env cells. Similarly, T-lymphoblasts were obtained by 5 days-stimulation by PHA (5 μ g/ml) and IL-2 (10 U/ml) and maintained in RPMI supplemented with 10% FCS, 1 mM sodium pyruvate and penicillin/streptomycin (100 U/ml).

Preparation of cytoplasts

HeLa Env cells were enucleated as described previously (Castedo et al., 1996; Chipuk et al., 2003). Briefly, cells were treated in 2 ml of supplemented Dulbecco's modified Eagle's medium (DMEM) containing cytochalasin B (15 μ g/ml; Calbiochem) and DNase I (80 U; Roche). Cell suspension was adjusted to a final concentration of $5-10 \times 10^6$ /ml and incubated at 37°C for 45 minutes before being layered onto a previously prepared discontinuous Ficoll[®] density gradient consisting in 3 ml of 100%, 1 ml of 90% and 3 ml of 55% Ficoll[®] in supplemented DMEM. 2 ml of cytochalasin-treated cell suspension was applied to a pre-equilibrated gradient and centrifuged for 1 hour in a pre-warmed Beckman SW41 rotor at 100,000 *g*. Cytoplasts were collected in the 90% Ficoll[®] layer, washed and resuspended in supplemented DMEM. Enucleation efficiency was determined 3 hours later by microscopic analysis of pre-stained cytoplasts with Hoechst 33342 (2 μ M; Sigma-Aldrich). For apoptosis induction, cytoplasts were subjected to 3 hours exposure to 1 μ M STS or 5 μ M ActD, then washed five times and co-cultured overnight with HeLa CD4 cells.

Immunofluorescence and flow cytometry

Different cells were cultured on coverslips coated with poly-L-lysine (PAA), pre-stained for 45 minutes with 5-chloromethylfluorescein diacetate (CellTracker[®] Green CMFDA, 15 μ M; Molecular Probes), 5- and 6-[[[4-chloromethyl]benzoyl]amino]tetramethylrhodamine (CellTracker[®] Red CMTMR, 15 μ M; Molecular Probes), 7-amino-4-chloromethylcoumarin (CellTracker[®] Blue CMAC, 20 μ M; Molecular Probes) or MitoTracker[®] Green (500 nM; Molecular Probes) and nuclei were stained with 2 μ M Hoechst 33342 (Sigma-Aldrich) before being examined using fluorescence microscopy. TUNEL staining was performed with a detection kit from Roche. The mitochondrial transmembrane potential ($\Delta\Psi_m$) was determined with 40 nM 3,3'-dihexyloxycarbocyanine iodide [DiOC₆(3); Molecular Probes] or tetramethylrhodamine methylester polychlorate (TMRM, 150 nM; Molecular Probes) (Castedo et al., 2002a). Specific antibodies for activated Bak (Bak Ab-1; Oncogene Research Products), cytochrome *c* (BD Pharmingen), activated caspase-3 (Casp-3 a; Cell Signaling Technology), phosphorylated-histone H2AX (Ser139; Upstate Cell Signaling) and phospho-Chk2 (Thr68; Cell Signaling Technology) were used on paraformaldehyde-fixed (4% w:v) cells; all were detected by a goat anti-mouse or goat anti-rabbit IgG-conjugated Alexa[®] Fluor from Molecular Probes (Boya et al., 2003). Cytofluorometric analyses were performed on FACS Vantage (Becton Dickinson) using 40 nM DiOC₆(3) for $\Delta\Psi_m$ quantification (Castedo et al., 2002a; Zamzami et al., 2000), 1 μ g/ml propidium iodide (PI) for determination of cell viability (Zamzami et al., 1995a) and an annexin V conjugated with fluorescein isothiocyanate kit (Bender Medsystems) for the assessment of phosphatidylserine (PS) exposure (Castedo et al., 1996). DNA content was quantified using 20 μ M Hoechst 33342 for 30 minutes at 37°C.

Electron microscopy

Cells were fixed for 1 hour at 4°C in 2.5% glutaraldehyde in phosphate buffer (pH 7.4), washed and fixed again in 2% osmium tetroxide

before embedding in Epon resin. Electron microscopy was performed with a transmission electron microscope (model EM902; Carl Zeiss MicroImaging, Inc.), at 80 kV, on ultrathin sections (80 nm) stained with uranyl acetate and lead citrate.

DNA fragmentation assays

For pulse field gel electrophoresis, DNA was prepared from agarose plugs (2×10^6 cells), followed by electrophoresis in a Bio-Rad Laboratories' CHEF-DR11 (1% agarose, TBE, 200 V, 24 hours, pulse wave 60 seconds, 120° angle). Comet assays were performed (using a kit from Trevigen) to detect double-stranded DNA breaks in HeLa Env cells or overnight in HeLa Env/CD4 syncytia (nonapoptotic adherent or apoptotic cells). Briefly, cells were immobilized in a bed of low melting point agarose, following a gentle cell lysis; cleaved DNA fragments migrated out of the cell under electrophoresis.

Determination of β -galactosidase activity

HeLa Env and HeLa CD4 cells were stably transfected with *Tat* and the *lacZ*, respectively, under the control of the HIV-1 long terminal repeat (LTR) and selected in medium containing 500 mg/ml G418. For the determination of β -galactosidase activity in situ, cells were fixed with a mixture of formaldehyde (0.37%) and glutaraldehyde (0.2%) in PBS solution for 5 minutes, then treated overnight with buffer containing 200 mM potassium ferrocyanide, 1 M $MgCl_2$ and 50 mg/ml X-Gal (Promega), and examined by contrast phase microscopy. Alternatively, co-cultured cells were lysed and assayed for β -galactosidase activity using the Enhanced β -galactosidase Assay kit (CPRG; Gene Therapy Systems) as well as a MRX II microplate reader (Dynex Technologies).

Contagious apoptosis in vivo

In vivo experiments were performed using 10×10^6 CellTracker[®] Red-pre-labeled Jurkat cells injected into the peritoneal cavity of athymic mice (*nu/nu*). Eight hours later, 25×10^6 HeLa Env or HeLa CD4 cells were injected and peritoneal cells were recovered after overnight incubation. Jurkat cells were analyzed by flow cytometric determination of $\Delta\Psi_m$ loss and phosphatidylserine exposure (Castedo et al., 2002a).

Results and Discussion

Contagious apoptosis induced by syncytium formation

HeLa cells exposed to a short pulse (1-3 hours) of staurosporine (STS) showed partial chromatin condensation with irregular nuclear contours when they were still adherent (Fig. 1A, Env, 3h STS). This 'pre-apoptotic chromatin condensation' (PACC) was not inhibited by addition of the broad-spectrum caspase inhibitor Z-VAD.fmk. However, PACC⁺ (that is STS-pulsed) cells were primed to undergo complete apoptosis. Even after thorough washing, the cells advanced to full-blown nuclear pyknosis during overnight culture, and this advancement to nuclear apoptosis was inhibited by Z-VAD.fmk (Fig. 1C). Healthy CD4⁺ cells fused with PACC⁺ cells expressing the HIV-1 Envelope (Env) upon co-culture. The efficacy of fusion of PACC⁺ HeLa Env cells incubated with normal CD4⁺ cells (or vice versa) was as high as that among untreated control cells, although the resulting syncytia were smaller than control syncytia (see below, Fig. 2B). In contrast, fully apoptotic cells failed to form heterokarya (not shown). Within the syncytia formed by co-incubating primed (PACC⁺) HeLa Env cells with normal HeLa CD4 cells (or vice versa, not shown), all nuclei entered apoptosis after

overnight culture (Fig. 1A-D), indicating that the apoptotic signal was transmitted from 'donor cells' (PACC⁺) to 'acceptor cells'. This 'contagious' advancement to apoptosis was rather efficient (>85% of syncytia) and, in all cases, all nuclei contained within syncytia entered apoptosis in a synchronized fashion (Fig. 1A,B). In contrast, in the presence of Z-VAD.fmk, this advancement to apoptosis was inhibited and nuclei stayed PACC⁺. The transmission of the apoptotic signal did not depend on the primary apoptosis inducer, given that induction of PACC in one of the two fusion partners with STS, actinomycin D, and the HIV-1-derived apoptogenic peptide Vpr52-96 yielded a similar 'contagion' of apoptosis (Fig. 1D). A synthetic peptide (C34), derived from the C helix of the HIV type 1 (HIV-1) gp41 envelope protein (Chan et al., 1998), completely abolished the fusion between HeLa Env and HeLa CD4 cells and blocked contagious apoptosis (not shown). Similarly, coculture of STS-pretreated HeLa CD4 cells (labeled with CellTracker Green[®]) and untreated HeLa CD4 cells (labeled with CellTracker Red[®]), did not result into fusion and did not cause the death of the untreated population. However, if fusion between STS-pretreated and untreated HeLa CD4 cells was enforced by the fusogenic compound polyethylene glycol, all nuclei contained within two-colored heterokarya exhibited an apoptotic chromatin condensation (Fig. 1E), as this was observed in the heterotopic interaction between STS-primed HeLa Env cells and untreated HeLa CD4 cells (Fig. 1A). Thus cell fusion, independently of the Env/CD4 interaction itself, is required for contagious apoptosis. Induction of full-blown contagious apoptosis correlated with caspase-3 activation, as detectable with an antibody recognizing the large subunit of proteolytically mature caspase-3 (Fig. 1F). In the presence of Z-VAD.fmk, when caspase activation was fully inhibited, mitochondrial changes associated with apoptosis still occurred, as indicated by the exposure of the N terminus of Bak (detectable with a conformation-specific antibody), the loss of the mitochondrial transmembrane potential [$\Delta\Psi_m$, detectable with the $\Delta\Psi_m$ -sensitive fluorochrome DiOC₆(3)] and the mitochondrial release of cytochrome *c* (detectable by immunofluorescence) (Fig. 1F). Altogether these data suggest that contagious apoptosis is propagated through a pathway that involves mitochondrion-dependent caspase activation.

Mechanistic differences between spontaneous and contagious syncytial apoptosis

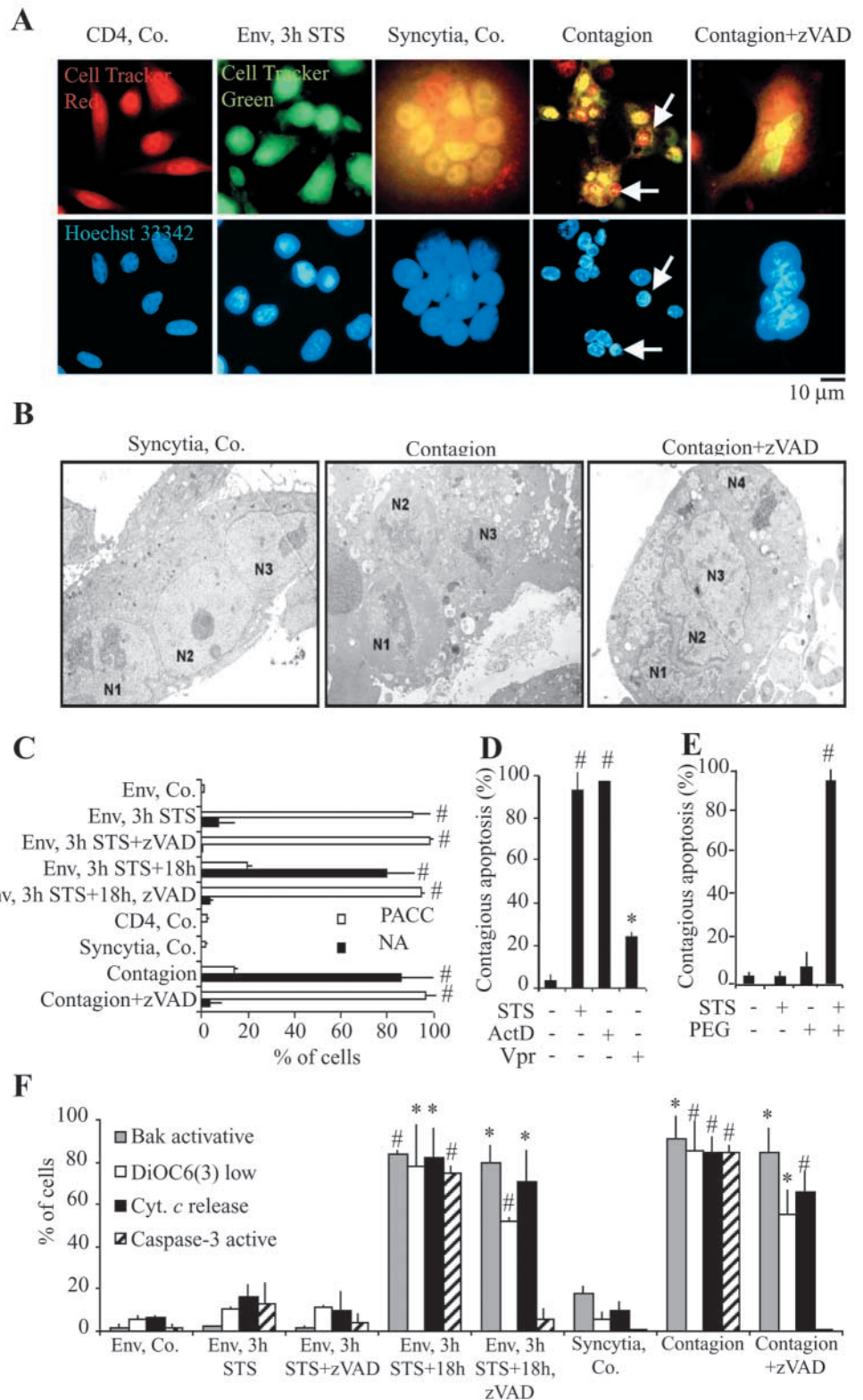
Syncytia formed by co-culture of normal HeLa Env and HeLa CD4 cells undergo apoptosis after a latency phase of more than 24 hours. In contrast, contagious apoptosis occurred much more rapidly, attaining approximately 50% of heterokarya within 4 hours (Fig. 2A). While spontaneously arising syncytia typically contained >10 nuclei, syncytia formed by co-incubation of STS-pretreated and control cells had mostly less than five nuclei (Fig. 2B). As detailed in the Introduction, syncytia arising from interactions between normal HeLa Env and HeLa CD4 cells spontaneously die through a complex signaling pathway that relies on the step-wise activation of a range of cellular regulators including cyclin B-dependent kinase-1 (Cdk1), mammalian target of rapamycin (mTOR), and p53 (Castedo et al., 2001; Castedo et al., 2002b; Perfettini et al., 2004). Pharmacological inhibitors of Cdk1 (roscovitine,

Fig. 1. Contagious apoptosis induced by syncytium formation.

(A) Representative immunofluorescent images of contagious apoptosis. HeLa cells were left untreated (Co.) or were treated with a 3-hour pulse of STS (3 μ M), leading to the acquisition of typical pre-apoptotic chromatin condensation (PACC). Syncytia were formed by overnight co-culture of untreated HeLa CD4 and HeLa Env cells (Syncytia, Co), or were obtained by co-culture of untreated HeLa CD4 cells and STS-pulsed HeLa Env cells, which were washed five times, either in the absence (Contagion) or in the presence (Contagion+zVAD) of the pan-caspase inhibitor Z-VAD.fmk (100 μ M). HeLa Env and CD4 were pre-stained with CellTracker[®] Green and CellTracker[®] Red, respectively, and fixed before nuclear staining with Hoechst 33342. Note that, in contagious apoptosis, nuclei from previously untreated HeLa CD4 cells (red) show extensive chromatin condensation (arrows).

(B) Transmission electron microscopy of control syncytia or syncytia generated with PACC⁺ Env cells. Note that contagious apoptosis is characterized by full-blown nuclear apoptosis with pyknosis of all nuclei (in the absence of Z-VAD). In the presence of Z-VAD, however, all nuclei showed PACC (partial chromatin condensation, irregular contours and rippled nuclear envelopes). N1, 2, 3 etc. indicate individual nuclei in each syncytium. (C) Quantification of PACC and nuclear apoptosis (NA), as determined by Hoechst 33342 staining, in HeLa Env induced by short-term exposure to STS alone (3h, STS), short-term STS exposure followed by washing and overnight culture (3h, STS+18h), control syncytia or contagion (induced as in A). Significance was calculated with the paired Student's *t*-test with respect to untreated controls. #*P*<0.005.

(D) Independence of contagious apoptosis from the initial inducer. HeLa Env cells were primed to PACC by a 3-hour treatment with STS (3 μ M), actinomycin D (ActD, 5 μ M) or the HIV-1-derived apoptogenic peptide Vpr 52-96 (3 μ M). Nuclear apoptosis mean \pm s.d., *n*=3) affecting co-cultured HeLa CD4 cells was determined as above. (E) Contagious apoptosis induced by polyethylene glycol-mediated fusion. HeLa CD4 cells were left untreated or were pre-treated with STS (3 μ M, for 3 hours followed by five washes), stained with CellTracker[®] Green and then co-cultured with CellTracker[®] Red-prestained cells, either without or after PEG-mediated fusion. Then, the percentage of CellTracker[®] Red-positive nuclei exhibiting chromatin condensation was measured as in A after 18 hours of co-culture. Statistical significance in D and E, #*P*<0.005, **P*<0.05. (F) Signs of MMP in contagious apoptosis. Individual cells or syncytia generated in D were subjected to $\Delta\Psi_m$ quantitation with DiOC₆(3), or were fixed, permeabilized and stained for the detection of activated Bak, the redistribution of cytochrome *c*, or the activation of caspase-3, as described in Materials and Methods. Significance was calculated with the paired Student's *t*-test with respect to untreated controls. **P*<0.05; #*P*<0.005.



purvalanol), mTOR (rapamycin, LY294002), or p53 (pifithrin α , PDTC), however, had no effect on contagious apoptosis (Fig. 2C), indicating that this type of apoptosis is not just an accelerated version of spontaneous syncytial apoptosis and rather obeys different principles.

Nuclear manifestations of contagious apoptosis are only partially caspase-dependent

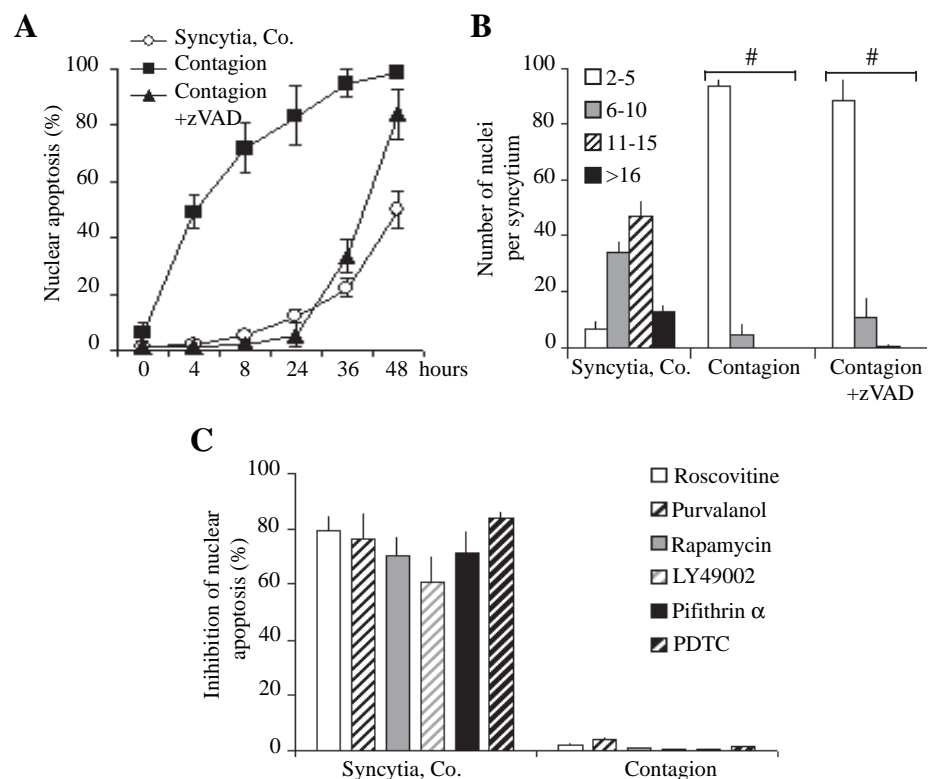
When healthy HeLa Env cells expressing the Tat protein were fused with HeLa CD4 cells expressing a Tat-transactivatable β -galactosidase reporter gene placed under the control of the LTR promoter, the resulting syncytia produced β -galactosidase, as detectable by cytohistochemical methods *in situ* (Fig. 3A) or by enzymatic methods (Fig. 3B). No such β -galactosidase production was observed when either of the two fusion partners was PACC⁺, and all syncytial nuclei evolved to nuclear apoptosis. Addition of Z-VAD.fmk, which prevented the development of full-blown apoptosis, did not restore the expression of β -galactosidase (Fig. 3A,B), pointing to a caspase-independent blockade of nuclear function. PACC⁺ (STS-pretreated) Env cells showed partial chromatinolysis, as determined by comet assay (Fig. 4A), yet lacked large-scale DNA fragmentation detectable by pulse-field electrophoresis (Fig. 4B), and were TUNEL negative (Fig. 5A). In contrast, when these cells underwent the transition to full-blown apoptosis, either as individual cells or after fusion with normal cells, their DNA underwent fragmentation to ~50 kbp pieces (Fig. 4b), and was TUNEL positive (Fig. 5A). Z-VAD.fmk prevented the advancement to nuclear apoptosis (Fig. 5A, see also Fig. 1C), correlating with a complete inhibition of ~50 kbp fragmentation (Fig. 4B) and a suppression of TUNEL positivity, both in single cells and in contagious apoptosis (Fig.

5A). Importantly, Z-VAD.fmk, however, did not prevent the 'contagion' of PACC from HeLa Env to HeLa CD4 nuclei occurring within syncytia, as detectable by chromatin staining (Fig. 1A) or electron microscopy (Fig. 1B). Indeed, when STS-primed, PACC⁺ HeLa Env cells were fused with untreated HeLa CD4 cells in the presence of Z-VAD.fmk, all the nuclei within the resulting syncytia exhibited PACC. Moreover, Z-VAD.fmk did not reduce the scores of comet assays (Fig. 4A). The comet assay measures the occurrence of DNA double strand breaks, which lead to so-called 'DNA foci', that is the accumulation of DNA repair proteins and cell cycle regulators in the proximity of DNA lesions (Banath and Olive, 2003; Castedo et al., 2004; Celeste et al., 2003). Accordingly, PACC correlated with the phosphorylation of histone H2AX (on serine 139) and that of Chk2 (on threonine 68), which accumulated in discrete nuclear speckles, detectable with phospho-neoepitope-specific antibodies (Fig. 5B). When contagion occurred within syncytia in the presence of Z-VAD.fmk, all nuclei exhibited such foci. To verify that the DNA foci were also present in nuclei from the 'recipient' cells, HeLa CD4 cells were pre-stained with CellTracker Red[®] and the fate of the nuclei within syncytia was followed. Phosphorylation of H2AX and Chk2 was also apparent in these 'recipient' nuclei. In summary, PACC involves caspase resistance, comet assay positivity, transcriptional silencing and DNA foci. PACC can be transmitted from donor to recipient nuclei, in a caspase-independent fashion.

Nuclei and mitochondrial DNA are dispensable for contagious apoptosis, which however is suppressed by Bcl-2

To determine which cellular component actually transmits

Fig. 2. Mechanistic differences between contagious apoptosis and spontaneous syncytial cell death. (A) Kinetic analysis of nuclear condensation observed in control syncytia or contagious apoptosis. Cells were stained as in Fig. 1A and nuclear apoptosis was measured as immunofluorescence at the indicated times after cellular fusion. (B) Effect of contagion on the syncytial size. HeLa CD4 cells were cultured overnight with healthy-(Syncytia, Co.) or PACC⁺-HeLa Env cells in the absence (Contagion) or presence of zVAD.fmk (Contagion+zVAD). Cells were stained as in Fig. 1A and the number of nuclei per syncytium was determined (mean \pm s.d., $n=4$). # $P<0.005$. (C) Differential effect of inhibitors on spontaneous syncytial death and contagious apoptosis. Cells were primed as in B and inhibitors were added prior to co-culture (roscovitine 10 μ M, purvalanol 3 μ M, rapamycin 1 μ M, LY294002 10 μ M, pifithrin- α 10 μ M and PDTC 50 μ M). Nuclear pyknosis was measured as in A after 72 hours of co-culture of control syncytia, and overnight co-culture for contagious apoptosis. Results are expressed as inhibition of nuclear apoptosis as compared to untreated control cultures (mean \pm s.d., $n=3$) # $P<0.005$.



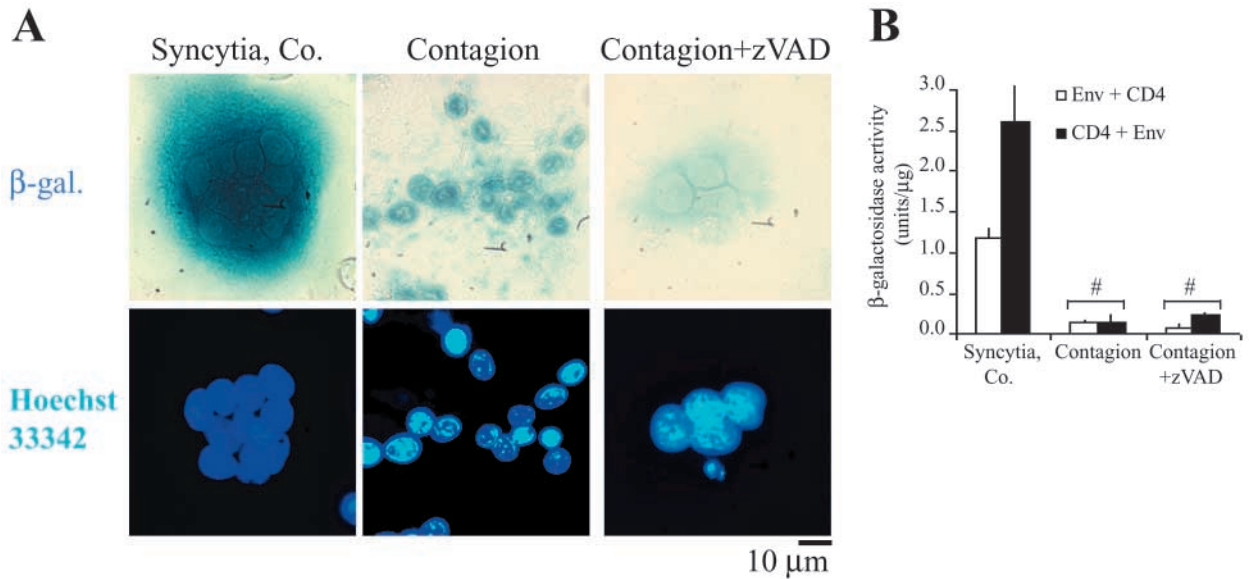


Fig. 3. Transcriptional silencing in contagious apoptosis, as determined by trans-complementation between a transactivator (Tat) and a promoter (LTR upstream of the β -galactosidase gene) in syncytia. HeLa Env cells expressing Tat and HeLa CD4 cells transfected with the β -galactosidase gene under the LTR promoter were co-cultured, followed by an in situ histochemical detection of β -Gal using X-Gal as a substrate (A) or by enzymatic methods applied to cell extracts (B). Results (mean \pm s.d. of triplicate samples) are representative of three independent experiments. # P <0.005.

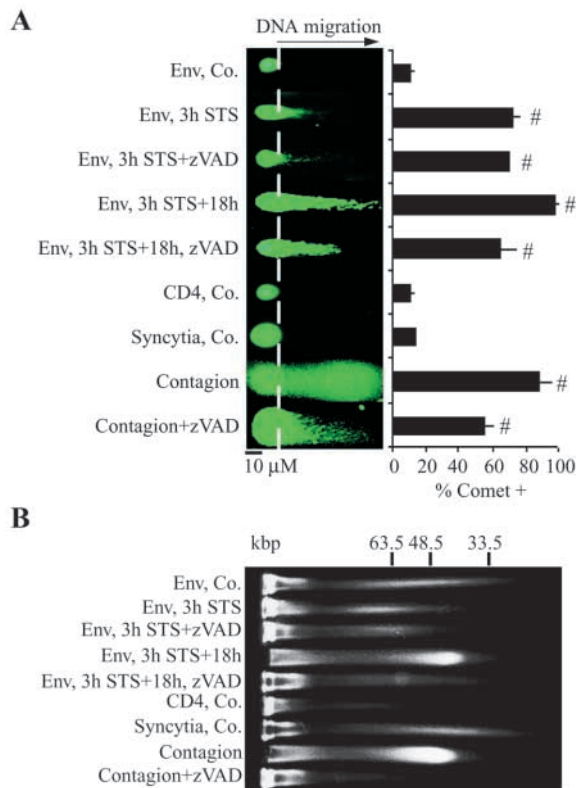


Fig. 4. DNA degradation and double strand breaks are associated with contagious apoptosis. (A) After the indicated regimes of STS/Z-VAD.fmk treatment, the frequency of comet assay-positive cells showing DNA double stranded breaks, was determined by microscopic observation (mean \pm s.d., $n=3$; # P <0.005) in a minimum of 200 cells for each point. (B). Nuclear DNA from samples analyzed in A was subjected to pulse-field gel electrophoresis. Results are representative of three independent experiments.

contagious apoptosis, HeLa Env cells were enucleated and the resulting cytoplasts were then treated with a sublethal STS dose, washed extensively, and fused with healthy HeLa CD4 cells. The nuclei of these latter cells readily advanced to PACC (in the presence of Z-VAD.fmk) or full-blown apoptosis (in the absence of Z-VAD.fmk) (Fig. 6A). Similar results were obtained when cytoplasts were pretreated with either STS or ActD. (Fig. 6B). Thus, the stimulus responsible for the transmission of the lethal signal is generated in cytoplasts and, as a consequence, must be cytoplasmic (non-nuclear). HeLa Env cells lacking mitochondrial DNA (ρ^0 cells) driven into PACC also stimulated the advancement of HeLa CD4 nuclei to nuclear apoptosis (Fig. 6C,D), indicating that neither nuclear nor mitochondrial DNA were required for the transmission of the lethal signal. In a further series of experiments, we introduced the prominent apoptosis inhibitor Bcl-2 into either donor (STS-pretreated HeLa Env) or acceptor (non-treated HeLa CD4) cells, prior to cell fusion. Although Bcl-2 failed to prevent the occurrence of PACC in Env cells treated with STS, such cells returned to a normal nuclear morphology after overnight culture (Fig. 7), suggesting that PACC is actually a reversible phenomenon. If present in donor or acceptor cells, Bcl-2 also abolished the transmission of the apoptotic signal to the acceptor nuclei, showing that both donor and acceptor nuclei were morphologically normal 18 hours after fusion. Very similar results were found when Bcl-2 was replaced by the cytomegalovirus-encoded vMIA, which also acts on mitochondria to inhibit apoptosis (Goldmacher et al., 1999; Poncet et al., 2004). The behavior of Bcl-2 and vMIA was in marked contrast to that of the baculovirus caspase inhibitor p35, which did not reverse the transmission of PACC (Fig. 7). Thus, a Bcl-2/vMIA-inhibited checkpoint determines the transmission of the apoptotic signal in this particular experimental system.

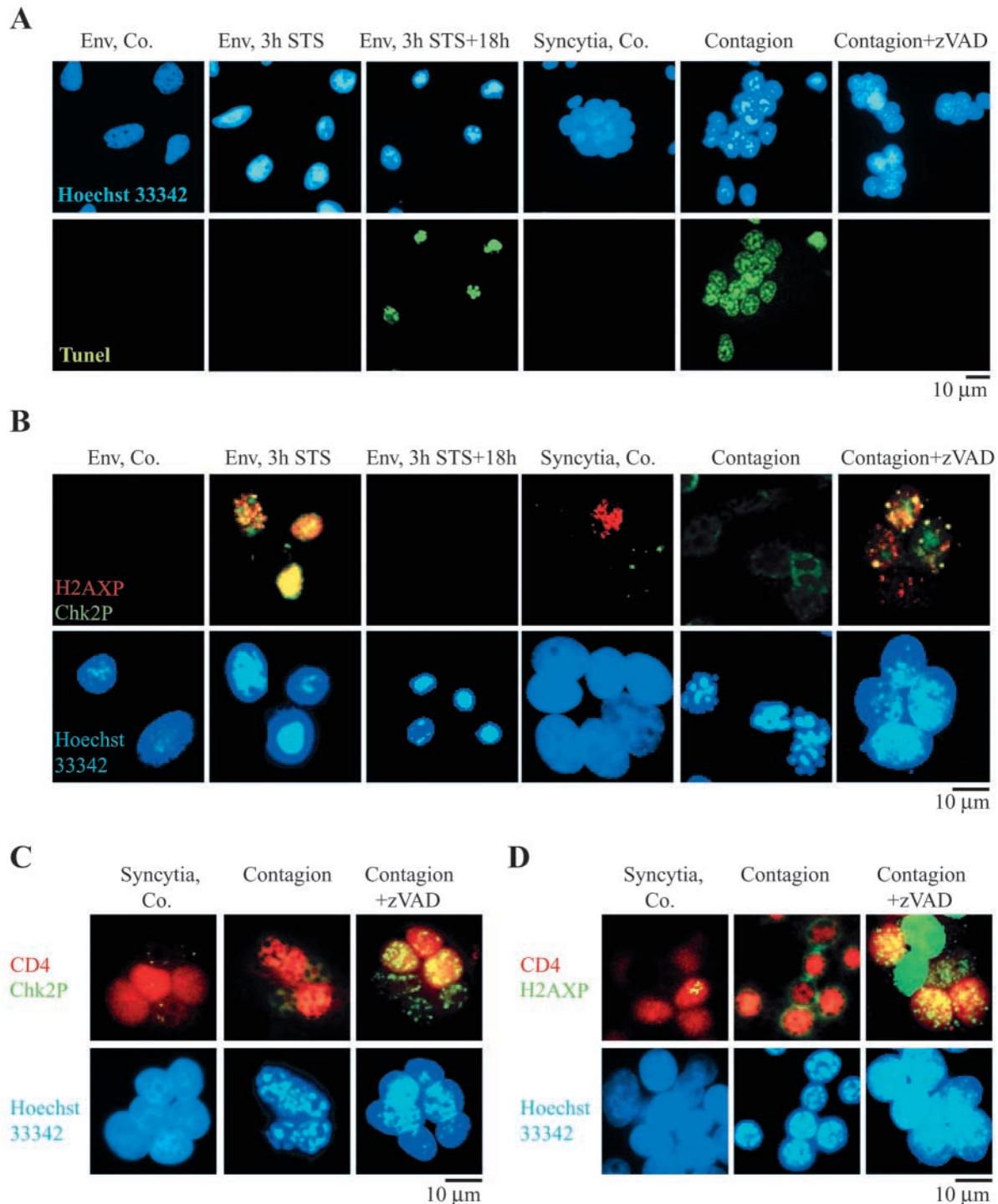


Fig. 5. Contagious apoptosis is associated with caspase-resistant foci of DNA lesions. (A) DNA fragmentation occurring during contagious apoptosis. Cells and syncytia generated as in Fig. 1 were subjected to simultaneous Hoechst 33342 and TUNEL staining. Note that only cells with full-blown chromatin condensation (but not those with PACC) are TUNEL-positive. (B) Representative images of cells stained with antibodies specifically recognizing phosphorylated H2AX (Ser 139, H2AXP) or Chk2 (Thr68, Chk2P) and counterstained with Hoechst 33342. (C,D) Transmission of H2AX and Chk2 phosphorylation to nuclei from HeLa CD4 cells. Prior to co-culture, healthy HeLa CD4 were stained with CellTracker[®] Red. After fusion with either untreated HeLa Env cells (Co.), STS-pulsed HeLa Env cells in the absence (Contagion), or presence of Z-VAD.fmk, the resulting syncytia were fixed, permeabilized and stained for the detection of Chk2P (C) or H2AXP (D), revealed with an anti-IgG Alexa[®] Fluor Green conjugate. Nuclei were counterstained with Hoechst 33342. This experiment was repeated twice, with similar results.

Contagious apoptosis as a mechanism of Env-mediated bystander killing

Two T-lymphoid cell lines (Jurkat and CEM) fuse with HeLa Env cells, while U937 do not fuse with HeLa Env and instead undergo transient interactions that involve transfer of plasma membrane lipids through a hemifusion-like process (Blanco et

al., 2003). STS-pulsed HeLa Env cells with PACC did induce killing of Jurkat or CEM cells, yet had no major apoptosis-inducing effect on U737 cells, which is in accord with the notion that fusion (rather than hemifusion) is required for cell induction (Fig. 8A). CD4⁺ lymphocytes (either resting or PHA/IL-2-activated lymphoblasts) from healthy donors could

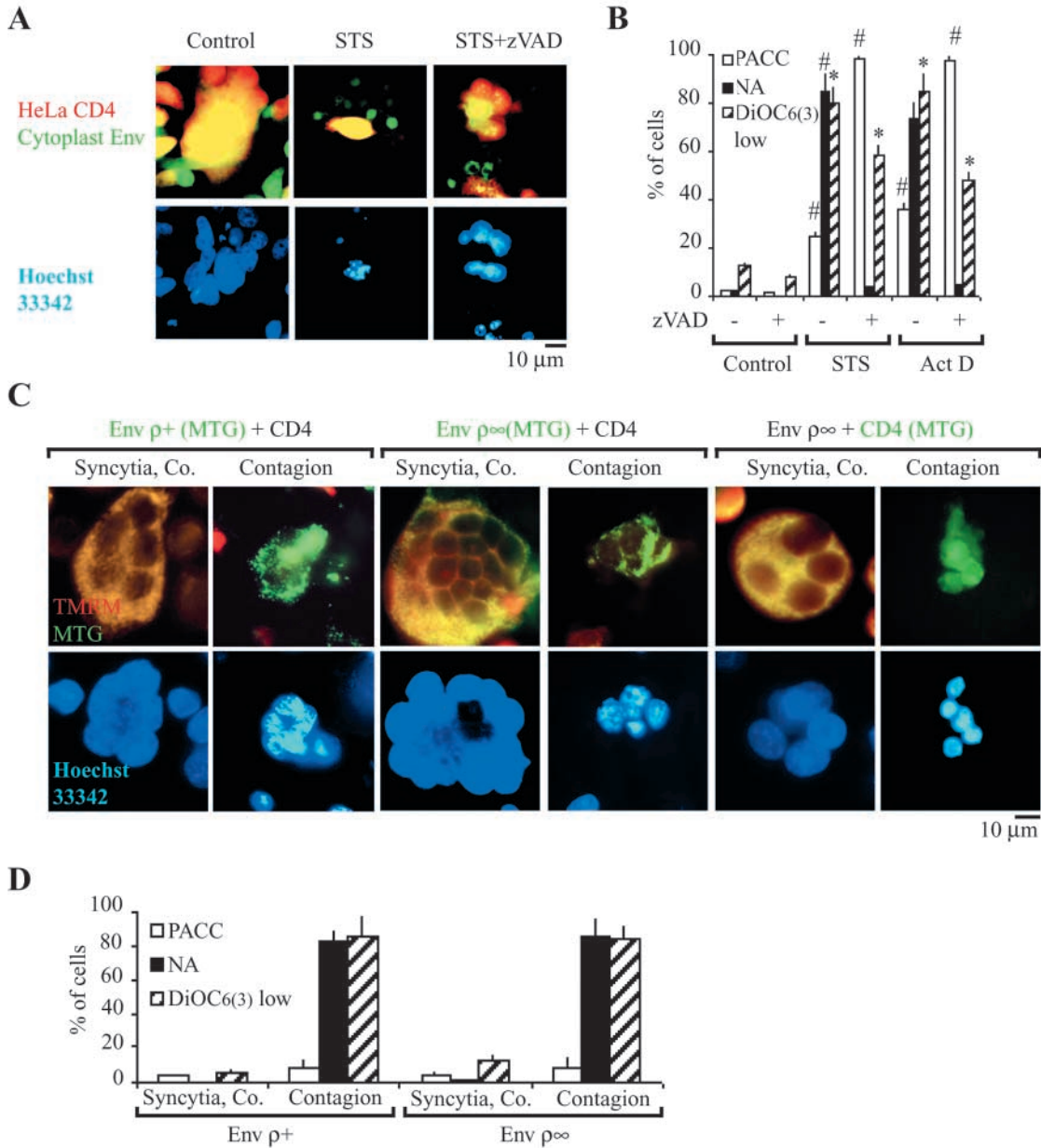


Fig. 6. Nuclei and mitochondrial DNA are dispensable for contagious apoptosis. (A) Representative immunofluorescence of contagious apoptosis triggered by enucleated HeLa Env cells. HeLa Env cytoplasts were pretreated or not with 1 μM STS, washed, and co-cultured overnight with healthy HeLa CD4. HeLa Env cytoplasts and normal HeLa CD4 were pre-stained with CellTracker[®] Green and CellTracker[®] Red, respectively, and syncytia were stained with the chromatin-specific dye Hoechst 33342. (B) Quantitative analysis of the data obtained as in A. HeLa Env cytoplasts were pretreated or not with STS (1 μM) or ActD (5 μM) and co-cultured with healthy CD4 cells in the presence or absence of zVAD.fmk. The phenotypic characteristics (mean ± s.d., n=3; *P<0.05; #P<0.005) of syncytia with PACC, i.e. nuclear apoptosis (NA) or low mitochondrial transmembrane potential ($\Delta\Psi_m$) were determined by staining with Hoechst 33342 or DiOC₆(3), respectively. (C) Representative images of syncytia formed by fusion of normal (ρ^+)HeLa Env cells mtDNA-depleted (ρ^-). HeLa Env or CD4 were pre-stained with MitoTracker[®] Green (MTG), as indicated, and $\Delta\Psi_m$ was assessed by TMRM staining. The yellow color indicates an overlap between the green (MTG) and red (TMRM) staining, observable in control syncytia, but not in contagious apoptosis in which the $\Delta\Psi_m$ is lost. (D) Quantitative analysis of data obtained in C. Nuclear apoptosis and loss of $\Delta\Psi_m$ were determined as previously. Values are means of two experiments ± s.d.

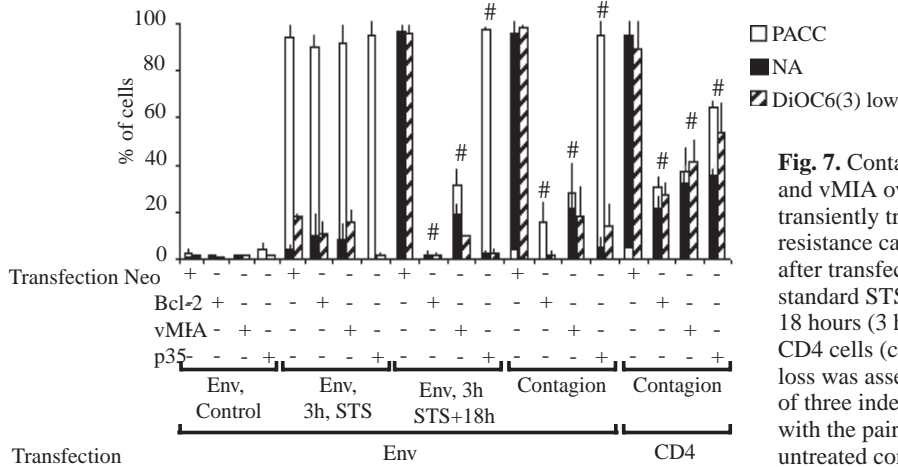
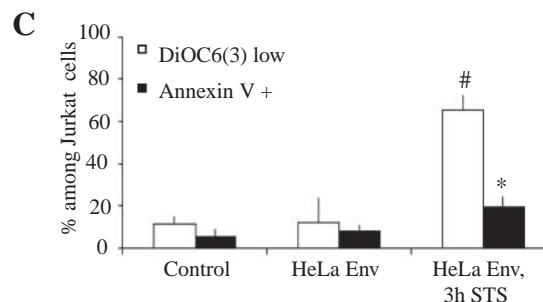
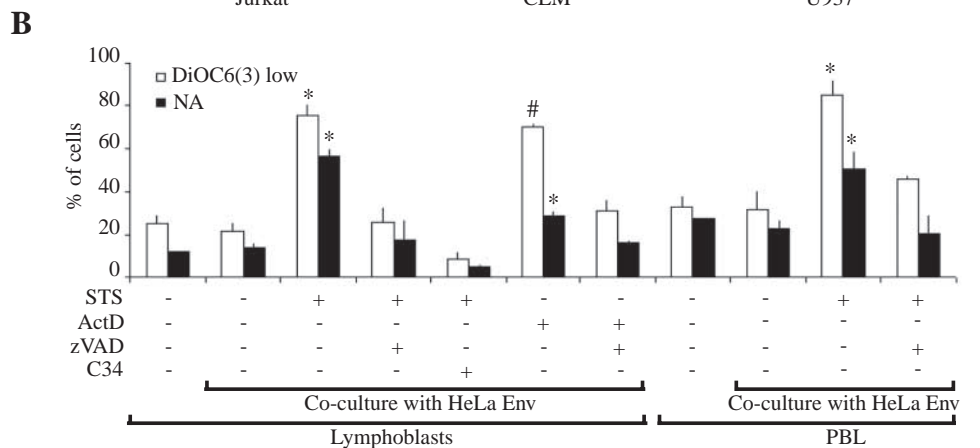
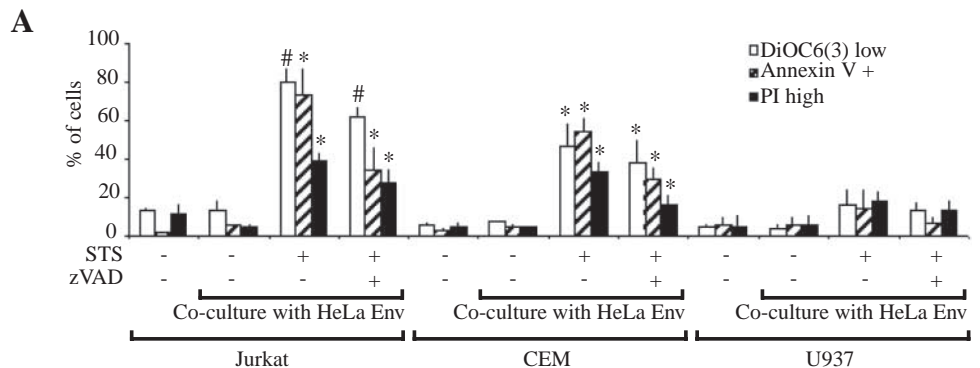


Fig. 7. Contagious nuclear apoptosis is suppressed by Bcl-2 and vMIA overexpression. Either HeLa Env or CD4 were transiently transfected with a vector expressing the neomycin resistance cassette only (Neo), Bcl-2, vMIA or p35. 24 hours after transfection, the HeLa Env cells were subjected to the standard STS pulse (3 h, STS), washed and cultured alone for 18 hours (3 h STS + 18 h) or co-cultured with untreated HeLa CD4 cells (contagion). The frequency of PACC, NA and $\Delta\Psi_m$ loss was assessed as in Fig. 6B, and results are given as means of three independent experiments. *P* values were calculated with the paired Student's *t*-test with respect to Neo controls untreated controls, #*P*<0.005.

Fig. 8. CD4⁺ T cells undergo contagious apoptosis both in vitro and in vivo.

(A) Contagious, fusion-induced apoptosis occurring in T-lymphoid cell lines in vitro. Jurkat, CEM and U937 cell lines were pre-stained with CellTracker[®] Red or CellTracker[®] Green and co-cultured overnight with HeLa Env at a ration of 2:1. Env cells were pretreated for 3 hours in presence or absence of STS (3 μ M) or ActD (5 μ M). Loss of $\Delta\Psi_m$, PS exposure and plasma membrane permeability were assessed by cytofluorometric analysis, while gating on the CellTracker[®] pre-labeled population. Data are means of three experiments \pm s.d. Significance was calculated with the paired Student's *t*-test with respect to untreated controls. **P*<0.05; #*P*<0.005. (B) HeLa Env cells triggered contagious apoptosis of primary human T cells. Freshly drawn peripheral blood lymphocytes (PBL) or T lymphoblasts (obtained by 5 days stimulation with PHA plus IL-2) were pre-stained with CellTracker[®] Red or CellTracker[®] Green, co-cultured overnight with CellTracker[®] Blue-pre-labeled HeLa Env cells and double-stained-positive cells were subjected to FACS analysis for $\Delta\Psi_m$ loss and nuclear apoptosis. Prior to co-culture HeLa Env were given a 3-hour incubation with STS or ActD (mean \pm s.d., *n*=2, **P*<0.05; #*P*<0.005), and the co-culture was performed in the presence or absence of 100 μ M zVAD.fmk or 50 nM fusion inhibitor C34 peptide, as indicated. (C) HeLa Env cells trigger contagious apoptosis of Jurkat cells in vivo. CellTracker[®] Red-pre-labeled Jurkat cells were injected into the peritoneal cavity of athymic mice (*nu/nu*). HeLa Env were left untreated or pulsed with STS (3 μ M), washed five times, and injected 8 hours after the Jurkat cells. After overnight incubation, peritoneal cells were recovered, and Jurkat cells (CellTracker[®] Red⁺) were analyzed cytofluorometrically to determine the $\Delta\Psi_m$ dissipation and PS exposure. Results are means for a minimum of five animals per group. **P*<0.05; #*P*<0.005.



fuse with healthy Env-expressing cells, yet failed to undergo nuclear apoptosis within a 18-hour culture period. However, when Env cells were driven into PACC (either with STS or with ActD), they readily induced the destruction of CD4⁺ lymphocytes and lymphoblasts (Fig. 8B). This effect was abolished by the use of the fusion inhibitor C34 (Fig. 8B) and was not observed when the lymphocytes were incubated with PACC⁺ HeLa CD4 cells (not shown). To demonstrate that contagious apoptosis can occur *in vivo*, CellTracker Red[®]-pre-labeled Jurkat cells were injected into the peritoneal cavity of athymic (*nu/nu*) mice. The instillation of STS-primed (but not untreated) HeLa Env cells (but not that of HeLa CD4 cells) induced a loss of $\Delta\Psi_m$ in a considerable number of the Jurkat cells (Fig. 8C), which is a sign of imminent apoptosis (Zamzami et al., 1995b). In conclusion, CD4⁺ T cells can undergo contagious apoptosis when exposed to dying Env-expressing cells, both *in vitro* and *in vivo*.

Concluding remarks

When HIV-1 Env-expressing cells are exposed to a short-term stress (STS, ActD, Vpr) that does not induce acute apoptosis, yet commits cells to undergo apoptosis later (even after withdrawal of the inducer by extensive washing), such cells can transmit the induction of apoptosis to their healthy CD4⁺ fusion partner. We have termed this fusion-mediated transmission of an apoptotic signal as 'contagious apoptosis' and characterized it to some detail. As an expression of commitment to apoptosis, the contagious fusion partner can show PACC with DNA double strand breaks, yet lack obvious signs of apoptosis, such as caspase-3 activation and mitochondrial changes indicative of MMP (Bak activation, cytochrome *c* release, $\Delta\Psi_m$ dissipation). Within hours, contagious apoptosis leads to the synchronized nuclear pyknosis in both the 'donor' cell (that is the stressed, apoptosis prone cell) and in the 'recipient' cell (that is the healthy, untreated fusion partner). This transmission of nuclear pyknosis correlates with the activation of caspase-3 and is fully inhibited by the broad-spectrum caspase inhibitor Z-VAD.fmk or the baculovirus-derived caspase inhibitor p35. Contagious apoptosis is accompanied by MMP, which, however, occurs independently from Z-VAD.fmk-inhibitable caspases (Fig. 1). Contagious apoptosis does not depend on cell cycle advancement or the action of the pro-apoptotic transcription factor p53 (Fig. 2), and instead correlates with an (caspase-independent) arrest of transcription (Fig. 3).

While massive chromatinolysis (measured by agarose gel electrophoresis in Fig. 4B and TUNEL staining in Fig. 5A) occurring in contagious apoptosis can be suppressed by Z-VAD.fmk, there are other, more subtle nuclear changes that occur in a caspase-independent fashion. Thus, STS-pulsed 'donor' cells have DNA double strand breaks (as determined by comet assay in Fig. 4A or by assessing H2AXP and Chk2P foci) and transmit such DNA double strand breaks to 'recipient' nuclei, even in the presence of Z-VAD.fmk (Fig. 5B-D). The morphological manifestation of DNA double strand breaks is PACC, that is a sub-apoptotic, partial, peripheral chromatin condensation affecting ruffled nuclei (Figs 1, 6). Thus, in the presence of Z-VAD.fmk, PACC appears to be contagious (Figs 1, 6), correlating with MMP (Fig. 1F).

Paradoxically however, DNA (including damaged DNA) is

not required to transmit the lethal signal in contagious apoptosis, as demonstrated by removal of nuclear DNA (by acute enucleation) or removal of mitochondrial DNA (by suppression of mitochondrial DNA replication) from the 'donor' Env-expressing cells (Fig. 6). However, suppression of MMP by Bcl-2 and vMIA could avoid the transmission of nuclear apoptosis and even that of PACC (observable in the presence of Z-VAD.fmk) (Fig. 7), pointing to the involvement of mitochondria (but not mitochondrial DNA, Fig. 6C,D) in the contagion of apoptosis. This latter observation indicates an interesting difference between caspase inhibition by Z-VAD.fmk and the effects of Bcl-2/vMIA. Z-VAD.fmk prevents the manifestation and transmission of nuclear apoptosis, yet has no effect on apoptosis-associated MMP or on the transmission of PACC. In contrast, Bcl-2 and vMIA, which inhibit MMP, do abrogate the transmission of PACC, pointing to an unexpected (and hitherto unexplained) cross talk between MMP and nuclear DNA lesions.

Irrespective of the detailed mechanisms of contagious apoptosis, our data indicate that this phenomenon could potentially affect CD4⁺ T cells in HIV-1 infection. Stressed, Env-expressing cells can kill CD4⁺ lymphoblastoid cell lines and primary T cells, as well as activated T lymphoblasts *in vitro*. Moreover, stressed Env⁺ cells can drive CD4⁺ cells into rapid apoptosis *in vivo* (Fig. 8). Although it has not been shown that this phenomenon also occurs *in vivo*, in HIV-1-infected individuals evolving toward AIDS, a plausible scenario would be that Env-expressing cells (which hence are HIV-1 infected) could transmit apoptosis to interacting CD4 cells. In conditions in which viral replication overwhelms the cellular defense response and triggers (pre-)apoptosis of the 'donor' Env⁺ cells, fusion with 'recipient' CD4⁺ cells would contribute to the depletion of CD4⁺ T lymphocytes by contagious apoptosis.

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