Replicative senescence enhances apoptosis induced by pemphigus autoimmune antibodies in human keratinocytes

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Abstract We have recently shown that skin lesions of the autoimmune disease pemphigus vulgaris are associated with Fas-mediated apoptosis. Here, we describe the induction of the Fas-dependent apoptosis pathway in cultured keratinocytes by pemphigus vulgaris autoantibodies (PV-IgG), as seen from a variety of cellular, morphological and biochemical parameters. All apoptotic characters appear stronger and faster in aged cultures than in young, showing increased susceptibility of senescent keratinocytes to PV-IgG-mediated apoptotic death and culture lesions. Together with immunosenescence, this phenomenon may explain the late onset of pemphigus disease.

Keywords: Apoptosis; Autoimmune disease; Fas; Skin; Replicative senescence

1. Introduction

Skin ageing is characterized by various cellular and morphological changes [1,2] that come with functional impairments such as slow wound healing [3], decreased protection against ultraviolet radiations [4], and increased susceptibility to skin cancer [5]. Most of these alterations can be related to skin senescence, which entails among others: reduced proliferation [3], increased oxidative stress [6] and reduced DNA repair capacity [7]. Cellular senescence can be induced in cultures by serial passaging or by oxidative stress, and these two kinds of models have been widely used to analyze the mechanisms of cellular ageing [8,9].

Programmed cell death (apoptosis) occurs in vivo for the needs of tissue turnover or elimination [10] and it contributes to anti-tumor protection, as does also cellular senescence [11,12]. These two processes are, in fact, associated in normal and pathological ageing [9,13] and their pathways overlap [14], suggesting that apoptosis might be associated to the senescent process itself. Yet, spontaneous apoptosis is not more frequent in senescent cultures than in young [15]. On the contrary, senescent human fibroblasts and keratinocytes were found to resist intrinsic [18] apoptosis induced by growth factor starvation [16] and ultraviolet irradiation [17], respectively. When the extrinsic pathway [18] of apoptosis was induced, however, senescent fibroblasts [19–21] and senescent keratinocytes [22] were found competent to apoptosis, and even more susceptible than their young counterparts. We previously showed that autoimmune antibodies from pemphigus vulgaris patients (PV-IgG) can induce the Fas-dependent pathway of apoptosis in human keratinocytes [23]. This was not really expected, since PV-IgG recognize mainly adherent junctional proteins (desmogleins 1 and 3) [24]. In this work, we show that cellular senescence enhances PV-IgG-induced apoptosis in human keratinocytes, providing further evidence that pemphigus lesional process may be driven by PV-IgG-induced apoptosis as we proposed before [23], and that aged epidermal cells may be more sensitive to it.

2. Materials and methods

2.1. Antibodies and reagents

Anti-Bcl-2 polyclonal, anti-Fas L polyclonal, anti-caspase 8 polyclonal and anti-p53 (DO-1) monoclonal antibodies were from Santa Cruz. Anti-Fas monoclonal was from Pharmingen, anti-involucrin monoclonal from Sigma, anti-Ki 67 monoclonal from Dako and anti-p16 monoclonal from Serotec. Enzyme-conjugated secondary antibodies were from Sigma (alkaline phosphatase) or Kirkegaard & Perry (peroxidase). FITC-conjugated goat anti-mouse, rhodamine red-conjugated goat anti-mouse and R-phytoerythrin-conjugated donkey anti-rabbit antibodies were from Jackson Immuno Research. Pemphigus-IgG (PV-IgG) was purified from patients' sera as previously described [23], Caspase substrates and inhibitors were from Calbiochem. The TUNEL apoptosis kit, propidium iodide and recombinant human interferon-γ (IFNγ) were from Promega.

2.2. Keratinocyte cultures

Primary keratinocyte cultures were obtained by trypsinization of newborn foreskins. Released cells were seeded and cultivated as previously described [22].

2.3. Biomarker diagnostic of cellular senescence in keratinocyte cultures

Keratinocyte suspensions were obtained from culture flasks by trypsinization and resuspension in PBS. Cell size was measured microscopically in a hemacytometer. Intrinsic fluorescence was analyzed at 480/560 nm by flow cytometry. Total protein content was determined as described [25]. Other markers were characterized by immunofluorescent labeling and flow-cytometric analysis, as follows. Suspended cells were fixed 15 min at 30 °C in PBS, 2% paraformaldehyde followed by cold methanol (67% final, 1 h at 4 °C). Cells were washed twice in PBS and incubated 2 h at 30 °C with the first (antigen-specific) antibody. They were washed twice in PBS and incubated 1 h at 30 °C with the second, FITC-labeled antibody. After two more

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washes in PBS, the cells were analyzed in a Becton–Dickinson flow cytometer and the average fluorescent signal was determined.

2.4. DNA fragmentation assay

Keratinocytes were scraped off from culture plates, suspended in 100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5, and incubated with 100 μg/ml proteinase K, 100 mM NaCl, 10 mM EDTA, 0.5% SDS, 10 mM Tris, pH 7.5, for 1 h at 50 °C. Lysates were subjected to phenol–chloroform (1:1) extraction and ethanol-precipitated. Pellets were dissolved in 1 mM EDTA and 10 mM Tris, pH 8.0, incubated for 1.5 min at 37 °C with 20 μg/ml DNAse-free RNAse, and analyzed by agarose gel electrophoresis.

2.5. Dot blots

Small drops (10 μl) of culture supernatants were spotted onto nitrocellulose paper. Blots were blocked in 10% milk in PBS, and incubated with a specific antibody in 5% milk, 0.1% Tween 20, 150 nM NaCl, and 50 mM Tris–HCl, pH 7.5, for 14 h at 4 °C. After serial washes, they were incubated with an alkaline phosphatase-conjugated, secondary antibody for 2 h at room temperature, washed again, and incubated with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in 0.1 M NaCl, 50 mM $MgCl_2$, and 0.1 M Tris–HCl, pH 9.2. Staining densities were analyzed using a ScanJet II CX/T gel scanner with the NIH Image V1.61 program.

2.6. Cellular ELISA

Cultured keratinocytes were detached in a trypsin–EDTA solution, washed and resuspended in PBS. Suspended cells were fixed 15 min at room temperature in 3% PFA, washed in PBS and counted in a hematocytometer. Samples of 5×10^5 cells were incubated 15 min with 0.1 M glycine, pH 7.5, and further permeabilized by 1% Triton X-100 in PBS, 30 min at room temperature. The cells were pelleted, washed, and pretreated 10 min in 3% H_2O_2. After serial washes, cell suspensions were incubated 1 h at room temperature in 5% BSA/PBS, then overnight at 4 °C with the first antibody, in the same buffer with 0.05% NaN_3. After serial washes in 0.05% Tween/PBS, cells were incubated with a second, peroxidase-conjugated antibody in 3% BSA/PBS, for 2 h at room temperature. After washings in 0.05% Tween, the pellet was resuspended and immobilized enzyme conjugates were revealed by a chromogenic substrate. Cell numbers were estimated by methylene blue staining as described elsewhere [26].

2.7. Caspase assay

The method was adapted from published procedures [27,28]. Keratinocytes were detached and resuspended as above. The suspension was homogenized in a Dounce homogenizer in 42 mM KCl, 1 mM dithiothreitol, 5 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 0.1 mg/ml BSA in 10 mM HEPES, pH 7.4, and submitted to complementary sonication. Protein concentration, evaluated by Bradford determination, was brought to 5–10 mg/ml in homogenization buffer. Enzyme activities were measured in samples containing 50 μg protein, 1 μM *N*-acetyl-Tyr–Val–Ala–Asp-amino-methyl-coumarin (*N*-Ac-YVAD-AMC) (caspase 1), or 1 μM *N*-acetyl-Asp–Glu–Val–Asp-amnio-methyl-coumarin (*N*-Ac-DEVD-AMC) (caspase 3), in extraction buffer and in 100 μl final volume. For specificity controls, 1 μM caspase 1 inhibitor *N*-acetyl-Tyr–Val–Ala–Asp-CHO (*N*-Ac-YVAD-CHO) or 1 μM caspase 3 inhibitor *N*-acetyl-Asp–Glu–Val–Asp-CHO (*N*-Ac-DEVD-CHO) was added. Released amino-methyl-coumarin was monitored at 20 °C, using 360 and 460 nm as excitation and emission wavelengths, respectively.

3. Results

3.1. Cell death and cell detachment, induced by PV-IgG or by Fas pathway inducers, are enhanced in aged keratinocytes

Keratinocytes from newborn foreskins were grown to confluence after 1–2 passages (young cells) or 4–5 passages (aged cells) as described in Section 2. The validity of passage 4 cells as senescent was confirmed by poor plating efficiency and quasi-absence of proliferation upon attempts of further passaging. Senescence-related markers were checked as shown in Table 1. Senescence-associated (SA) β-galactosidase [29], p16 [30], protein content per cell [26] and intrinsic fluorescence related to lipofuscin accumulation [31] were significantly increased, while the proliferation marker Ki 67 [32] was decreased, and the keratinocyte early differentiation marker involucrin [33] remained unchanged, a pattern clearly featuring replicative senescence. The young and aged cultures were incubated with 1.5 μg/ml of Fas receptor-specific monoclonal antibody (anti-Fas) combined with 10 ng/ml of IFNγ, or with 2.5 mg/ml of immunoglobulins from pemphigus vulgaris patients (PV-IgG), or with 50 μg/ml of cycloheximide (CHX), a control inducer activating the Fas-dependent pathway of apoptosis [34]. After 5 days, the three treatments caused cell

Table 1
Enhancement of senescence markers in aged keratinocyte cultures

Biomarker	Relative increase
SA β-galactosidase	2.5 ± 0.2
P16	2.1 ± 0.5
Protein content per cell	2.0 ± 0.4
Intrinsic fluorescence at 480/560 nm	2.0 ± 0.2
Average cell size	1.6 ± 0.3
Involucrin	1.1 ± 0.1
Ki 67	0.6 ± 0.1

Relative increase is the ratio of parameters determined in keratinocytes at passage 4 versus passage 2. Specific markers were determined by immunofluorescent labeling and flow cytometric analysis as described in Section 2. Intrinsic fluorescence at 480/560 nm measures the age-related accumulation of lipofuscin in cells [31].

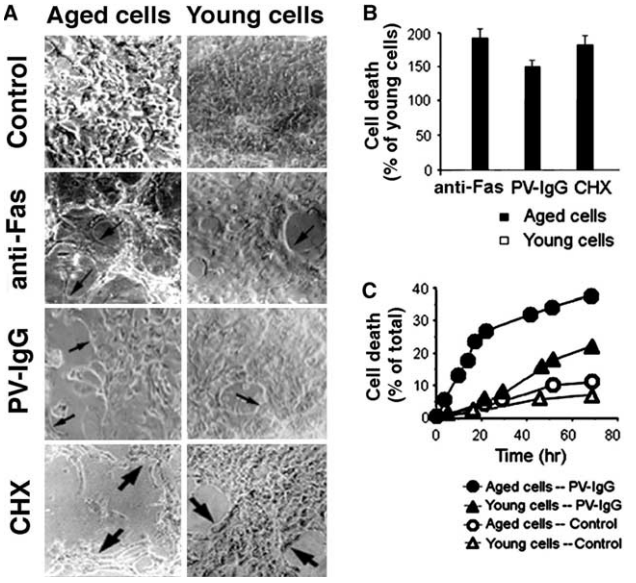

Fig. 1. Replicative senescence increases keratinocyte sensitivity to cell death. (A) Keratinocytes were grown to confluency after two passages (young cells) or four passages (aged cells) and incubated five days with 1.5 μg/ml monoclonal anti-Fas + 10 ng/ml IFNγ, or with 2.5 g/ml PV-IgG, or with 50 μg/ml CHX, or with 4 mg/ml N-IgG as control. Boundaries of in vitro "lesions" are pointed by arrows. Magnification 200×. (B) After three days of PV-IgG treatment, dead cells were counted by trypan blue staining. Dead cell ratios in aged cultures were averaged from four independent experiments, taking the values for their young counterparts as 100%. (C) Percentages of dead cells were measured by trypan blue staining at various times in young and aged cultures.

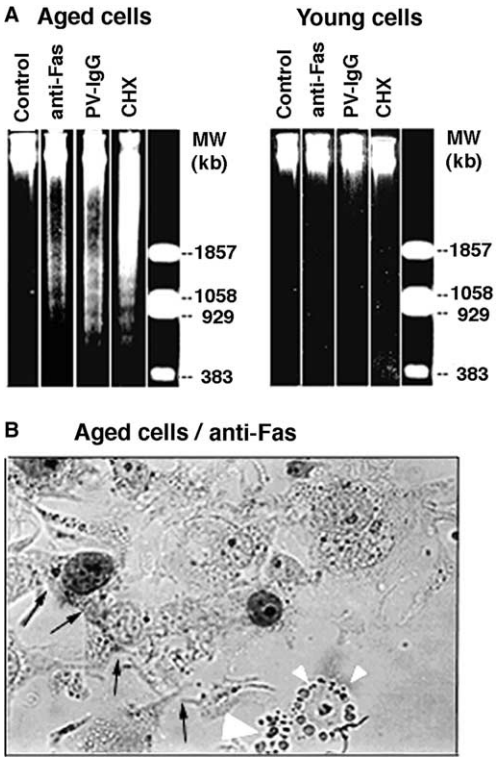

Fig. 2. Induction of apoptotic appearance in cultured keratinocytes. Human keratinocytes were grown and treated as in Fig. 1A. (A) DNA was extracted and 2 μg of DNA samples was electrophoresed on agarose gels. (B) This phase-contrast micrography shows typical apoptotic characteristics induced by anti-Fas antibody in aged keratinocytes. Magnification 1000×.

death and detachment, forming cleared areas that were larger and more abundant in aged cultures than in young. Such lesions were absent in untreated cultures, or in cultures treated by IFNγ alone, or by immunoglobulins from healthy donors (Fig. 1A). Dead cells were counted in situ after 3 days (i.e., before lesion formation) using trypan blue vital staining. There were 1.5 to 2 times more dead cells in aged cultures than in young, depending on the inducer (Fig. 1B). No cell killing was observed in control samples, treated with 4 mg/ml IgG from healthy donors (N-IgG). Furthermore, kinetic measurements showed that cell death induction by PV-IgG was faster in aged cultures (Fig. 1C), in accordance with the observation that inducers of Fas-mediated apoptosis kill aged cells more readily [22].

3.2. *DNA fragmentation and morphological changes, induced by PV-IgG or by Fas pathway inducers in keratinocytes, are enhanced in aged cells*

Keratinocyte cultures were treated as above, DNA was extracted, and analyzed by electrophoresis. DNA fragmentation and characteristic 190 bp ladders appeared in aged cell extracts treated with either of the three inducers, but not in young cells, and not in untreated controls (Fig. 2A). Under microscope, trypan blue revealed more dead cells in aged cultures, as well as cleared areas consecutive to cell detachment, surrounded by morphologically altered cells (Fig. 2B) and typical apoptotic features, like blebbing membranes (small white arrowheads) or apoptotic bodies (large white arrowhead).

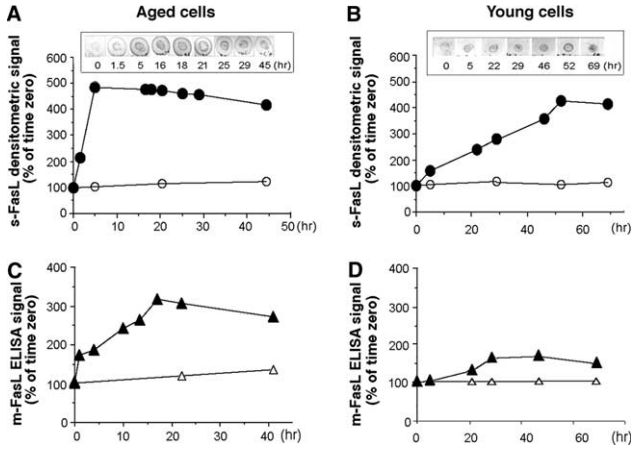

Fig. 3. Fas L induction by PV-IgG is enhanced in aged keratinocytes. Young (passage 2) and aged (passage 4) keratinocytes were grown to confluence in 24-well plates and treated by PV-IgG as above. Soluble and membranal forms of Fas L were monitored by specific antibody binding. (A) and (B) s-Fas L was detected in dot blots of growth medium as described in Section 2. Relative staining was measured by densitometry and plotted versus time of treatment. (C) and (D) m-Fas L amounts were determined by cellular ELISA. In this figure, solid symbols stand for treated cells and open symbols for controls treated with 4 mg/ml N-IgG.

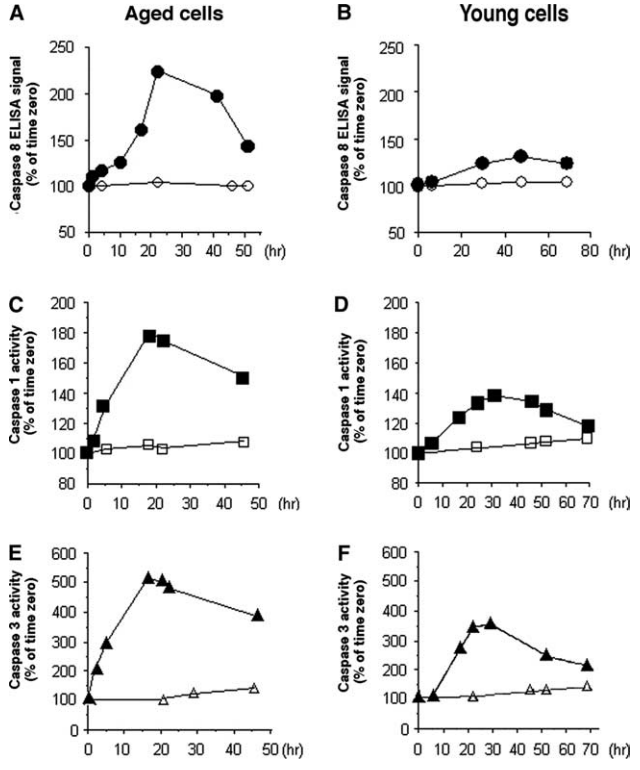

Fig. 4. Caspase induction by PV-IgG is enhanced in aged keratinocytes. Young and aged keratinocytes were grown to confluence and treated by PV-IgG as above. (A) and (B) Amounts of caspase 8 protein were determined by cellular ELISA. Caspase 1 (C, D) and caspase 3 (E, F) were assayed in cellular extracts using fluorogenic substrates as described in Section 2. 100% activity represent 76.7 RFU/h for caspase 1 and 104 RFU/h for caspase 3. Solid symbols stand for treated cells and open symbols for uninduced controls treated with 4 mg/ml N-IgG.

3.3. PV-IgG-mediated Fas L induction is enhanced and accelerated in aged cells

Fas L is the natural activator of Fas receptor, found either as a type-II transmembrane protein (m-Fas L), or as a soluble, proteolytically derived fragment (s-Fas L). Both forms have been ascribed the capacity to induce apoptosis [35]. We made kinetic measurements of Fas L appearance, following PV-IgG treatment, in cellular bodies and in cell supernatant, assaying s-Fas L by dot blots and m-Fas L by cellular ELISA. s-Fas L concentration increased 5-fold after 5 h of treatment in aged keratinocyte cultures (Fig. 3A), whereas the same increase took 50 h in young cultures (Fig. 3B). In parallel, m-Fas L ELISA signals were increased 3.2-fold after 17 h in aged cultures (Fig. 3C) and only 1.7-fold after 30 h in young cultures (Fig. 3D).

3.4. PV-IgG-mediated caspase induction is accelerated and amplified in aged keratinocytes

Caspases are a group of cystein proteases involved in different apoptotic steps. Caspases 8 and 10 activate the extrinsic death pathway, and caspase 9 activates the intrinsic, mitochondrial pathway [18], while caspase 1 works mainly as a cytokine precursor activator, and caspase 3 as an executor of apoptosis [36], We measured the amounts of caspase 8 by cellular ELISA at different times, in keratinocyte cultures under PV-IgG treatment The increase in caspase 8 was 2-fold larger and 2-fold faster in aged cells than in young (Fig. 4A and B), and consistently caspase 1 and caspase 3 activities increased more in aged cell than in young cell lysates (Fig. 4C–F). This showed that the apoptotic process was induced in its different stages and to a larger extent in aged keratinocytes.

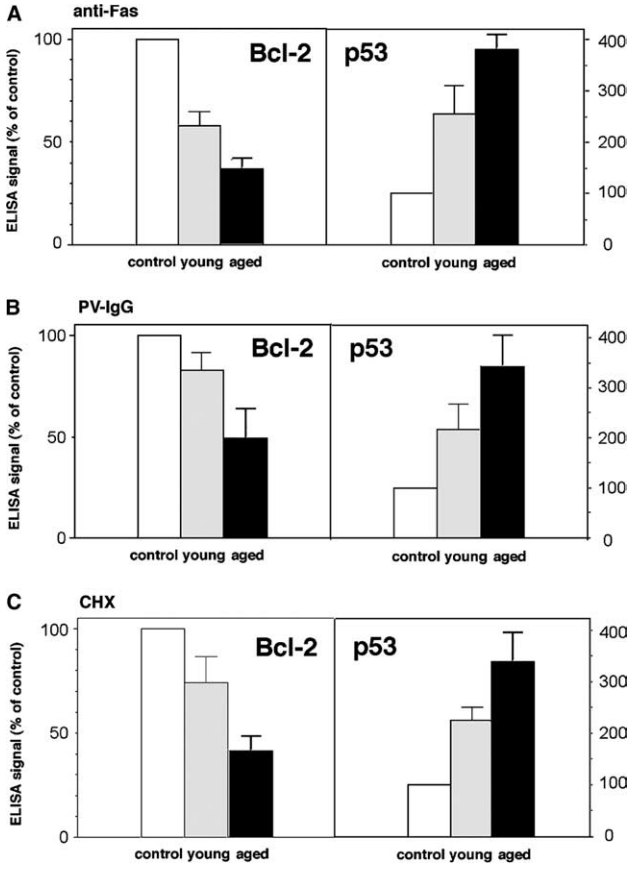

Fig. 5. Regulation ofBcl-2 and p53 by PV-IgG is enhanced in aged keratinocytes. Young and old keratinocytes were grown to confluence and treated by PV-IgG as above for 3 days. Amounts of Bcl-2 and p53 proteins were determined by cellular ELISA in four independent experiments. In each experiment, 100% corresponded to the untreated control.

3.5. PV-IgG-mediated induction of intrinsic apoptosis is enhanced in aged keratinocytes

We investigated the fate of Bcl-2 and p53, two proteins involved in the mitochondrial pathway of apoptosis as protective and inducing effector, respectively [18], Aged and young keratinocyte cultures were treated for 3 days with anti-Fas antibody, PV-IgG or CHX [34]. Using cellular ELISA, we observed that Bcl-2 was down-regulated and p53 was up-regulated, in accordance with their respective roles in the apoptotic process. Consistently, this effect was enhanced in aged cells by the three apoptosis inducers used (Fig. 5).

3.6. Aged keratinocytes are resistant to protection against apoptotic death

Apoptosis can be inhibited by specific reagents that prevent its activation steps or inhibit its effector enzymes. Caspase 1 inhibitor YVAD-CHO inhibits cell death in different cell types [37,38], and anti-FasL antibodies prevent Fas-mediated apoptosis in vivo and in vitro [39]. Keratinocytes were treated 4 days with anti-Fas or PV-IgG, in the presence or in the absence of caspase 1 inhibitor. Fig. 6A and B compares death scores observed in young and aged keratinocytes treated by anti-Fas or by PV-IgG, taking the scores of uninduced controls as 100%. In aged cells, the level of dead cells was higher, and

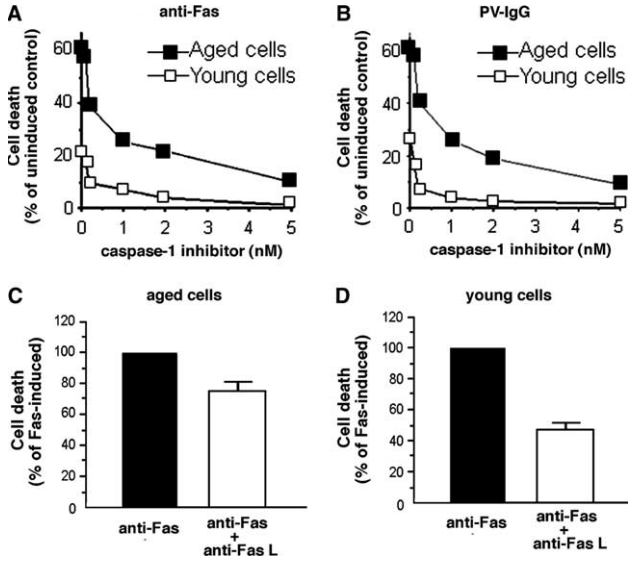

Fig. 6. Apoptotic death is more difficult to suppress in aged cells. (A) and (B) Young and aged keratinocytes were grown to confluence and treated by monoclonal anti-Fas or by PV-IgG as above for 4 days, with various concentrations of caspase 1 inhibitor. Death ratios were determined by trypan blue staining. Control values obtained in the absence of treatment (for anti-Fas) or in the presence of 4 mg/ml N-IgG (for PV-IgG) were taken as 100%. Solid symbols stand for aged cells and open symbols for young cells. (C) and (D) Cells were incubated with monoclonal anti-Fas in the presence or in the absence of anti-Fas L antibodies. Death ratios were measured in four independent experiments. In each experiment, the value obtained without anti-Fas L was taken as 100%.

decreased only partially with increasing concentrations of caspase 1 inhibitor, showing that the inhibitor was poorly protective. Then, keratinocyte cultures were treated with 1.5 μg/ml anti-Fas and 4 μg/ml anti-Fas L antibodies for four days. The death scores were $74 \pm 6\%$ in aged cells and $44 \pm 4\%$ in young cells, taking controls without anti-Fas L as 100% (Fig. 6C and D). These results show that aged cells are not only more prone to apoptotic death, but their apoptotic course is also more difficult to suppress.

4. Discussion

The apoptotic equilibrium is known to be altered in a variety of aged tissues. Excessive apoptosis is associated with functional decline in aged heart, liver, kidney, immune system and brain [40], and with neurodegenerative and autoimmune diseases [41]. Balance between proliferation and apoptosis is lost in the development of tumors [41], an age-dependent phenomenon. Finally, oncogenes and cell cycle regulators contribute to the apoptotic pathway [42,43], suggesting that apoptosis may be linked to tumorigenesis and cellular senescence.

First attempts to induce apoptosis in aged cells suggested that human fibroblasts [16] and keratinocytes [15,17] were resistant to apoptosis at senescence. In all these experiments, however, the inducing stimuli (growth factor deprivation, calcium shock, UV irradiation) activated the intrinsic pathway of apoptosis, mediated via the mitochondrial system [18]. When the extrinsic, cell signaling-mediated pathway was addressed, senescent cells were found competent for, and even sensitized to, apoptosis induction, both in human fibroblasts [21] and in keratinocytes [22]. These data restored consistency with in vivo evidence that Fas-mediated apoptosis is enhanced in age-related disorders [13,41,44], as well as in normal ageing [45].

We previously showed that autoimmune PV-IgG can induce apoptosis in human keratinocytes [23]. In this work, we compared this effect in young and aged cell cultures, where senescence of aged samples was characterized using biomarkers of cellular senescence and differentiation. Replicative senescence increased keratinocyte propensity to apoptose, upon PV-IgG as upon anti-Fas treatment, and the two inducers caused similar symptoms. Hence, we can conclude that PV-IgG activates the extrinsic apoptosis pathway, the putative cause of the lesional process in pemphigus [23], more readily in senescent cells. This finding is consistent with our previous observation that senescent keratinocytes are more sensitive to Fas-mediated apoptosis [22]. It is noteworthy that p53 and Bcl-2, two participants of the intrinsic apoptosis pathway, were also mobilized in the PV-IgG-induced process, possibly because they contributed to a mitochondrial amplification loop [18] secondary to activation of the Fas pathway.

A wealth of data convincingly suggests that cellular senescence is associated with, and involved in, organismal ageing [9]. Senescence markers are overexpressed in aged tissues [29]. Fibroblasts from old donors [46], or from patients with premature ageing syndromes [47], or from donors submitted to photoageing [48], have a shorter life span when transplanted in vitro. Mutations that enhance cellular senescence cause premature ageing and early death in engineered mice [49,50]. We therefore speculate that increased cellular senescence in aged epidermis, and higher susceptibility of senescent cells to PV-IgG-induced apoptosis, may be a cause of the increased frequency and severity of pemphigus disease observed in the elderly [51], on top of immunosenescence that enhances autoimmunity [52]. Furthermore, the fact that senescent cells are more sensitive to the extrinsic apoptosis pathway turns out to be a properly shared by different cell types [21,22]. Since many degenerative and autoimmune pathologies seem to proceed via a Fas-mediated apoptotic pathway [13,41,44], age-relatedness of these diseases may also be attributed to the increased sensitivity of senescent cells to cell signaling-induced apoptosis. This highlights the perspective that regulators of extrinsic apoptosis may be used to control these disorders, not only for the treatment of acute symptoms, but also to address the more general propensity of aged tissues to apoptose upon stimulation by external signals, which may be a cause for many age-related diseases.

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References

[1] West, M.D. (1994) Arch. Dermatol. 130, 87–95.
[2] Yaar, M., Eller, M.S. and Gilchrest, B.A. (2002) J. Invest. Dermatol. Symp. Proc. 7, 51–58.
[3] Gerstein, A.D., Phillips, T.J., Rogers, G.S. and Gilchrest, B.A. (1993) Dermatol. Clin. 11, 749–757.
[4] Gilchrest, B.A., Stoff, J.S. and Soter, N. (1982) J. Invest. Dermatol. 79, 11–15.
[5] Wei, Q. (1998) J. Invest. Dermatol. Symp. Proc. 3, 19–22.
[6] Chen, Q.M. (2000) Ann. N.Y. Acad. Sci. 908, 111–125.
[7] Moriwaki, S., Ray, S., Kraemer, K.H. and Grossmann, L. (1996) Mutat. Res. 364, 117–123.
[8] Toussaint, O., Medrano, E.E. and von Zglinicki, T. (2000) Exp. Gerontol. 35, 927–945.
[9] Campisi, J. (2001) Exp. Gerontol. 36, 607–618.
[10] Ellis, R.E., Yuan, J.Y. and Horvitz, H.R. (1991) Annu. Rev. Cell. Biol. 7, 663–698.
[11] Parkinson, E.K., Munro, J., Steeghs, K., Morrison, V., Ireland, H., Forsyth, N., Fitzsimmons, S. and Bryce, S. (2000) Biochem. Soc. Trans. 28, 226–233.
[12] Nagata, S. and Golstein, P. (1995) Science 267, 1449–1456.
[13] Muradian, K. and Schachtschabel, D.O. (2001) Z. Gerontol. Geriatr. 34, 441–446.
[14] Itahana, K., Dimri, G. and Campisi, J. (2001) Eur. J. Biochem. 268, 2784–2791.
[15] Norsgaard, H., Clark, B.C. and Rattan, S.I.S. (1996) Exp. Gerontol. 31, 563–570.
[16] Wang, E. (1995) Cancer Res. 55, 2284–2292.
[17] Chatuverdi, V., Qin, J.Z., Denning, M.F., Choubey, D., Diaz, M.O. and Nickoloff, B.J. (1999) J. Biol. Chem. 274, 23358–23367.
[18] Gross, A. (2001) IUMBMB Life 52, 231–236.
[19] Tepper, C.G., Seldin, M.F. and Mudryj, M. (2000) Exp. Cell Res. 260, 9–19.
[20] Seluanov, A., Gorbunova, V., Falcovitz, A., Sigal, A., Milyavsky, M., Zurer, I., Shohat, G., Goldfinger, N. and Rotter, V. (2001) Mol. Cell. Biol. 21, 1552–1564.
[21] DeJesus, V., Rios, I., Davis, C., Cheng, Y., Calhoun, D., Zaker, Z. and Hubbard, K. (2002) Exp. Cell Res. 274, 92–99.
[22] Wang, X., Brégégère, F., Soroka, Y., Khiayat, A., Redziniak, G. and Milner, Y. (2003) Mech. Ageing Dev. 125, 237–249.
[23] Wang, X., Brégégère, F., Frusic-Zlotkin, M., Feinmesser, M., Michel, B. and Milner, Y. (2003) Apoptosis 9, 131–143.

[24] Stanley, J.K. (1997) J. Dermatol. 24, 741–743.
[25] Crissman, H.A., Oka, M.S. and Steinkamp, J.A. (1976) J. Histochem. Cytochem. 24, 64–71.
[26] Brégégère, F., Soroka, Y., Bismuth, J., Friguet, B. and Milner, Y. (2003) Exp. Gerontol. 38, 619–629.
[27] Enari, M., Talanian, R.V., Wong, W.W. and Nagata, S. (1996) Nature 380, 723–726.
[28] Suzuki, A., Iwazaki, M. and Wagai, N. (1997) Exp. Cell Res. 233, 48–55.
[29] Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M. and Campisi, J. (1995) Proc. Natl. Acad. Sci. USA 92, 9363–9367.
[30] Rheinwald, J.G., Hahn, W.C., Ramsey, M.R., Wu, J.Y., Guo, Z., Tsao, H., De Luca, M., Catricala, C. and O'Toole, K.M. (2002) Mol. Cell. Biol. 22, 5157–5172.
[31] Porta, E.A. (2002) Ann. N.Y. Acad. Sci. 959, 57–65.
[32] Schwarting, R., Gerdes, J., Niehus, J., Jaeschke, L. and Stein, H. (1986) J. Immunol. Methods 90, 65–70.
[33] Watt, F.M. (1983) J. Invest. Dermatol. 81, 100s–103s.
[34] Tang, D., Lahti, J.M., Grenet, J. and Kidd, V.J. (1999) J. Biol. Chem. 274, 7245–7252.
[35] Schmitz, I., Kirchhoff, S. and Krammer, P.H. (2000) Int. J. Biochem. Cell Biol. 32, 1123–1136.
[36] Grutter, M.G. (2000) Curr. Opin. Struct. Biol. 10, 649–655.
[37] Suzuki, A., Iwasaki, M., Kato, M. and Wagai, N. (1997) Exp. Cell Res. 233, 41–47.
[38] Hisahara, S., Takano, R., Shoji, S., Okano, H. and Miura (2000) J. Neural Transm. Suppl. 58, 135–142.
[39] Suzuki, N., Ichino, M., Mihara, S., Kaneko, S. and Sakane, T. (1998) Arthritis Rheum. 41, 344–353.
[40] Joaquin, A.M. and Gollapudi, S. (2001) J. Am. Geriatr. Soc. 49, 1234–1240.
[41] Fadeel, B., Orrenius, S. and Zhivotovsky, B. (1999) Biochem. Biophys. Res. Commun. 266, 699–717.
[42] Bringold, F. and Serrano, M. (2000) Exp. Gerontol. 36, 607–618.
[43] Zhang, Y. and Herman, B. (2002) Mech. Ageing Dev. 123, 245–260.
[44] Kawakami, A. and Eguchi, K. (2002) Med. Electron. Microsc. 35, 1–8.
[45] Campisi, J. (2003) Exp. Gerontol. 38, 5–11.
[46] Stanulis-Praeger, B. (1987) Mech. Ageing Dev. 38, 1–48.
[47] Martin, G.M., Sprague, C.A. and Epstein, C.J. (1970) Lab. Investig. 23, 86–92.
[48] Gilchrest, B.A. (1980) J. Gerontol. 35, 537–541.
[49] Rudolph, K.L., Chang, S., Lee, H.W., Blasco, M., Gottlieb, G.J., Greider, C. and De Pinho, R.A. (1999) Cell 96, 701–712.
[50] Donehower, L.A. (2002) J. Cell Physiol. 192, 23–33.
[51] Udey, M.C. and Stanley, J.R. (1999) JAMA 282, 572–576.
[52] Stacy, S., Krolick, K.A., Infante, A.J. and Kraig, E. (2002) Mech. Ageing Dev. 123, 975–985.