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Differential calcium response in HeLa and HeLa-Fas cells by cytotoxic T lymphocytes☆,☆☆

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Abstract

We constructed a CD95 overexpressing HeLa cell line which was extremely sensitive towards CD95 mediated apoptosis. In these CD95 overexpressing cells, CD95 blocks the nuclear calcium signal induced by perforin positive and CD95 ligand positive killer cells. This phenomenon is highly relevant in states of inflammatory syndromes such as systemic inflammatory response syndrome (SIRS) and sepsis which are associated with a high probability to reactivate latent viruses due to a functional deficiency of cytotoxic effectors.

Keywords: Apoptosis; Ca^{2+} release; CD95 overexpression; HeLa cells; Cytotoxic T lymphocytes

The CD95 (Fas/APO-1) death receptor induces apoptosis after binding of its natural ligand (CD95 ligand, CD178) or an agonistic monoclonal antibody [1,2]. This 45 kDa glycosylated type I transmembrane molecule belongs to the TNF/nerve growth factor (NGF) family of receptors [1]. The CD95 (Fas/Apo-1) ligand is a 40 kDa type II trans-membrane protein and a member of the TNF family [1,3]. Upon CD95 stimulation, a cytoplasmic complex of proteins including caspase 8 is formed and the activation signal is transferred to other proteins which execute cell death via a differential involvement of mitochondria to finally activate caspase 3 and endonucleases [4]. In another pathway, CD95 leads to p21-activated kinase (PAK) kinase activation, the activity of which modulates the translocation of phosphatidylserine to the outer surface of the cell membrane, allowing the binding of annexin [5]. However, the physiological relevance of CD95 has remained a matter of discussion especially since CD95 appears to modulate Ca^{2+} signalling in T lymphocytes [6]. Increases of cytoplasmic Ca^{2+} elicit a number of selective responses and the nature of the Ca^{2+} signal decides whether the activation of transcription factors such as NFκB and c-Jun N-terminal kinase (JNK) or NFAT occurs [7]. Moreover, Ca^{2+} oscillations are involved in apoptosis induced by chemotherapeutics [8], through the cytoplasmic reassembly of the actin cytoskeleton and subsequent appearance of plasma membrane blebbing [9] and a number of other events including nuclear condensation and fragmentation [10].

In cellular cytotoxicity assays, non-cloned cytotoxic lymphocytes induce Ca^{2+} waves in the cytoplasm which is immediately followed by a nuclear Ca^{2+} peak in wildtype HeLa cells. By contrast the cytotoxic lymphocytes induce only cytoplasmic Ca^{2+} release in the CD95 overexpressing HeLa-Fas cells. HeLa-Fas cells are distinct from HeLa wildtype cells by their high sensitivity against CD95-mediated apoptosis.

Materials and methods

Construction of a CD95 overexpressing HeLa cell line. The CD95 cDNA was cloned from a cDNA library of activated Jurkat cells

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(stimulated with PMA, Stratagene). PCR primers were used from the sequence of the human CD95 gene [11]. The sequence of the upper primer was 5′CACTCTAGACCAAGCTTTGGATTCCA3′, the sequence of the lower primer was 5′AACCATGCTGGGCATCTG GA3′. PCR was performed on a "RoboCycler" (Stratagene) using 1 μl-DNA (1 μg/μl, isolated from the T-cell cDNA library), 1 μl of each primer (10 pmol/μl), 22 μl H_2O, and 25 μl Master-Mix (Qiagen). The following cycling conditions were chosen: 1 × 94 °C, 4 min, 40 × 94 °C, 1 min, 58–69 °C (gradient), 2 min, 72 °C, 2.5 min, and 1 × 72 °C, 5 min. The resulting 1015 bp product was isolated, subcloned into pCR3.1(+) (Invitrogen, Karlsruhe, Germany), and designated as hfas-3/4. The construct was sequenced using the dye-terminator technology (PE Applied Biosystems, Darmstadt, Germany).

HeLa cells (ATCC CCL-2) were cultured using RPMI medium (Invitrogen, Karlsruhe, Germany) supplemented with 1% (v/v) L-glutamine (200 mM Invitrogen, Karlsruhe, Germany Technologies), 1% penicillin/streptomycin (10,000 U/ml penicillin and 10 μg/ml streptomycin (Invitrogen, Karlsruhe, Germany), and 10% (v/v) heat inactivated fetal calf serum (FCS, Invitrogen, Karlruhe, Germany) further designated as complete medium. Approximately 4×10^5 cells/well were transfected with 5 μg hfas-3/4 DNA using the Superfect-technology (Qiagen, Hilden, Germany) as recommended by the manufacturer. After 24 h, the antibiotic Geneticin (Invitrogen, Karlsruhe, Germany) was added in a concentration of 200 μg/ml. Positive clones were isolated by limiting dilution on 96 well plates. CD95-RNA was detected by quantitative RT-PCR method using a LightCycler (Roche, Penzberg, Germany). The sequence for the upper primer was 5′CAAAAGTGTTAATGCCCAAGT3′, for the lower primer 5′AAGCCACCCC AAGTTAGA3′. The PCR was performed using a LightCycler amplification Kit SYBR Green I (Roche, Penzberg, Germany) as described by the manufacturer. Briefly, 2 μl SYBR Green, 13 μl H_2O, 1.6 μl $MgCl_2$, 1 μl of each primer (5 pmol/μl), 0.4 μl Enzyme Mix, and 1 μl total RNA (approximately 100 ng) were mixed and amplified under the following cycling conditions: 1 × 55 °C, 10 min, 1 × 95 °C, 30 s, 45 × 95 °C, 1 s, 54 °C, 10 s, 72 °C, 15 s, 1 × 97 °C, 0 s, 65 °C, 20 s, 99 °C, 0 s, and 1 × 40 °C, 1 min. All PCR positive clones were used for further characterization in apoptosis assays. The CD95 overexpressing HeLa cell clone used for the following studies was termed HeLa-Fas and compared to a pCR3.1(+) vector transfected control HeLa cell clone.

Flow cytometry. CD95 expression was tested by FITC-labelled Apo-1 (Alexis Biochemicals, Lausen, Switzerland) and PE-labelled DX-2 antibodies (Becton Dickinson Europe Heidelberg, Germany). The cytoplasmic expression of CD95 ligand (CD178) (clone G247, Pharmingen via Becton Dickinson Europe, Heidelberg, Germany), anti-perforin (clone 2δG9 Pharmingen via Becton Dickinson Europe, Heidelberg, Germany), and anti-granzyme B (clone CLB-GB11 from Pelicluster purchased via Tebu, The Netherlands) in killer cells was tested concomitantly with antibodies against the surface antigens CD8, CD56, and CD16 (all purchased from Becton Dickinson Europe, Heidelberg, Germany).

Determination of soluble CD95 ligand (sCD178) by ELISA. Soluble CD95 ligand was determined in culture supernatants (24 h following subculture and a cell density of 1×10^6 lymphoblasts/ml of cytotoxic effector cells) as well as in lysates of IL-2 propagated cell lines by the CD95 ligand specific ELISA (MBL, Japan purchased via Beckman–Coulter, Krefeld, Germany).

Crystal violet assay. Induction of CD95 mediated apoptosis of approximately 10^5 cells (in 96 well plates) was with an agonistic monoclonal CD95 antibody (2R2, Alexis Biochemicals, Lausen, Switzerland) with 0.1 μg/ml and time points as indicated. After incubation, living cells were stained with crystal violet as described [12,13].

Caspase inhibition. Approximately 10^5 cells were preincubated for 2 h with 10 μM Z-VAD-Fmk (BioCat, Heidelberg, Germany). Then CD95 mediated apoptosis was induced with 1 μg/ml of a monoclonal agonistic CD95 antibody [14] in the presence of 10 ng/ml protein A as crosslinker with time points as indicated in the presence of Z-VAD-Fmk and assayed as above.

Source of cellular effector lymphocytes. Natural killer (NK-) effector lymphocytes from Ficoll-separated blood cells were activated by high dose IL-2 (10^3 IE/ml) for 3 days, followed by continuous culture in complete medium containing 100 IU IL-2/ml. The tumor NK cell line NK-92 was generated from a patient with NK leukemia. NK-92 [15] was a gift from Torsten Tonn (Blutspendedienst, Frankfurt, Germany).

Cytotoxicity assays. Cellular cytotoxicity was tested by established chromium release assays using 51-chromate (130 μCi sodium chromate for 1 h labelled) target cell and HeLa-cell layers in 96-well round bottomed (to test non-adherent target cells such as K562) and 96-well flat bottomed microtiter plates to test adherent target cells (10^4 K562 and 5×10^3 HeLa or HeLa-Fas per well) that were incubated with titrated amounts of effector cells and release of the isotope was determined after 4 and 16 h using a β plate scintillation counter (Perkin-Elmer/LKB, Freiburg, Germany). Percent cytolysis was documented and lytic units (LU) were calculated by regression analysis to normalize cytotoxicity to defined $E{:}T$ ratios. One LU is defined as the reciprocal value of the amount of effector cells necessary to lyse 25% of 10^7 target cells [16].

Calcium release by confocal laser scanning microscopy. Adherent target cells (HeLa and HeLa-Fas) were seeded in 35 mm cover glass dishes, washed with pre-warmed Hank's balanced salt solution (HBSS), and then incubated with 2 μM Fluo-4-AM (Molecular Probes, purchased via Mobitec, Goettingen, Germany) for 30 min, washed again, and observed in an upright confocal laser scanning microscope (Leica TCS-NT, Heidelberg, Germany) equipped with an argon–krypton laser. For determination of Fluo-4 fluorescence the excitation wavelength was 488 nm and emission wavelengths were monitored by >515 nm band pass filters. The calcium signals were recorded in the continuous scan mode using a 63×/1.23 oil-immersion objective. Twenty microliters of a suspension of non-labelled effector cells (1×10^7 cells/ml) was added close to the observation focus, carefully preventing agitation. After 10 min of continuous recording, the killer cell suspension was washed off and cell layers were incubated for 2 h in complete medium at 37 °C, 5% CO_2 in an incubator. The cell layer was then stained with 25 μM propidium iodide (PI) for 1 min and washed. The cell layer was then examined by two color analysis using excitation at 488 and 568 nm, respectively, and emission wavelengths at >525 and >600/630 nm. PI staining and morphological examinations were adequate to distinguish apoptotic from necrotic cell death in HeLa and HeLa-Fas cells.

Results

The expression of CD95 on the surface of stably transfected HeLa-Fas cells and vector transfected control HeLa cells was documented by flow cytometric analysis of surface stained HeLa and CD95 overexpressing HeLa-Fas cells using the APO-1 monoclonal antibody (Fig. 1). Compared to vector transfected HeLa cells, HeLa-Fas cells show a strong CD95 expression on the cell surface. Similar results were obtained by using the anti CD95 antibody DX-2 (data not shown).

The functional activity of the CD95 death receptor of the HeLa-Fas cells was demonstrated by staining with crystal violet after stimulation with an agonistic monoclonal antibody at different time points (Fig. 2). A fast induction of cell death was observed in HeLa-Fas cells

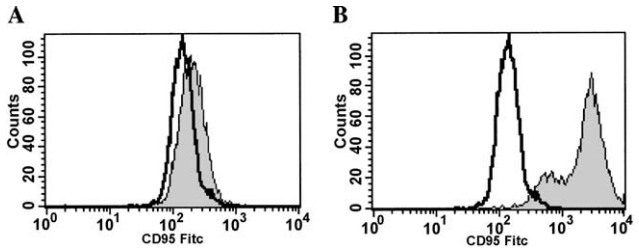

Fig. 1. Flow cytometric analysis of wildtype HeLa and CD95 overexpressing HeLa-Fas cells. CD95 expression was measured in mock (A) or CD95 transfected (B) HeLa cells. Binding of fluorescently labelled mouse isotype control (solid line) and anti CD95 specific monoclonal APO-1-FITC (light gray filled histogram) is shown.

(Fig. 2B). No such cell death was induced in HeLa control cells (Fig. 2A). In comparison, L929-APO cells, which also overexpress CD95 [17], died significantly slower than HeLa-Fas cells (Fig. 2C). Essentially the same results were obtained by using a different agonistic monoclonal anti CD95 antibody [14] in the presence of protein A (data not shown).

Cell death was caspase dependent, since it was specifically blocked by caspase inhibitors. The pan-caspase inhibitor Z-VAD-Fmk completely inhibited CD95 mediated apoptosis (Fig. 2D), whereas the caspase-8 inhibitor Z-IETD-CHO only partially inhibited CD95-mediated apoptosis at a 10-fold higher concentration

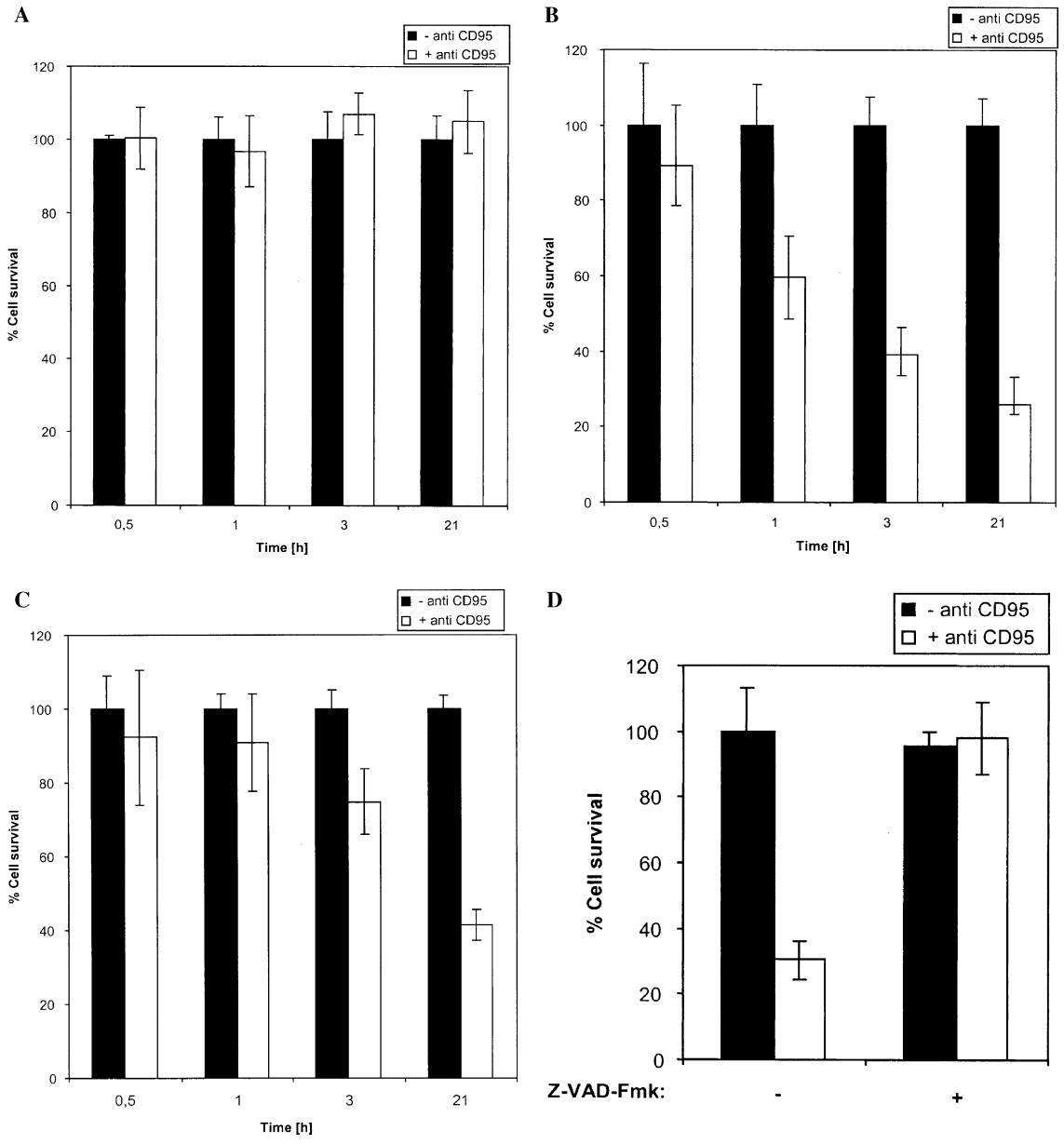

Fig. 2. Induction and inhibition of apoptosis. CD95-mediated apoptosis was induced for 0.5, 1, 3, and 21 h in HeLa-control (A), HeLa-Fas (B), and L929-APO (C) cells (white bars) in comparison to non-induced controls (black bars). (D) CD95-mediated apoptosis was induced for 2 h in HeLa-Fas cells (white bars) in the absence or presence of Z-VAD-Fmk as indicated in comparison to non-induced controls (black bars). In all experiments cell survival was measured by crystal violet staining. The mean values of quadruplicate assays with standard deviations are presented.

Table 1
Cellular cytotoxicity of IL-2 activated and propagated cell lines

Effector	Cellular cytotoxicity % lysis [lytic units, LU] of target cells			
	K562	K562-EGTA	HeLa	HeLa-Fas
868-IL-2-cultures	34 [3852]	24 [1055]	34 [131]	83 [1185]
NK-92 leukemic cell line	45 [6002]	−2 [0]	−1.2 [0]	0.5 [0]

IL-2 activated and propagated cell lines were generated from Ficoll-isolated peripheral blood cells by 7–14 days of in vitro culture. Lymphocyte blasts were tested for their lytic activity against the classical NK target K562 in the presence and absence of EGTA in order to distinguish Ca^{2+} dependent and Ca^{2+} independent cytolyses. HeLa and HeLa-Fas were tested as radioactively labelled confluent target cell layers. Radioactivity released from the target was a measure for cytotoxic activity. NK-92 cell line served as a perforin positive control.

Table 2
Antigen expression pattern of cytolytic effector cell lines

	% CD56	% CD16	% Perforin*	% CD95 ligand (CD178)*	% CD95
868-IL-2-culture	68	15	15	28	49
NK-92 leukemic cell line	85	7.2	>63	>70	0.2

Expression of NK-relevant antigens was tested by flow cytometry of either surface labelled (antiCD56, antiCD16, and antiCD95) or *cytoplasmic antigens (anti-perforin, anti-CD-95 ligand CD178). CD95 ligand expression was further verified by quantifying soluble CD95 ligand in the culture fluid by the MBL Elisa.

(data not shown). The partial inhibition could be due to the reversible inhibition of CHO-modified caspase inhibitors.

In routine cytotoxicity assays, HeLa and HeLa-Fas cells were different regarding susceptibility against cellular cytolysis as detected by chromium release (Table 1). For comparative experiments, we chose the IL-2 propagated T cell line 868-IL-2 which contained CD95 ligand positive as well as perforin positive NK effectors and the NK cell line NK-92 lacking CD95 ligand (Table 2). Both cell lines, 868-IL-2 and NK-92, induced chromium release in the MHC-class I negative K562 target cells. In the presence of the Ca^{2+} chelator EGTA, however, lysis was observed only with 868-IL-2 (Table 1). This indicates that lysis by NK-92 which was established from a patient with NK leukemia [15] was restricted to the Ca^{2+} dependent perforin mechanism. When HeLa cell lines were tested as targets, the leukemic cell line NK-92 neither lysed HeLa wildtype nor HeLa-Fas cells. This indicates that the activating recognition receptors of NK-92 are either not activated or blocked by NK inactivating receptors. Thus NK-92 was not suitable to study further. However, 868-IL-2 lysed both, HeLa wildtype and HeLa-Fas cells. The cytotoxicity of 868-IL-2 against the CD95 overexpressing targets was more than two-fold as compared to HeLa wildtype cells (Table 1). Phenotypically, 868-IL-2 contained CD95 ligand positive effectors as well as those which lacked CD95 ligand and expressed perforin and granzyme B (Table 2). Due to polyclonality of this effector population not all killer cells were blocked by the HLA-class I antigens expressed by HeLa cells.

For life imaging experiments, HeLa target cells were labelled with the Ca^{2+} indicator Fluo-4-AM and an increase in their cytoplasmic Ca^{2+} levels was monitored after addition of non-labelled 868-IL-2 to the target cell layer (Fig. 3A). Following contact between effector and target, the cytoplasmic Ca^{2+} signal is rapidly translocated to the cell nucleus in HeLa wildtype cells. These Ca^{2+} oscillations finally culminate in extensive blebbing of the plasma membrane (red arrow in Fig. 3A; see http://www.uni-ulm.de/klinik/anaesthesie/experi for avi. presentation). The velocities of Ca^{2+} signal oscillations are probably due to the frequencies of contact between a killer effector and a given target cell. Blebbing may culminate in the release of plasma membrane vesicles (data not shown). Following a post-incubation time of the HeLa target cell layer for 2 h and subsequent staining with the DNA sensitive dye PI demonstrates that the killer lymphocytes not only induced extensive blebbing which remains prominent in most of the HeLa target cells after PI staining, but also cell shrinkage and nuclear condensation, which are hallmarks of apoptosis (Fig. 4A).

By contrast, the same killer cell line, 868-IL-2, induces a different Ca^{2+} signal in the CD95 overexpressing HeLa-Fas target cells. Here the rise of Ca^{2+} appears to be restricted to the cytoplasm and no nuclear signal occurs (Fig. 3B, see http://www.uni-ulm.de/klinik/anaesthesie/experi for life imaging avi.series). Again, individual attacks by the cytotoxic lymphocytes generate individual Ca^{2+} oscillations (arrow). Blebbing was less prominent in HeLa-Fas cells, but the majority of the nuclei of HeLa-Fas cells are even more fragmented than in HeLa wildtype cells (Fig. 4B).

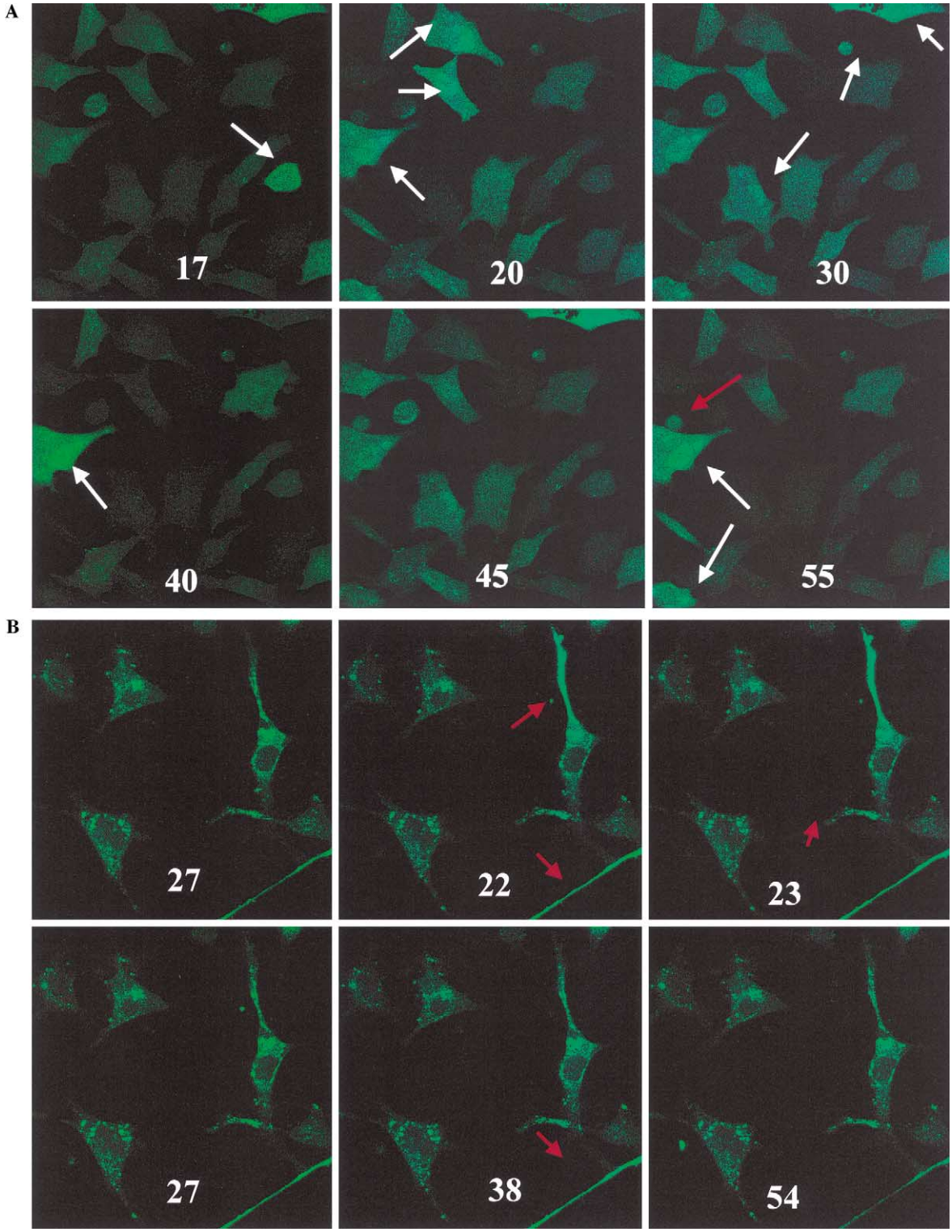

Fig. 3. Cytotoxicity of HeLa and HeLa-Fas monolayers by 868-IL-2 lymphoblasts. (A) Monolayers of HeLa were labelled with Fluo-4-AM, and green fluorescence signals were monitored by continuous laser scanning microscopy, immediately after the addition of 868-IL-2 activated lymphoblasts. The frequency of individual images recording the fluorescence signals in the cytoplasm of the target cells and nuclei was almost 1 image/s. Numbers in individual images indicate seconds after addition of the effector lymphocytes. White arrows indicate Ca^{2+} release in individual target cells. Following several attachments with the killer lymphoblasts, target cells show blebbing of the plasma membrane (red arrow) (HeLa-868-IL-2.avi). (B) Monolayers of HeLa-Fas labelled with Fluo-4 and the same 868-IL-2 effector cells were added (see Fig. 3A for further details). Ca^{2+} release was restricted to the cytoplasm and is most prominent in the elongations of the cell. Red arrows mark cytoplasmic protrusions with extensive Ca^{2+} responses (HeLa-Fas-868-IL-2.avi).

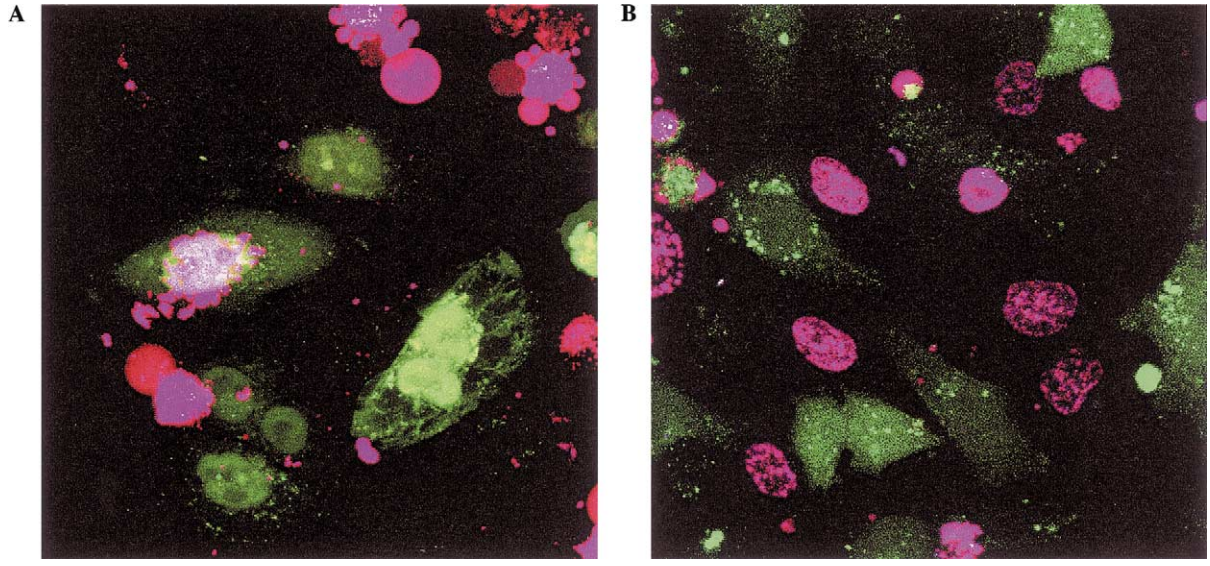

Fig. 4. PI staining of HeLa monolayers following cytotoxicity assays. (A) Fluo-4 labelled HeLa monolayers after cytotoxicity assays with 868-IL-2 (30 min), 2 h post incubation and staining with PI (25 μM). (B) HeLa-Fas monolayers after cytotoxicity assays with 868-IL-2 (30 min), 2 h post incubation and staining with PI (25 μM).

Discussion

In the present investigation a stably transfected, CD95 overexpressing HeLa cell line (HeLa-Fas) has been described and functionally tested in both, apoptosis inducer assays as well as in cellular cytolysis. According to the results presented here, HeLa and HeLa-Fas cell lines constitute a suitable pair to test and evaluate the induction of apoptosis by NK cells via CD95-dependent and independent mechanisms. Moreover, this CD95 overexpressing HeLa-Fas clone is more sensitive to CD95 mediated apoptosis than other cell lines described so far (Fig. 2). The modulation of susceptibility to cell death by CD95 expression is an important event to explain cell death and tissue destruction during SIRS and sepsis in the presence or absence of inflamnmatory cytokines, infectious agents, and/or cytotoxic cells [18]. As expected, the mechanism of CD95 induced killing has been shown to be caspase dependent in HeLa-Fas cells (Fig. 2).

In cellular cytolysis assays, HeLa and HeLa-Fas cells are both susceptible to cytolysis by IL-2 activated effectors of healthy donors. We chose the polyclonal 868-IL-2 cell line derived from lymphocytes of a healthy blood donor since perforin-dependent effectors as well as CD95 ligand expressing effectors were both present and active (Tables 1 and 2). The perforin dependent killers require the presence of Ca^{2+} in the medium to induce polymerization of perforin monomers in the target membrane [19,20], whereas CD95 ligand positive effectors induce cytolysis via CD95 recognition and this recognition event is Ca^{2+} independent [20]. Historically, the functional relevance of perforin versus CD95 dependent cellular cytotoxicity has been demonstrated by the generation of perforin knockout mice [21–24].

Since sphingomyelinase is activated upon crosslinking of the CD95-antigen [25], the involvement of ceramide regulating cytochrome c release from mitochondria appears to be at least one of the Ca^{2+} dependent, cytoplasmic processes [8]. Alternatively, the cytoplasmic Ca^{2+} signal may be indicative for reorientation of the cytoskeleton [26]. These latter events occur in both, effector and target cells upon cell cell contact (for review see [27]).

Clearly, the Ca^{2+} oscillations induced by CD95 ligand positive effectors are distinct from those elicited by perforin-positive killing, in that they do not induce a nuclear Ca^{2+} signal in the target and induce less prominent apoptosis (Figs. 3 and 4). Immediately after the cytoplasmic Ca^{2+} increase, the Ca^{2+} signal migrates to the nucleus. This sequence is repeated in a given HeLa target cell, whenever a killer cell gains contact. After multiple cycles of Ca^{2+} oscillation, membrane blebbing is the first morphological sign of apoptosis in HeLa control cells (Fig. 4A). In contrast, CD95 overexpressing HeLa-Fas cells seem to undergo apoptosis very quickly until a final stage since nuclear fragmentation occurs more rapidly in HeLa Fas cells, which express the expected apoptotic phenotype. The morphology of the dying HeLa cells (Fig. 4A) is similar to a caspase independent cell death that has been described for a caspase 8 deficient Jurkat-derived cell line [28].

In addition, the CD95 expressing target cells appear to lack the nucleus associated Ca^{2+} oscillations. Here, Ca^{2+} release induced by CD95-ligand positive effector cells is restricted to the cytoplasm. Stimulation by the

killer lymphocyte induces Ca^{2+} release from intracellular stores such as the endoplasmic reticulum and mitochondria (Fig. 3B). Remarkably, the strongest Ca^{2+} signal is detected in the cell protrusions of transfected HeLa-Fas cells. These results are reminiscent of the observation by Lepple-Wienhues and co workers who found that CD95 expression blocked a certain Ca^{2+} signal in cytotoxic effectors. Although Ca^{2+} activated release channels [6] have not been studied in our experimental setting, the similar blockade of a defined Ca^{2+} signal in HeLa cells should be followed further. A molecularly similar type of inhibition in Ca^{2+} responsiveness by CD95 has been initially described in patients with glioblastoma [29] but then also in other tumors [30].

The mechanism of CD95 activation does not solely act as an inducer of apoptosis but it appears to be a major player in immune regulation as well. This argument is further substantiated by the finding that cellular cytotoxicity is more often due to granule exocytosis than CD95/CD95 ligand interactions [31].

In summary, the present investigation describes a CD95 overexpressing HeLa cell clone which is highly sensitive to CD95 induced apoptosis. Two important pathways of apoptosis induced by cytotoxic lymphocytes involve the perforin/granzyme and/or CD95 induced death pathways. The pair of HeLa wildtype and HeLa-Fas is suitable to prove and further study patterns of Ca^{2+} release response involved in target cell apoptosis.

Acknowledgments

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References

[1] S. Nagata, Apoptosis by death factor, Cell 88 (1997) 355–365.
[2] P.H. Krammer, CD95's deadly mission in the immune system, Nature 407 (2000) 789–795.
[3] T. Suda, T. Takahashi, P. Golstein, S. Nagata, Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family, Cell 75 (1993) 1169–1178.
[4] C. Scaffidi, S. Fulda, A. Srinivasan, C. Friesen, F. Li, K.J. Tomaselli, K.M. Debatin, P.H. Krammer, M.E. Peter, Two CD95 (APO-1/Fas) signaling pathways, EMBO J. 17 (1998) 1675–1687.
[5] T. Rudel, G.M. Bokoch, Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2, Science 276 (1997) 1571–1574.
[6] A. Lepple-Wienhues, C. Belka, T. Laun, A. Jekle, B. Walter, U. Wieland, M. Welz, L. Heil, J. Kun, G. Busch, M. Weller, M. Bamberg, E. Gulbins, F. Lang, Stimulation of CD95 (Fas) blocks T lymphocyte calcium channels through sphingomyelinase and sphingolipids, Proc. Natl. Acad. Sci. USA 96 (1999) 13795–13800.
[7] R.E. Dolmetsch, R.S. Lewis, C.C. Goodnow, J.I. Healy, Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration, Nature 386 (1997) 855–858.
[8] J. Chandra, E. Mansson, V. Gogvadze, S.H. Kaufmann, F. Albertioni, S. Orrenius, Resistance of leukemic cells to 2-chlorodeoxyadenosine is due to a lack of calcium-dependent cytochrome *c* release, Blood 99 (2002) 655–663.
[9] R.M. van Gorp, S. Heeneman, J.L. Broers, N.M. Bronnenberg, M.C. van Dam-Mieras, J.W. Heemskerk, Glutathione oxidation in calcium- and p38 MAPK-dependent membrane blebbing of endothelial cells, Biochim. Biophys. Acta 1591 (2002) 129–138.
[10] G. Ermak, K.J. Davies, Calcium and oxidative stress: from cell signaling to cell death, Mol. Immunol. 38 (2002) 713–721.
[11] A. Oehm, I. Behrmann, W. Falk, M. Pawlita, G. Maier, C. Klas, M. Li-Weber, S. Richards, J. Dhein, B.C. Trauth, H. Ponstingl, P.H. Krammer, Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen, J. Biol. Chem. 267 (1992) 10709–10715.
[12] N. Itoh, S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, S. Nagata, The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis, Cell 66 (1991) 233–243.
[13] H. Hug, M. Enari, S. Nagata, No requirement of reactive oxygen intermediates in Fas-mediated apoptosis, FEBS Lett. 351 (1994) 311–313.
[14] B.C. Trauth, C. Klas, A.M. Peters, S. Matzku, P. Moller, W. Falk, K.M. Debatin, P.H. Krammer, Monoclonal antibody-mediated tumor regression by induction of apoptosis, Science 245 (1989) 301–305.
[15] J.H. Gong, G. Maki, H.G. Klingemann, Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells, Leukemia 8 (1994) 652–658.
[16] E.M. Schneider, I. Lorenz, M. Muller-Rosenberger, G. Steinbach, M. Kron, G.E. Janka-Schaub, Hemophagocytic lymphohistiocytosis is associated with deficiencies of cellular cytolysis but normal expression of transcripts relevant to killer-cell-induced apoptosis, Blood 100 (2002) 2891–2898.
[17] K. Schulze-Osthoff, P.H. Krammer, W. Droge, Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death, EMBO J. 13 (1994) 4587–4596.
[18] A. Heininger, U. Vogel, C. Aepinus, K. Hamprecht, Disseminated fatal human cytomegalovirus disease after severe trauma, Crit. Care Med. 28 (2000) 563–566.
[19] E. Rouvier, M.F. Luciani, P. Golstein, Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity, J. Exp. Med. 177 (1993) 195–200.
[20] H. Takayama, H. Kojima, N. Shinohara, Cytotoxic T lymphocytes: the newly identified Fas (CD95)-mediated killing mechanism and a novel aspect of their biological functions, Adv. Immunol. 60 (1995) 289–321.
[21] H. Kojima, N. Shinohara, S. Hanaoka, Y. Someya-Shirota, Y. Takagaki, H. Ohno, T. Saito, T. Katayama, H. Yagita, K. Okumura, et al., Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes, Immunity 1 (1994) 357–364.
[22] D. Kagi, F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, P. Golstein, Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity, Science 265 (1994) 528–530.
[23] B. Lowin, M. Hahne, C. Mattmann, J. Tschopp, Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways, Nature 370 (1994) 650–652.
[24] C.M. Walsh, M. Matloubian, C.C. Liu, R. Ueda, C.G. Kurahara, J.L. Christensen, M.T. Huang, J.D. Young, R. Ahmed, W.R. Clark, Immune function in mice lacking the perforin gene, Proc. Natl. Acad. Sci. USA 91 (1994) 10854–10858.
[25] M.G. Cifone, D. Botti, C. Festuccia, T. Napolitano, E. del Grosso, G. Cavallo, M.A. Chessa, A. Santoni, Involvement of

phospholipase A2 activation and arachidonic acid metabolism in the cytotoxic functions of rat NK cells, Cell. Immunol. 148 (1993) 247–258.

[26] J. Delon, N. Bercovici, R. Liblau, A. Trautmann, Imaging antigen recognition by naive CD4+ T cells: compulsory cytoskeletal alterations for the triggering of an intracellular calcium response, Eur. J. Immunol. 28 (1998) 716–729.

[27] J.D. Robertson, J. Chandra, V. Gogvadze, S. Orrenius, Biological reactive intermediates and mechanisms of cell death, Adv. Exp. Med. Biol. 500 (2001) 1–10.

[28] H. Matsumura, Y. Shimizu, Y. Ohsawa, A. Kawahara, Y. Uchiyama, S. Nagata, Necrotic death pathway in Fas receptor signaling, J. Cell Biol. 151 (2000) 1247–1256.

[29] L.A. Morford, L.H. Elliott, S.L. Carlson, W.H. Brooks, T.L. Roszman, T cell receptor-mediated signaling is defective in T cells obtained from patients with primary intracranial tumors, J. Immunol. 159 (1997) 4415–4425.

[30] K. Staveley-O'Carroll, E. Sotomayor, J. Montgomery, I. Borrello, L. Hwang, S. Fein, D. Pardoll, H. Levitsky, Induction of antigen-specific T cell anergy: an early event in the course of tumor progression, Proc. Natl. Acad. Sci. USA 95 (1998) 1178–1183.

[31] M. Yasukawa, H. Ohminami, J. Arai, Y. Kasahara, Y. Ishida, S. Fujita, Granule exocytosis, and not the Fas/Fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4(+) as well as CD8(+) cytotoxic T lymphocytes in humans, Blood 95 (2000) 2352–2355.