p53 and thymic 'death by neglect': thymic epithelial cell-induced apoptosis of CD4⁺8⁺ thymocytes is p53-independent

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Abstract

The involvement of the tumor suppressor protein, p53, in thymic epithelial cell-induced apoptosis of CD4⁺8⁺ (double positive) thymocytes, was studied in an *in vitro* model consisting of a thymic epithelial cell line (TEC) and thymocytes. p53 expression was not augmented in double positive (DP) thymocytes upon co-culturing with TEC, although extensive apoptosis was observed. In the same cells, p53 expression was upregulated in response to low ionizing irradiation, which was accompanied with massive apoptosis. Moreover, TEC induced apoptosis in two DP thymomas, derived from p53^(-/-) mice, and in a double positive thymoma clone expressing mutant p53. The extent and kinetics of TEC-induced apoptosis was not affected by the status of p53 in the thymocytes tested. We conclude that thymic epithelial cell-induced apoptosis of immature DP thymocytes is p53-independent and apparently, involves a different apoptotic pathway than that triggered by DNA damage. *Cell Death and Differentiation* (2000) 7, 241–249.

Keywords: apoptosis; p53; tumor suppressor protein; double positive thymocytes; thymic epithelial cells; death by neglect

Abbreviations: TEC, thymic epithelial cell line; TE, thymic epithelial cells; RadLV, radiation leukemia virus; TcR, T cell receptor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; DP, double positive; DN, double negative; PI, propidium iodide; GC, glucocorticoids; WT, wild type

Introduction

Somatic rearrangements of genes encoding the TcR generate a repertoire of T cell clones that recognize a large variety of antigens. Since this process is stochastic, it also yields cells

with non-functional receptors as well as receptors potentially capable of recognizing self antigens. Maturation of such cells and their emigration to secondary lymph nodes might lead to autoimmune disorders or progression to malignant lymphomas. This outcome is, however, prevented by intrathymic selections that eliminate thymocytes expressing useless or harmful receptors, thus ensuring that only T cells making a 'legitimate' TcR will become immunocompetent.

The two major signals that trigger intra-thymic selections are TcR engagement and glucocorticoids (GC), both delivered by thymic epithelial cells.^{1–4} TcR cross-linkage eliminates cells expressing receptors with strong affinity to self antigens.^{5,6} GC, on the other hand, facilitate positive selections of cells expressing TcR with intermediate affinity to self antigens, by antagonizing their TcR-evoked apoptotic death.^{7–9} GC also play a role in the elimination of cells expressing aberrant or irrelevant receptors through a TcR-independent process denoted 'death by neglect'.^{5,10}

Thymic selection operates on maturing DP thymocytes. These cells are highly sensitive to DNA damage and respond to it by elevating p53 expression.^{11,12} Initially identified as a tumor suppressor, p53 plays a pivotal role in maintaining the integrity of the genome, by allowing either DNA repair or elimination of cells with excessive DNA damage. Normally, the p53 protein is latent inactive and short lived. However, upon exposure to stress signals, such as DNA damage or hypoxia, p53 is activated by a conformational change and its protein level increases due to structural stabilization.^{13,14}

An important function of p53 is to trigger apoptosis. In thymocytes, p53 is mandatory for the induction of an early apoptotic response to DNA damage, as indicated by the resistance of thymocytes derived from p53-null mice to irradiation-induced apoptosis.^{11,12,15} The high frequency of thymic lymphomas in p53-null mice,^{12,16–19} suggests an involvement of p53 in regulating the genesis of the T lymphoid lineage. One of the postulated roles of p53 in the thymus is to block further differentiation of double negative (DN) thymocytes that failed to rearrange their TcR locus into a transcriptionally competent gene.^{20,21} This assumption assigns a function for p53 in controlling the maturation of DN thymocyte precursors. Whether p53 plays a role in the maturation of DP thymocytes has not been established. However, p53 involvement in the response to TcR engagement or GC was excluded by the demonstration of non-impaired apoptosis in p53^(-/-) thymocytes responding to phorbol ester (PMA) and calcium ionophore (which mimic TcR signaling) and to dexamethasone.^{11,12}

We examined the role of p53 in the apoptosis of DP thymocytes induced by thymic epithelial cells. We have previously described an *in-vitro* experimental system based on co-culturing thymic epithelial cells with thymocyte

subsets. Using this system, we have shown that DP cells are the only thymocyte subset that undergoes apoptosis in response to primary or transformed thymic epithelial cells (TEC), in the absence of an exogenous antigen. The apoptosis of DP thymocytes or thymic lymphomas, induced by TEC, was TcR-independent, MHC non-restricted and CD95-independent.^{22,23} Apparently, GC is one, albeit not the single, mediator of the response.¹⁰ Based on kinetic studies, we concluded that the effect of thymic epithelial cells could not be fully attributed to GC secretion.¹⁰ We therefore assumed that thymic epithelial cells may deliver additional, as yet unidentified apoptotic signals. While a correlation between elevated p53 expression in thymocytes and their responsiveness to TEC has been reported,²⁴ the involvement of p53 in this apoptotic death pathway remains unclear. Here we demonstrate that p53 is not involved in apoptosis of DP thymocytes induced by TEC.

Results

p53 expression in DP thymocytes responding to TEC

We tested whether TEC affect the expression of p53 in DP thymocytes. We used DP thymocytes exposed to low ionizing irradiation as a positive control, since they undergo p53-dependent apoptosis in response to DNA damage.^{11,12,25} Changes over time in p53 expression were measured following irradiation of C57BL/6 thymocytes. Irradiated thymocytes were harvested at various time intervals and each sample was divided in two, for measuring p53 expression and apoptosis. In one set of samples, p53 expression was determined by Western blot analysis. As demonstrated in Figure 1, p53 level in the cells increased, peaked 1–2 h post irradiation and declined afterward. To correlate the increase in p53 levels with apoptosis, we analyzed the cell cycle status of the parallel sample by flow cytometry. Owing to chromatin condensation and loss of degraded DNA, apoptotic cells display reduced DNA content

determined by PI fluorescence intensity, as compared to viable cells.²⁶ Five hours after irradiation, a small population of sub G₀ cells was detected (6%), which steadily increased and reached 50% 24 h following irradiation (Figure 2a). Concomitantly with the appearance of sub-G₀ cells, the G2/M fraction decreased and eventually disappeared. Apoptotic cells were also detected by staining with FITC-conjugated Annexin-V. The percent of Annexin-V positive cells increased from 27% in the untreated controls to 98% in irradiated cells 24 h following irradiation (Figure 2b). The per cent apoptosis determined by Annexin-V staining exceeded that evaluated by PI staining, since Annexin-V detects an early stage of apoptosis (expression of phosphatidylserine) that precedes DNA degradation.

Changes in p53 expression in thymocytes co-cultured with TEC could not be evaluated by Western blotting, since TEC express high levels of p53 (data not shown). We took advantage of the fact that TEC adhere tightly to polystyrene surfaces and do not express CD4 or CD8, and collected the non-adherent cells from the co-culture. The cells were stained for CD8, CD4 and either anti-p53 or PI, and then analyzed by flow cytometry. We could thus correlate the cells' phenotype with their expression of p53 and apoptosis. We first applied three color flow cytometry to the irradiated positive controls: Thymocytes were harvested at various time intervals following irradiation and tested for p53 and DNA content. p53 expression in gated DP cells (R3 in Figure 3a), peaked at 1.5 h following irradiation (Figure 3b), and after 24 h 65% of the cells apoptosed as indicated by the number of cells at sub G₀ (data not shown). Next, we evaluated p53 expression and apoptosis in thymocytes responding to TEC. No increase in p53 expression was detected in gated DP thymocytes within 24 h of co-culture with TEC (Figure 4a). The proportion of sub G₀ cells increased to 83% following overnight incubation with TEC, as compared to 34% in the untreated control cells incubated alone (Figure 4b).

TEC-induced apoptosis does not require functional p53

The possible involvement of p53 in TEC-induced killing of DP cells was further investigated, drawing on the p53^(-/-) DP thymic lymphomas, KO52DA20 and KO5236B5.²⁷ KO5236B5 cells co-cultured with TEC underwent apoptosis, as demonstrated by elevation in sub-G₀ (Figure 5) and Annexin-V positive cells (data not shown). Co-culture of TEC with KO52DA20 cells also resulted in apoptosis of the latter (data not shown).

Another thymic lymphoma tested was PD1.6. It is characterized as descending from immature DP thymocytes.²² PD1.6 cells express high levels of p53, as compared to DP thymocytes (Figure 6a). Elevated p53 expression is often associated with a mutated gene. Indeed, immunoprecipitation of ³⁵S-methionine-labeled PD1.6 lysates, with PAb246 and PAb240, which recognize wild-type and mutant p53, respectively,^{13,27–30} revealed that p53 of PD1.6 was mutated: p53 was immunoprecipitated from PD1.6 with PAb240 but not with PAb246,

Figure 1 Elevated p53 expression in thymocytes exposed to low ionizing irradiation. Irradiated thymocytes (400 cGy) were cultured for various time intervals. Lysates (30 µg/lane) were resolved on 8.5% SDS–PAGE. Gels were subjected to immunoblotting using anti-p53 polyclonal antibody (PAb 248). ECL was developed with Horse Radish Peroxidase (HRP)-conjugated anti-mouse IgG. Relative protein levels were determined by densitometry. The presented experiment is one representative out of at least three independent experiments

whereas PAb246, but not PAb240 immunoprecipitated p53 from Con A-activated splenocytes. PAb248 precipitated p53 from both splenocytes and PD1.6 (Figure 6B).

Upon co-culturing of PD1.6 and TEC, no elevation of p53 expression was observed in the former cells (Figure 7), even though they underwent apoptosis (Figure 5). Since PD1.6 cells were cloned and uniform it was sufficient to identify it by staining with anti-Thy 1.2 antibodies. A comparative analysis demonstrated that both KO5236B5 and PD1.6 were sensitive to TEC-induced death, to the same extent as normal thymocytes (Figure 8), even though the cell lines were relatively resistant to irradiation-induced death (Figure 8). These results argue against the involvement of p53 in TEC-induced apoptosis of thymocytes.

Discussion

P53 is a central mediator of the apoptotic response to cellular stress. It has been implicated in the response of thymocytes to DNA damage, but not to TcR cross-linkage or GC.^{11,12} These responses were measured in p53-null mice. It is still possible that other forms of apoptotic mechanisms might be disrupted in these mice, but their effect was not detected due to compensatory mechanisms in the thymus. The high incidence of thymic lymphomas in p53-null mice, and the fact that many such lymphomas are CD4⁺8⁺,^{16–19,27} indicated that p53 may play a role in the elimination of immature DP thymocytes during thymic development. This prompted us to study the possible involvement of p53 in thymic epithelial cell-elicited apoptosis of DP thymocytes.

Figure 2 Irradiation-induced apoptosis of thymocytes. Irradiated thymocytes (400 cGy) were cultured for the indicated times periods, and then either fixed with methanol and stained with 2 μ g PI (a) or stained with FITC-conjugated Annexin V (b). The presented experiment is one representative out of at least three independent experiments

The epithelium is a major component of the thymic stroma. Thymic epithelial cells are in close proximity to maturing thymocytes and control their development by virtue of their involvement in TcR-dependent negative and positive selection, and in the TcR-independent 'death by neglect'. They might therefore transduce various signals, the two of which reported so far are TcR cross-linkage and GC.^{1-5,10} To ensure the maturation of positively selected T cells only, thymic epithelial cells induce a TcR-mediated apoptosis in self-reactive thymocytes on the one hand, and a TcR-independent apoptosis, in useless thymocytes

expressing TcR with low affinity to self antigens, on the other hand.

We focused on the TcR-independent apoptotic pathway, induced by thymic epithelial cells in the absence of an exogenous antigen. This interaction apparently reflects the process of 'death by neglect' which accounts for the elimination of the vast majority of DP thymocytes during intrathymic maturation.²³ We have previously demonstrated that a thymic epithelial cell line (TEC), as well as primary thymic epithelial cells, can activate a TcR-independent apoptotic death of thymo-

Figure 3 Upregulation of p53 expression in irradiated CD4⁺8⁺ thymocytes. **(a)** Gating on DP thymocytes. Thymocytes were stained with PE-conjugated anti-CD4 and biotinylated anti-CD8 followed by streptavidin-Cy 5. **(b)** Elevated p53 expression in irradiated CD4⁺8⁺ thymocytes. Thymocytes were irradiated (400 cGy) and incubated (5×10^5 /ml) under standard conditions for the indicated time intervals. The cells were stained with PE-conjugated anti-CD4 and biotin-labeled anti-CD8, followed by streptavidin-Cy5. The cells were then fixed with methanol and stained with FITC-conjugated anti-p53 antibodies. Histograms of gated CD4⁺8⁺ thymocytes are presented. (— Unstained cells; - - - untreated cells; irradiated cells). The bar at the bottom of the chart represents mean fluorescence intensity of p53 expressed by irradiated CD4⁺8⁺ thymocytes. The presented experiment is one representative out of three independent experiments

Figure 4 TEC-induced apoptosis is not accompanied by elevation of p53 expression in CD4⁺8⁺ thymocytes. Thymocytes were incubated with TEC for the indicated time intervals. Non-adherent cells were collected and samples were divided for measuring p53 expression and apoptosis. **(a)** P53 expression in CD4⁺8⁺ thymocytes activated by TEC. Cells were stained with PE-conjugated anti-CD4 and biotin-labeled anti-CD8, followed by streptavidin-Cy5. Methanol fixed cells were stained with FITC-conjugated anti-p53 antibodies. The results of gated CD4⁺8⁺ thymocytes are presented (Control - - - ; co-cultured with TEC ———). The bar at the bottom of the chart represents mean fluorescence intensity of p53 expressed by CD4⁺8⁺ thymocytes activated by TEC. **(b)** TEC induced apoptosis in CD4⁺8⁺ thymocytes. Non-adherent cells in the co-culture were collected and stained with FITC-conjugated anti-CD8 and biotin-labeled anti-CD4 followed by streptavidin-Cy5. Cells were then fixed and stained with PI. The sub-G₀ fraction of gated CD4⁺8⁺ thymocytes incubated overnight with TEC is presented. Data presented are the mean ± S.E. of three different experiments

cytes or thymic lymphomas with an immature character.²²

GC hormones have been implicated in positive selection of thymocytes.^{7–9} We have previously shown that GC partially mediate TEC-induced apoptosis of DP thymocytes.¹⁰ GC may be secreted by TEC, which possess all the enzymes required for their biosynthesis.⁸ Our data, however, suggested that GC is not the sole mediator and

that TEC deliver additional, as yet unidentified signals, which participate in apoptosis of DP cells¹⁰ (and unpublished data).

The involvement of p53 in TEC-evoked death of DP thymocytes was addressed by correlating changes in the level of p53 in the thymocytes with their apoptotic response. These parameters were tested in three settings: (1) DP thymocytes expressing wild type (WT)

Figure 5 TEC induced apoptosis of CD4⁺8⁺ thymomas. KO5236B5 and PD1.6 co-cultured with TEC. Non adherent cells were collected after overnight co-culture with TEC. Cells were stained with FITC-conjugated anti Thy1.2, fixed and stained with PI. Per cent sub-G₀ of gated Thy1.2⁺ cells is presented. The presented experiment is one representative out of three independent experiments

p53; (2) DP thymic lymphomas originating from p53-null mice; and (3) A DP thymic lymphoma clone expressing a mutant p53. Upregulation of p53 in response to irradiation served as a positive control.

Our results demonstrate that TEC induce apoptosis of WT DP thymocytes without affecting the level of p53. This stands in contrast to the profound elevation of p53 expression in the same cells responding to DNA damage. The lack of correlation between p53 expression and the induction of TEC-induced apoptosis, together with the efficient apoptosis induced by TEC in p53-null or in a thymoma expressing a mutant p53, suggest that p53 is not mandatory for TEC-induced apoptosis in DP thymocytes and thymic lymphomas. The possible involvement of other members of the p53 family such as p51, p73 and p63, is not known and cannot be excluded.

Our results are at variance from those of Schreiber *et al*²⁴ who found an involvement of p53 in TEC-induced apoptosis of thymocytes. Their study, however, was based on a correlated expression using bulk thymocytes. In addition, p53 expression in their study, measured by Western blot analysis, could have been attributed to residual TEC cells, which express high level of p53.

p53 is frequently mutated in malignant cells.^{31,32} It has been demonstrated that certain p53 mutations such as p53val135 or mutations in the zinc-binding domains L2 and L3, render cells more resistant to death induced by DNA damage.^{33–35} The mutated p53 expressed in PD1.6 did not protect it from TEC-induced apoptosis, but it did confer heightened resistance against irradiation-induced death. This suggests that the TEC-induced apoptotic pathway is distinct from that induced by DNA damage.

Our present findings, that rule out the involvement of p53 in thymocyte apoptosis induced by TEC, expand the magnitude of p53-independent apoptotic pathways in the

thymus. This conclusion agrees with our previous demonstration that TEC-induced apoptosis of thymocytes is independent of CD95,²³ which is a target for upregulation by p53.

A general conclusion drawn from these and other findings is that a wide range of apoptotic pathways evoked in DP thymocytes are p53-independent.

Materials and Methods

Cells and co-culture

Thymocytes were obtained from 6–8-week-old C57BL/6 female mice (Harlan Sprague Dawley, Indianapolis, IN, USA). PD1.6 is a thymic lymphoma clone generated by transformation with Radiation Leukemia Virus (RadLV).³⁶ KO52DA20 and KO5236B5 are thymic lymphomas derived from p53 knockout mice that were kindly provided by Dr. A Strasser.²⁷ TEC, an SV40 transformed thymic epithelial cell line of a (C57BL/6 × Balb/c)F1 mouse,³⁷ was kindly provided by Dr. A Kruisbeek (NIH, Bethesda, MD, USA).

TEC were harvested with trypsin-versene and seeded at 1.5×10^5 cells/ml 24 h prior to experimentation. Thymocytes or thymic lymphoma clones (3×10^5 cells/ml) were cultured overnight in 10 ml (92 × 17) tissue culture dishes or 25 V/C tissue culture flasks (Nunc, Roskilde, Denmark), with or without TEC. Following overnight incubation of TEC and lymphocytes or lymphomas, non-adherent cells were collected. Half of each sample was used to evaluate p53 expression and the other half to measure the extent of apoptosis.

Reagents and antibodies

Biotin-, Fluorescein-isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal antibodies (mAb) specific for CD4, CD8, Thy1.2 or mouse IgG were purchased from Pharmingen Inc. (San Diego, CA, USA). Cy-5 Streptavidin was purchased from Jackson Immuno Research (Baltimore, MD, USA). Anti-murine P53 Pab421, PAb246, PAb240 and PAb248 mAbs, were used as culture supernatant of the respective hybridomas. FITC-conjugated anti-p53 was generated in the lab from affinity-purified PAb421 and PAb248 mAbs and used as a mixture. FITC-conjugated Annexin-V was purchased from Boehringer Mannheim GmbH Biochemicals (Mannheim, Germany). Propidium Iodide (PI) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Apoptosis assay

Apoptotic death was detected either with the DNA intercalating agent propidium iodide (PI), which determines cellular DNA content, or by staining with Annexin-V. The latter detects an early apoptotic step by its ability to bind phosphatidylserine which redistributes from the inner leaflet of the plasma membrane to the outer one. Both methods were combined with two colors staining by fluorescent antibodies specific for CD4 and CD8, in order to identify CD4⁺8⁺ cells in the co-culture. Following overnight incubation with or without TEC, non-adherent cells were harvested and stained. For detection of apoptotic cells by a loss of DNA content (sub-G₀), cells were stained for 1 h with FITC-conjugated anti-CD8 and biotin-labeled CD4, followed by 15 min staining with streptavidin-Cy5. The cells were then fixed with methanol and stained with 2 μg PI.^{26,28} In these experiments the cell cycle data were recorded from cells stained with both green and dark red (Cy-5) fluorescence ('gating' on CD4⁺8⁺). Detection of apoptosis with

Figure 6 (a) PD1.6 express higher level of p53 as compared to DP thymocytes. Cells were stained with PE-conjugated anti-CD4 and biotin-labeled anti-CD8, followed by streptavidin-Cy5. Fixed cells were stained with FITC-conjugated anti-p53 (PAb421 and PAb248) antibodies. (— Unstained control; — FITC-conjugated PAb421+PAb248). (b) PD1.6 cells express a mutant p53. PD1.6 and Con A-activated spleen cells were metabolically labeled with 35 S-methionine. Cell extracts were pre-cleared by immunoprecipitation with irrelevant antibody (PAb5D7), and then immunoprecipitated with PAb246, which recognizes only WT p53 (lanes 3 and 7), PAb240 which recognizes only mutant p53 (lanes 2 and 6) or with PAb248 which recognizes both WT and mutant p53 (lanes 4 and 8). Lanes 1–4: activated spleen. Lanes 5–8: PD1.6. In lanes 1 and 5 cells were immunoprecipitated with control antibody PAb5D7

Figure 7 p53 expression in PD1.6 is not upregulated by TEC. PD1.6 cells were incubated in tissue culture flasks precoated with TEC. Non-adherent cells were harvested at the indicated time points, stained with biotin-labeled anti-Thy1.2 followed by streptavidin-Cy5. Fixed samples were stained with FITC-conjugated anti-p53 (PAb421 and PAb248). (— Unstained PD1.6; - - - untreated PD1.6; — TEC stimulated PD1.6). Mean fluorescence intensity of TEC-activated, non-adherent PD1.6 cells prior to and following activation is shown in the right box. The presented experiment is one representative out of three independent experiments

Figure 8 TEC-induced apoptosis of KO5236B5 (p53-null), PD1.6 (mutated p53) and thymocytes (WT p53). Following overnight incubation with TEC, non-adherent cells were harvested. Cells were stained with biotinylated anti-Thy1.2, followed by streptavidin-Cy5, and then with FITC-conjugated Annexin-V. Per cent Annexin-V-positive cells of gated Thy1.2-positive cells (above control staining) is presented. Data presented is one representative out of three independent experiments

Annexin-V was performed by staining with PE-conjugated anti-CD4 and biotin-labeled anti-CD8, followed by streptavidin-Cy5. The cells were then stained for 15 min with Annexin-V-FITC (20 μ l/ml) according to the directions of the manufacturer. The results were recorded from cells stained with both PE and Cy-5. Flow cytometric analysis was performed using a Becton-Dickinson two lasers FACSCalibur. The flow rate was 100 cells per second and 1×10^4 cells were collected per sample. Data were analyzed by the Cellquest software.

p53 expression

Western blot analysis Cell lysates were resolved by 8.5% SDS-PAGE (10 μ g/lane) and transferred to nitrocellulose membranes. Membranes were probed for 1 h with the anti-p53 monoclonal antibody PAb248 (diluted 1:200). P53 was detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG by an enhanced chemiluminescence (ECL) (Super Signal-chemiluminescence substrate, Pierce, Rockford, IL, USA).

Flow cytometry Non-adherent cells were stained for 1 h with PE-conjugated anti-CD4 and biotin-labeled anti-CD8, followed by 15 min staining with streptavidin-Cy5. The cells were then fixed in methanol as described above and stained for 30 min with 1 μ g FITC-conjugated anti-p53 antibodies PAb248 and PAb421 and analyzed by flow cytometry.^{13,29,30} Cells stained with both red (PE) and dark red (Cy5) fluorescence were gated on and green fluorescence (p53 expression) of cells was recorded.

Metabolic labeling and immunoprecipitation

5×10^6 PD1.6 cells or mitogen stimulated spleen cells (2.5 μ g/ml ConA for 24 h), were 'starved' for 30 min (37°C) in Methionine-free DMEM and labeled with ³⁵S-methionine (100 μ Ci/plate) (2 h, 37°C). Cell extracts were incubated with 50 μ l non-specific hybridoma supernatant ('pre-clear') (30 min, 4°C), then with protein A-sepharose

(10 min, 4°C). Following centrifugation, supernatants were incubated with 60 μ l anti-p53 antibodies (60 min, 4°C), and then with protein A-sepharose (10 min, 4°C). Following centrifugation pellets were resuspended in 30 μ l sample buffer, boiled and loaded on 8.5% polyacrylamide gel, transferred to nitrocellulose and exposed to phosphorimager or X-ray film.

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