The amyloid β peptide Aβ (25–35) induces apoptosis independent of p53

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Abstract Apoptosis of neuronal cells apparently plays a role in Alzheimer's disease (AD). The amyloid beta (Aβ) peptide derived from β-amyloid precursor protein is found in AD brain in vivo and can induce apoptosis in vitro. While p53 accumulates in cells of AD brain, it is not known if p53 plays an active role in Aβ-induced apoptosis. We show here that inactivation of p53 in two experimental cell lines, either by expression of the papillomavirus E6 protein or by a shift to restrictive temperature, does not affect apoptosis induction by Aβ (25–35), indicating that Aβ induces apoptosis in a p53-independent manner.

Key words: Alzheimer's disease; Amyloid beta (25–35); p53; HPV-16 E6; HPV-16 E7; Apoptosis

1. Introduction

Programmed cell death or apoptotic cell death is regularly detected in the brain of human Alzheimer's disease (AD) patients [1,2] and it has been suggested that apoptosis plays an important role in the degeneration of neuronal tissue observed in AD [3,4]. While differential expression of various apoptosis-regulating genes was recently demonstrated in brains of AD patients versus control groups [5], final proof for this hypothesis is still missing. A degradation product of the β-amyloid precursor protein (βAPP), referred to as β-amyloid protein or Aβ, is found in the neuronal plaques typical for AD (for review, see [6]). It was shown that addition of purified Aβ peptide and of its active fragment Aβ (25–35) can induce apoptosis in a variety of mammalian cell types in vitro [7–11]. These findings raise the possibility that Aβ may be responsible for apoptotic cell death observed in the brains of AD patients. This is supported by some transgenic mouse models for AD, which display neuronal loss associated with Aβ deposition (for example, see [12,13]).

From many studies in non-neuronal cell types, in particular fibroblasts and epithelial cells, a key role for p53 as regulator of apoptotic cell death has been inferred (for review, see [14]). In particular, activation of p53 in response to various stress stimuli leads to increased expression of several proapoptotic genes, such as Bax, IGFBP-3 and Fas (for review, see [15]). Circumstantial evidence suggests that p53 may also play a role in apoptotic death occurring in the brain of AD patients. Thus, it was shown that p53 expression is increased in the brain of AD patients [16] and in the brain of Aβ transgenic mice [12]. However, a functional role for p53 in Aβ-induced apoptosis is not established by these studies. To address this question, we used two experimental cell systems, derived from normal human epithelial cells and from human osteosarcoma cells, respectively, which have been established previously to perform a functional analysis of p53 in a defined genetic background. Since it was shown before that Aβ (25–35) can induce apoptosis in various non-neuronal cell types [7,9–11,17], the analysis of the effects of Aβ (25–35) in our cellular system should allow a study of the role of p53 in Aβ-induced apoptosis. The results of our experiments demonstrate that Aβ (25–35) induces apoptosis in human keratinocytes as well as the Saos-2 osteosarcoma cell line and suggest that p53 is not essential for Aβ-induced apoptosis.

2. Materials and methods

2.1. Cell culture

Primary human keratinocytes were isolated from human newborn foreskin and infected with the pLXSN retroviral vector expressing the HPV-16 E6 and E7 genes, respectively, as described [18]. Individual clones were selected from these cultures and expression of the viral genes confirmed at the mRNA and protein level (Whitaker et al., manuscript in preparation). Keratinocytes were cultured in keratinocyte growth medium (Promocell, Heidelberg, Germany). Saos-2 and X4.4 cells (gifts from C. Larsen, Poitiers, France) were cultured in DMEM (Sigma, Vienna, Austria) with 10% fetal calf serum.

2.2. Aβ (25–35)

For this study we used the active aggregated fragment of Aβ, Aβ (25–35) (Sigma, Vienna, Austria). This peptide induces apoptosis in a variety of cell types [19]. It was dissolved in water (stock solution: 1 mg/ml).

2.3. Assessment of apoptosis

Cells growing in 75 cm^2 culture flasks were harvested by a 5–10 min exposure to trypsin–EDTA (Life Technologies, Paisley, UK) and washed twice. Cells ($1–5\times10^5$) were then seeded into the wells of six-well plates (Costar Corning, NY, USA) and cultured for 24 h in growth medium. Thereafter, the cells were washed and Aβ (25–35) was added to the culture medium at concentrations of 2, 5 and 10 μg/ml. The cells were incubated for 24 h. For the assessment of apoptosis, cells were harvested by application of trypsin–EDTA (Life Technologies, Paisley, UK), washed twice in phosphate buffered saline (PBS) and then gently resuspended in 200 μl propidium iodide (PI; Sigma, Vienna, Austria) with Triton X-100 (0.1%; Sigma, Vienna, Austria). The PI fluorescence of individual nuclei was measured using a FACS scan flow cytometer (program CellQuest, Becton Dickinson, Mountain View, USA). Red fluorescence due to PI staining of DNA was expressed on a logarithmic scale and the forward scatter (FSC) of particles was measured simultaneously. Five thousand events were measured on the scatter gate. All measurements were performed under identical instrumental settings. This technique identifies a population of apoptotic nuclei that has a lower fluorescence intensity than diploid nuclei but is clearly distinct from debris and non-viable cells. The number of apoptotic nuclei is expressed as a percentage of the total number of events. Nuclear labelling with propidium iodide is a quick and reliable method for the assessment of apoptosis which is used by many laboratories [20–22]. Apoptosis detection by Annexin V

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binding was carried out by using Annexin V (human recombinant, Alexis Biochemicals, Läufelfingen, Switzerland) according to the manufacturer's recommendations.

2.4. Western blotting

Equal amounts of protein (20 μg pro lane) were separated on a 12.5% SDS–PAGE gel. After wet transfer on a PVDF membrane (Boehringer Mannheim, Germany), filters were blocked with non-fat milk and incubated with primary monoclonal antibodies Anti-p53 (clone Do-1, Santa Cruz Biotechnologies, Heidelberg, Germany), Anti-Bax (B-9, Santa Cruz Biotechnologies), Anti-Bcl-2 (Clone 124, Boehringer Mannheim), p21(WAF-1) (Santa Cruz Biotechnologies) and cdk4 (Transduction Laboratories, Hamburg, Germany). The filters were then incubated with a secondary, horseradish peroxidase-conjugated, goat anti-mouse antibody (Dako, Glostrup, Denmark) and the protein bands were visualized with a chemiluminescent substrate detection system ECL (Amersham, Vienna, Austria). The quantification of Western blots was performed by densitometric scanning of the autoradiograms; expression of M2 pyruvate kinase served as an internal standard and loading control.

3. Results and discussion

To determine the role of p53 in Aβ-mediated apoptosis, human keratinocytes were used in which p53 had been inactivated by stable expression of the E6 oncogene encoded by human papillomavirus type 16 (HPV-16) from a E6-expressing retroviral vector ([18]; Whitaker, manuscript in preparation). It has previously been shown that E6 triggers degradation of p53 via the ubiquitin–proteasome pathway [23], an activity which depends on a third protein, E6-AP which serves as specific ubiquitin ligase for p53 (for recent review, see [24]). E6-expressing keratinocytes in early culture can grow indefinitely but, unlike many p53-deficient tumor cells, do not display a grossly transformed phenotype. To determine the role of p53 for Aβ-induced apoptosis under physiological conditions, the effects of Aβ on E6-expressing keratinocytes were compared with results obtained in primary keratinocytes. As a further control, human keratinocytes expressing the E7 gene of HPV-16 were used. It has previously been shown that E7 binds and inactivates the proteins encoded by the retinoblastoma gene family (for review, see [25]). E7 does not reduce the stability of p53, but rather leads to its accumulation and p53 may well play a role for the well-documented induction of apoptosis by HPV-16 E7 [26]. When the p53 status of the three cell types was compared by Western blotting, we found that, as expected, p53 was easily detected in primary keratinocytes but absent from E6-expressing cells (Fig. 1) and expression of p21 followed the expression pattern of p53. The levels of both p53 and p21 were significantly induced in E7-expressing cells, in keeping with the reported ability of E7 to induce stabilization of p53 [26].

To directly determine the ability of Aβ to induce apoptosis in our keratinocyte model, the cells were incubated with increasing concentrations of an amyloidogenic peptide. While Aβ (1–42) is the amyloidogenic peptide that is usually observed in AD brains, its experimental use is hampered by its limited in vitro stability in an aggregated, fibrillary state. A shorter peptide, referred to as Aβ (25–35), is currently used in most of the published literature since it is aggregated and reasonably stable in vitro. Furthermore, it was shown that Aβ (25–35) and Aβ (1–42) display very similar biological activity in vitro (for example, see [10,17,27,28]). When Aβ (25–35) was added to primary keratinocytes, a significant and dose-dependent increase in the percentage of apoptotic cells

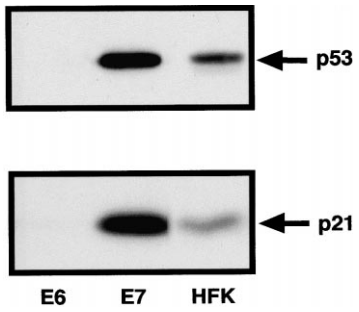

Fig. 1. The expression of p53 and p21 in primary keratinocytes (HFK) and in cells immortalized with HPV-16 E6 or E7 protein is shown in a Western blot. HPV-16 E6 immortalized keratinocytes do not contain detectable p53 and p21 proteins.

was observed (Fig. 2A), resulting in a nearly five-fold increase in apoptotic cell death at an Aβ (25–35) concentration of 10 μg/ml. As shown in Fig. 2B, more than 30% of the total cell population undergo apoptosis at 24 h after addition of Aβ (25–35), indicating that Aβ (25–35) provides a strong apoptotic signal for human keratinocytes, as was observed in other cell types before [7,9–11,17]. To address the role of p53 for Aβ-induced apoptosis, the experiment was repeated with the E6-expressing keratinocyte line, which does not contain detectable amounts of p53 protein (Fig. 1). Surprisingly, a strong apoptotic effect was induced by Aβ (25–35) also in this cell line (Fig. 2A and B), which is similar to the effect seen in primary keratinocytes expressing normal levels of p53. When Aβ (25–35) was added to E7-expressing cells, expressing elevated levels of p53 protein, again a strong apoptotic effect was observed, further supporting the conclusion that the expression level and function of p53 is irrelevant for Aβ-induced apoptosis. Aβ-induced apoptosis was also analyzed by the Annexin V detection system, which is based on the exposure of phosphatidylserine to the extracellular medium, an early event in apoptosis (for review, see [29]). Using this method, again a p53-independent induction of apoptosis by Aβ (25–35) was observed (Fig. 2C).

The results shown above indicate that inactivation of p53 by HPV-16 E6 does not affect Aβ-induced apoptosis, while E6 does suppress apoptosis induced by other stimuli [30–32], most likely by inactivating p53, a key regulator of apoptosis. While specific inactivation of p53 by E6 appears to be a normal consequence of HPV infection, it is now clear that E6 also affects the function of other cellular proteins different from p53 (for review, see [33]). We therefore also wanted to analyze the role of p53 in Aβ-mediated apoptosis in a different cellular background. While the data shown in Fig. 2 clearly indicate that p53 is dispensable for Aβ-mediated apoptosis in our keratinocyte model system, we were interested to know if short term modulation of p53 function may affect apoptosis

Table 1
The influence of Aβ (25–35) (10 μg/ml) on the induction of apoptosis in X4.4 and Saos-2 cells at 32 and 37°C, respectively

	32°C		37°C	
	Co	Aβ (25–35)	Co	Aβ (25–35)
X4.4	1.7 ± 0.4	14.5 ± 1.2	1.8 ± 0.7	11.2 ± 3.7
Saos-2	1.5 ± 0.1	19.2 ± 1.5	2.4 ± 0.7	12.0 ± 0.8

Results represent the percentages of apoptotic cells ($\bar{x} \pm$ S.E.M., $n = 5$) after a 24 h incubation.

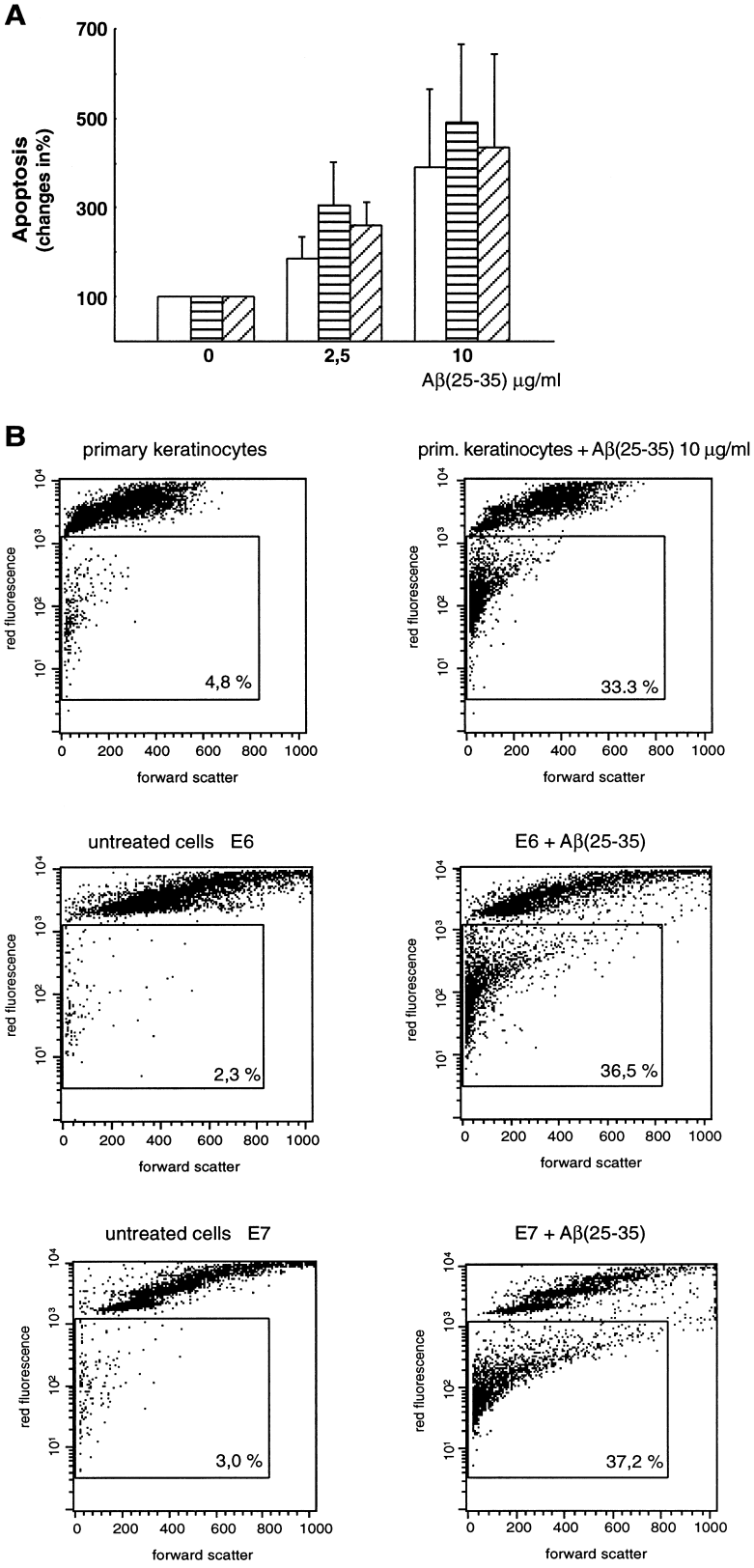

Fig. 2. A: The influence of the expression of p53 on the induction of apoptosis by Aβ (25–35) (2, 5 and 10 µg/ml) in primary keratinocytes or cells immortalized with HPV-16 E6 and E7 protein. The keratinocytes immortalized with HPV E6 (horizontally hatched bars) do not contain detectable p53 protein. In contrast primary keratinocytes (open bars) and keratinocytes immortalized with E7 protein (diagonally hatched bars) produce p53. The error bars represent $\bar{x} \pm$ S.E.M. ($n = 6$). B: A representative experiment of a FACS flow-cytometry analysis on the induction of apoptosis by Aβ (25–35, 10 µg/ml) in primary keratinocytes or cells immortalized with HPV-16 E6 and E7. Analysis was performed by determination of forward scatter (*x*-axis) and propidium iodide staining (*y*-axis). C: FACS flow-cytometry analysis by annexin V staining. Aβ (25–35, 10 µg/ml) was added to keratinocytes immortalized with HPV-16 E6 and HPV-16 E7, respectively. Apoptosis was analyzed by determination of propidium iodide staining (*x*-axis) and annexin V binding (*y*-axis), respectively.

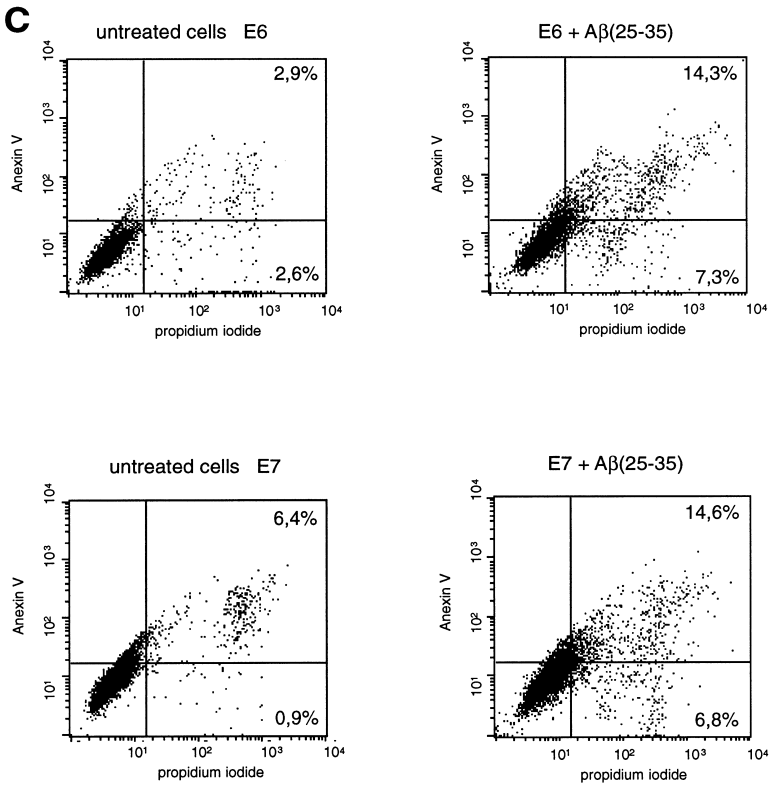

Fig. 2 (*continued*).

induction by Aβ. To this end, a cell line with inducible p53 function was used.

We used the p53 gene from *Xenopus laevis* (Xl-p53) which behaves as a temperature-sensitive mutant of p53 [34]. Saos-2 cells, a p53-deficient human osteosarcoma cell line, had been stably transfected with an expression vector for Xl-p53, to yield the cell line X4.4. Saos-2 and X4.4 cells were grown at 37°C, where Xl-p53 is inactive. Upon shifting the temperature to 32°C, Xl-p53 becomes fully active [34]. Cells were grown at 37°C or shifted to 32°C for 24 h, to activate Xl-p53 function. Activation of p53 function was monitored by determining the expression of p21(WAF-1), a well-established target gene for p53-dependent trans-activation [35]. As is shown by Western blot analysis in Fig. 3, expression of p21(WAF-1) was strongly induced by the temperature shift in X4.4 but not the parental Saos-2 cells, confirming the functional activation of the temperature-sensitive p53 protein. Subsequently, Aβ (25–35) was added and the amount of apoptosis determined. This experiment revealed that in the absence of any functional p53 (i.e. in cells grown at 37°C), Aβ (25–35) induces a 5–6-fold increase of apoptosis in both Saos-2 and X4.4 cells. When shifted to 32°C, there was no significant change in the susceptibility to Aβ (25–35)-induced apoptosis (Table 1). These results clearly indicate that p53 function is not involved in Aβ-mediated apoptosis, in agreement with the results obtained in keratinocytes (Fig. 2).

The data reported here indicate that apoptosis induced by Aβ (25–35) is not dependent on p53, suggesting that Aβ can induce apoptosis via a p53-independent pathway. Our findings would suggest that the increased levels of p53 in AD brains [16] are not linked to apoptosis induced by Aβ. It is conceivable that other proteins are responsible for Aβ-induced cell death in AD brain tissue. Bax, a protein that promotes apoptosis, was also found at increased concentrations in dystrophic neurites of senile plaques [36] and the Bax-related protein Bcl-2 is overexpressed in reactive glial cells surrounding senile plaques [37]. Kim et al. [38] reported that Aβ (25–35) decreases Bcl-2 mRNA expression, as well the ratio of the anti-apoptotic and pro-apoptotic proteins Bcl-xl/Bcl-xs. To study a potential role of Bax/Bcl-2 for apoptosis induction in our experimental system, the expression of both genes was analyzed by Western blot. We found that expression of the Bax protein is slightly increased upon Aβ (25–35)-treatment in both the E6-expressing and E7-expressing keratinocytes; in contrast, expression of Bcl-2 is not detectable in either cell line (Fig.

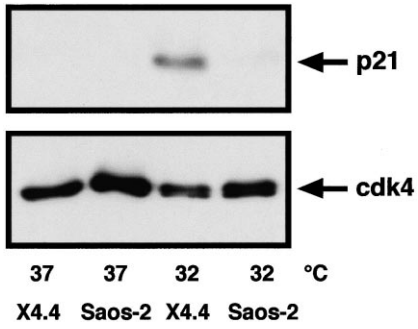

Fig. 3. X4.4 and Saos-2 were grown at 37°C or shifted to 32°C for 24 h, as indicated. The expression of p21(WAF-1) was determined by Western blot. The p53 protein from *Xenopus laevis* is not recognized by the p53 antibodies available to us, which are directed to the human protein. As a loading control, Western blots were reprobed with antibodies to cdk4.

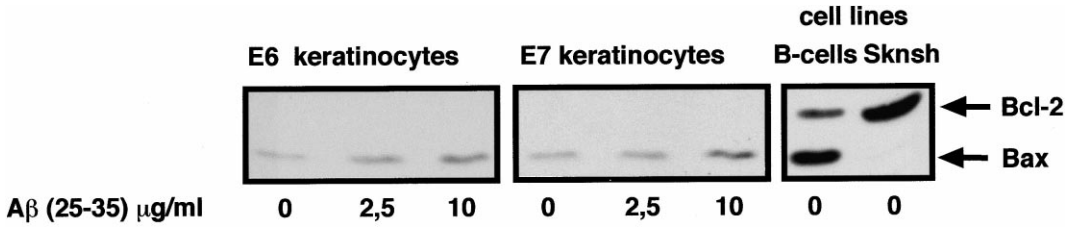

Fig. 4. Effects of Aβ (25–35) on the expression of Bax and Bcl-2. Aβ (25–35) was added in increasing concentrations to keratinocytes immortalized with HPV-16 E6 and HPV-16 E7, as indicated. Expression of Bax and Bcl-2 was determined by Western blot. Treatment by Aβ (25–35) at 2.5 μg/ml increased Bax expression in the E6-expressing keratinocytes to 169% (±39%) of the original level, and in E7-expressing keratinocytes to 156% (±10%) of the original level. In contrast, expression of Bcl-2 was not detectable in either cell line. As positive controls, extracts derived from a neuroblastoma cell line (Sk-n-sh; obtained from ATCC, Manassas, VA, USA) and EBV-transformed B-lymphocytes [39] were used.

4). These results imply a moderate increase of Bax in Aβ (25–35)-induced apoptosis in immortalized keratinocytes. While our present results indicate that apoptosis may occur via p53-independent pathways in the AD brain, more work is required to unravel signalling pathways leading to Aβ-induced apoptotic cell death.

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