Apoptosis induced by denied adhesion to extracellular matrix (anoikis) in thyroid epithelial cells is p53 dependent but fails to correlate with modulation of p53 expression

Mario Vitale[a,*], Tiziana Di Matola[a], Maurizio Bifulco[a,b], Adele Casamassima[a], Gianfranco Fenzi[c], Guido Rossi[a,d]

[a] *Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università Federico II, Via S. Pansini 5, 80131 Naples, Italy*
[b] *Dipartimento di Medicina Sperimentale e Clinica, Università di Catanzaro, Catanzaro, Italy*
[c] *Dipartimento di Endocrinologia ed Oncologia Molecolare e Clinica, Università Federico II, Via S. Pansini 5, 80131 Naples, Italy*
[d] *Centro di Endocrinologia ed Oncologia Sperimentale, C.N.R. Via S. Pansini 5, 80131 Naples, Italy*

Received 1 October 1999

Abstract In normal epithelial cells, impaired cell-matrix contact leads to induction of programmed cell death, a process that has been termed 'anoikis'. We investigated the role of p53 and other apoptotic proteins in anoikis in thyroid epithelial cells. Western blot analysis demonstrated that neither p53 nor Bcl-2, Bcl-XL and Bax protein expression changed during anoikis. However, loss of endogenous p53 activity in cells transfected with a dominant-negative mutated p53 inhibited anoikis demonstrating the involvement of p53-dependent processes. The phosphatase inhibitor sodium orthovanadate opposed anoikis when added to the cells within 6 h, suggesting a role for phosphorylated proteins.

Key words: Apoptosis; Anoikis; p53; Thyroid cell

1. Introduction

Normal epithelial cells require attachment to the extracellular matrix (ECM) to survive [1]. Normal epithelial cells undergo a type of apoptosis named 'anoikis' when attachment to ECM is denied, whereas the anchorage dependence is reduced or totally absent in transformed cells [2,3]. The response to denied ECM attachment appears to be cell type-specific. While non-transformed primary fibroblasts in suspension undergo cell cycle arrest, Myc/Ras and E1A/Ras transformation sensitizes to apoptosis while Src transformation renders fibroblasts resistant to anoikis [4,5]. In contrast, transformation renders epithelial cells resistant to this type of apoptosis. It is now clear that some apoptotic pathways initiate at the cell surface from membrane receptors such as Fas/APO1 and TNFR-1 and that the p53 tumor suppressor gene plays a crucial role in some forms of apoptosis like that induced by irradiation or following treatment with some chemotherapeutic compounds [6]. Recently, the p53 tumor suppressor gene has been suggested to modulate survival of transformed fibroblasts and an immortalized mammary epithelial cell line in suspension [4]. In Ha-ras-transformed murine embryonic fibroblasts, Western blot analysis demonstrated an increased p53 protein level after a few hours of culture in suspension. We have shown that TAD-2, a human epithelial cell line originated from normal fetal thyroid cells, is a suitable model to study thyroid cell-ECM interaction [7].

In the present study we demonstrate that thyroid epithelial cells undergo apoptosis through a p53-dependent pathway when integrin-mediated adhesion to ECM is denied, that it is not induced by p53 over-expression itself nor by regulation of Bcl-2, Bcl-XL or BAX, but rather it is dependent on phosphorylation events involving proteins others than p53 and Bcl-2.

2. Materials and methods

2.1. Cell cultures and transfections

Immortalized human fetal thyroid cells (TAD-2) were generously donated by Dr. T.F. Davies, Mount Sinai Hospital (New York, NY, USA) and cultured in a 5% CO_2 atmosphere at 37°C, in RPMI medium supplemented with 10% fetal calf serum (FCS). Medium was changed every 3–4 days. Cells were detached by 0.5 mM EDTA in calcium- and magnesium-free phosphate buffered saline (PBS) with 0.05% trypsin. TAD-2 cells were transfected by calcium phosphate precipitation with pRSVneo alone or with pLTRp53cG containing the temperature-sensitive dominant-negative p53 gene mutated at codon 135 (gift of Dr. A. Levin, Princeton University, Princeton, NJ, USA), generating several TAD53 cell clones. Stably transfected cell were selected and grown with G-418 sulfate (Gibco BRL, Gaithersburg, MD, USA).

2.2. Estimation of apoptotic cells

Analysis of DNA fragmentation in cells undergoing anoikis was performed as described previously in the same cell line [7] and the percent apoptotic cells was determined by flow cytometry. Briefly, cytofluorimetric estimation of apoptosis was performed as follows: floating and adherent cells were trypsinized, collected and washed in cold PBS. Cells were fixed in 70% cold ethanol for 30 min. Ethanol was removed by two PBS washes and cells were incubated in PBS, 50 μg/ml propidium iodide, 10 μg/ml deoxyribonuclease-free ribonuclease A for 1–3 h at 4°C. Cells were then analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA, USA). The percent apoptotic cells was calculated by dividing the number of cells displaying red fluorescence lower than the G0-G1 diploid peak by the total number of cells × 100.

2.3. Antibodies and Western blot analysis

Mouse monoclonal antibody to p53 was purchased from Transduction laboratories (Lexington, KY, USA); mouse monoclonal antibody to Bcl-2 and rabbit polyclonal antibodies to Bcl-X and Bax were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Cells were washed in cold PBS and lysed for 10 min at 4°C with 1 ml of lysis buffer (50 mM Tris, pH 7.4, 0.5% NP40, 0.01% SDS) containing protease inhibitors. Lysates from adherent cells collected by scraping and lysates from floating cells were centrifuged at 12 000 × *g* for 15 min at 4°C. The protein concentration in cell lysates was determined by Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA) and 50 μg of total

*Corresponding author. Fax: (39) 81-7701016.
E-mail: mavitale@cdsunina.it

PII: S0014-5793(99)01512-4

protein from each sample was boiled for 5 min in Laemmli sample buffer (125 mM Tris pH 6.8, 5% glycerol, 2% SDS, 1% β-mercaptoethanol and 0.006% bromophenol blue). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Hybond-ECL Nitrocellulose, Amersham, Rainham, UK). Acrylamide concentration was 12% for p53 and Bcl-XL, 15% for Bcl-2 and Bax. Membranes were blocked by 5% non-fat dry milk, 1% ovalbumin, 5% FCS and 7.5% glycine. and after three washes, the membranes were incubated for 1 at 4°C with 0.5 μg/ml of mouse monoclonal or rabbit polyclonal primary antibodies in PBS. After three washes, filters were incubated for 1 h at 4°C with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Bio-Rad) diluted 1:2000 in PBS, Tween-20. After a final wash, protein bands were detected by an enhanced chemiluminescence system (Amersham). For quantitation, relative intensities of hybridization signals were measured at 560 nm with a gel scan apparatus (PDI, New York).

2.4. Statistics

Student's *t*-test for paired samples was performed where indicated. Differences at $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of inhibition of protein synthesis on anoikis

Some kinds of apoptosis require protein synthesis while others do not and programmed cell death can even be accelerated by inhibitors of protein synthesis [8,9]. We therefore investigated the effect of the protein synthesis inhibitor cycloheximide on anoikis of thyroid epithelial TAD-2 cells (Fig. 1). A total of 5×10^5 adherent cells or cells in suspension in agar-coated plates were incubated in the absence or in the presence of 0.06 μM cycloheximide and apoptosis was determined by flow cytometry after 24, 48 and 72 h. This very low concentration of cycloheximide was not toxic in adherent cells. In cells maintained in suspension in the presence of cycloheximide, inhibition of apoptosis reached approximately 80% at 48 h, then the effect of cycloheximide became less evident, giving 50% inhibition of anoikis at 72 h. Macromolecular synthesis may be required to accomplish the apoptotic pathway to achieve overexpression or de novo expression of pro-apoptotic proteins, while in slow apoptosis, protein synthesis could be required to maintain the steady state of apoptotic proteins.

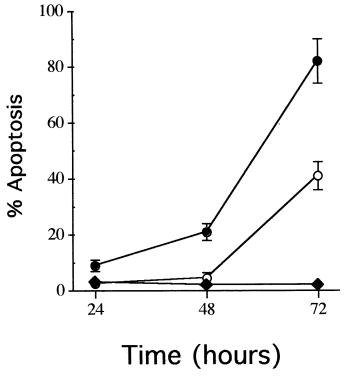

Fig. 1. Effect of cycloheximide on cell death induced by denied adhesion. Cells in suspension in agar-coated plates were incubated in the absence (●) or in the presence (○) of 0.06 μM cycloheximide and apoptosis was determined by flow cytometry at the indicated times. Apoptosis in adherent cells was not induced by cycloheximide at this low concentration (♦). Results are reported as percent apoptosis from three separate experiments.

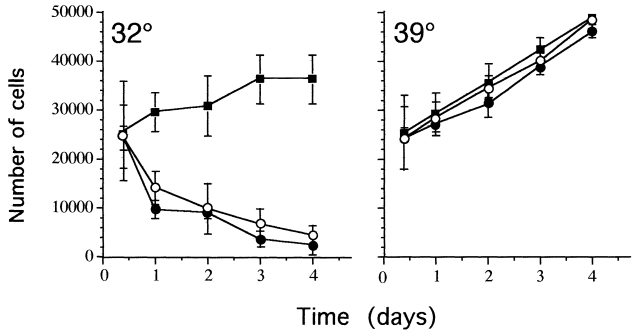

Fig. 2. Proliferation curves of TADneo (■), TADp53pool (○), and TAD53-3 (●). The cells were seeded and cultured at 32°C (left panel) or at 39°C (right panel). After 6 h the plates were washed to remove floating cells and adherent cells were cultured in fresh medium. At the indicated times, the number of cells in triplicate wells was determined. The TAD53-4 clone is not reported in the diagram because it was superimposed on TAD53-3.

3.2. p53 is involved in suspension-induced apoptosis

p53 tumor suppressor gene is crucial in some but not all forms of apoptosis. To determine whether p53 is required for apoptosis to occur in thyroid cells lacking adhesion to ECM, we stably transfected TAD-2 cells with a vector encoding a temperature-sensitive murine p53 protein (p53cG) that displays a dominant-negative effect at 39°C and wild-type activity at 32°C. Several TADp53cG stable cell clones were generated by G-418 selection of TAD-2 cells co-transfected with pLTRp53cG and pRSVneo expression vectors. At 32°C, overexpression of the active form of p53cG determined cell death in monolayer cultures, while proliferation curves of p53cG-expressing cell mutants and control cells expressing only neomycin (TADneo) cultured in adherent conditions at 39°C were identical (Fig. 2).

We then assayed the dominant-negative effect of mutated p53 expression in TADp53cG mutants at 39°C. Various TADp53cG clones and TADneo cells were treated for 24 h at 39°C with 70 μg/ml of etoposide, a potent antineoplastic drug that inhibits topoisomerase II and induces a p53-dependent apoptosis in most cell types, and apoptosis was deter-

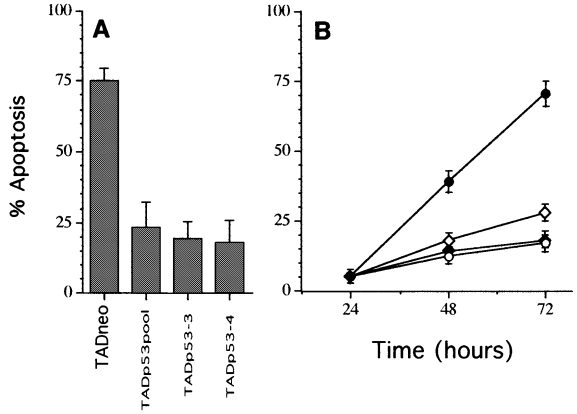

Fig. 3. Involvement of p53 in suspension-induced apoptosis. A: TADneo and TADp53 cell mutants were cultured for 24 h in the presence of etoposide and apoptosis was estimated by flow cytometric analysis. B, TADneo (●), TADp53-pool (■), TADp53-3 (○) and TADp53-4 (□) were cultured in suspension for the indicated times and apoptosis was determined.

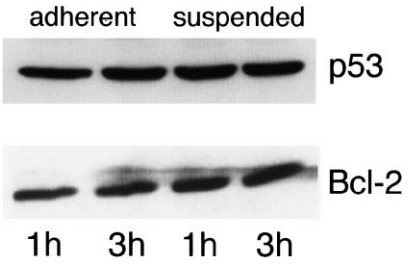

Fig. 4. Western blot analysis of p53 and Bcl-2 expression in adherent and suspended cells. TAD-2 cells were plated in uncoated or agar-coated plates and cultured in full medium. After 1 h and 3 h, adherent and suspended cells were collected and p53 and Bcl-2 expression was determined as described in Section 2. The level of both proteins remained unchanged.

mined by flow cytometry (Fig. 3A). TADneo cells were very sensitive to etoposide, which induced apoptosis in 75% of the cells, demonstrating that endogenous p53 was present and that this cell line is sensitive to p53-dependent apoptosis. TADp53cG mutants displayed up to 66% inhibition of etoposide-induced apoptosis, demonstrating inhibition of endogenous p53 activity. TADp53cG mutants were cultured in suspension for 72 h at 39°C and apoptosis was determined (Fig. 3B). TADp53cG clones maintained in suspension displayed a lower apoptosis, as compared to TADneo cells, demonstrating that transcriptional activity of p53 is involved in anoikis.

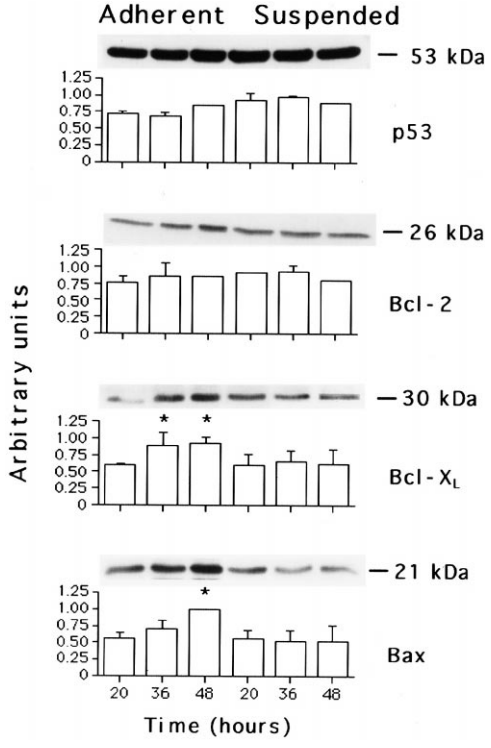

Fig. 5. Western blot analysis of p53, Bcl-2, Bcl-XL and Bax expression in adherent and suspended cells. TAD-2 cells were cultured in uncoated or agar-coated plates for 20 h, 36 h and 46 h. Representative immunoblots of each protein are shown. Mean and standard deviation of relative expression of each protein were also determined by scanning densitometry of 3–5 immunoblots. In each diagram, a value of 1 arbitrary unit was assigned to the highest density measured. $*P < 0.05$.

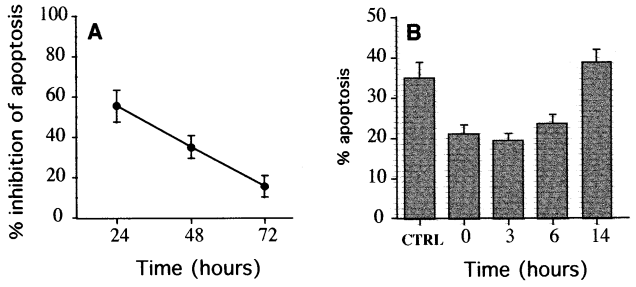

Fig. 6. Effect of sodium orthovanadate on anoikis. A: TAD-2 cells were plated in agar-coated plates in the presence of 50 μM sodium orthovanadate and percent apoptosis was determined by flow cytometry after the indicated times of culture. B: The cells were cultured in suspension for 48 h in the presence of sodium orthovanadate added to the cells immediately after plating or after 3, 6 or 14 h. Percent apoptosis was determined by flow cytometry in three independent experiments.

3.3. *Expression of p53, Bcl-2, Bax and Bcl-XL is not regulated in anoikis*

Western blot analysis of different pro- and anti-apoptotic proteins was performed in thyroid cells cultured in a monolayer or in suspension onto agar-coated plates. Analysis of protein expression within the first 3 h showed that both the anti-apoptotic Bcl-2 protein and its transcriptional regulator p53 were expressed in adherent cells at a constant level (Fig. 4). The level of these two proteins remained unchanged also 1 h and 3 h after transferring cells to suspension. Western blot analysis of p53, Bcl-2, Bax and Bcl-XL was then extended to TAD-2 cells cultured in monolayer or in suspension up to 48 h. Fig. 5 shows for each protein a representative immunoblot and the mean ± S.D. of densitometric analysis of four independent experiments from 20 to 48 h. Clear expression of p53 and Bcl-2 proteins was detectable in all samples. p53 and Bcl-2 protein expression was unchanged at all times in both adherent and floating cells undergoing apoptosis. Also a shift of their molecular weight was not observed, indicating that posttranslational modification of these two proteins did not occur. Also Bcl-XL and Bax corresponding bands were clearly visible by immunoblot. A slightly increased expression of both proteins was detected after 36 h and 48 h of monolayer culture (Bcl-XL reached approximately a 1.8-fold and Bax a 2.0-fold increase). In cells cultured onto agar-coated plates and undergoing apoptosis, protein expression did not change, always remaining at the lower level. These results demonstrate that loss of adhesion to ECM does not induce apoptosis through a variation of the level of p53 and proteins under its transcriptional control (Bcl-2 and Bax) nor Bcl-XL.

3.4. *Inhibition of endogenous phosphatases inhibits anoikis*

To investigate whether phosphorylation events are required for the execution of anoikis, we studied the effect of the phosphatase inhibitor sodium orthovanadate on anoikis of thyroid epithelial cells. TAD-2 cells in suspension were cultured in the presence or absence of 50 μM sodium orthovanadate for 24, 48 and 72 h (Fig. 6A). The inhibitory effect of sodium orthovanadate was stronger at 24 h (57%) than at 48 h and only a modest effect was visible by 72 h of treatment (18%), indicating that sodium orthovanadate produced a delay of apoptosis. We determined the time required by denied adhesion to trigger an apoptotic pathway no more sensitive to orthovanadate.

Cells were cultured in suspension for 48 h in the presence of 50 μM sodium orthovanadate, added to the cells immediately after plating or after different times (Fig. 6B). Flow cytometry demonstrated that orthovanadate was unable to inhibit apoptosis when added 14 h after transferring the cells to suspension, thus indicating that at that time phosphorylation events had ended their role and an execution pathway was activated.

4. Discussion

We previously showed that the TAD-2 cell line is a good model to study anoikis in epithelial cell and that this cell line is sensitive to both p53-independent apoptosis (lovastatin) [10] and p53-dependent apoptosis (etoposide, present paper). Our study indicates that, like in transformed fibroblasts, in epithelial thyroid cells an excess of wild-type-like p53 induced apoptosis whereas inactivation of endogenous p53 inhibited anoikis. However, in contrast to transformed fibroblasts [4], modulation of p53 expression was not observed at any time during anoikis, indicating that to accomplish its apoptotic pathway, denied adhesion does not require variations of expression of p53 itself nor of p53-modulated apoptotic factors. This difference between fibroblasts and epithelial cells may be attributed to the different response to the microenvironment of these two cell types, since transformation sensitizes fibroblasts to anoikis whereas it renders epithelial cells resistant [5]. As predicted by the observation that p53 expression was unchanged, also Bcl-2 and Bax proteins, whose genes are known to be under the transcriptional control of p53, remained constant during apoptosis. Bcl-2, Bax and Bcl-XL, proteins belonging to the Bcl-2 family, constitute a critical intracellular checkpoint of apoptosis. Homo- or heterodimerization of these proteins, together with other members of the Bcl-2 family, determines their availability to participate in apoptosis, and the overall ratio of death agonists to antagonists dictates the susceptibility to a death stimulus. Overexpression of Bcl-2 protects CHO cells from anoikis, demonstrating that although modulation of the (Bcl-2, Bcl-XL)/Bax ratio does not occur in thyroid cells, resetting of the Bcl-2 checkpoint also regulates this type of apoptosis in epithelial cells [11].

Variation of protein expression is not the only mechanism by which the Bcl-2 checkpoint can be reset and posttranslational mechanisms also appear to be involved. Serine phosphorylation inactivates Bcl-2 in response to some apoptotic stimuli [12,13], whereas hypophosphorylated Bad, a distantly related Bcl-2 protein, heterodimerizes with Bcl-XL, neutralizing its protective effect and promoting cell death [14,15]. The data presented in our study support a critical role of phosphorylation in anoikis. The apoptotic factors involved are other than Bcl-2 and p53 as Western blot analysis failed to show a shift of molecular weight. Activated phosphoinositol 3-kinase (PI-3K) has been proposed to be involved in protection from different types of apoptosis, including anoikis. In PC12 cells and in fibroblasts, the PI-3K inhibitors wortmannin and LY294002 induce apoptosis, whereas constitutively activated PI-3K or its target, the protein kinase B/Akt (PKB/Akt), block anoikis in MDCK cells [16]. Thus, protein kinases activated by ECM are candidates to transfer the signal originated from the extracellular matrix to apoptotic factors, thereby suppressing apoptosis and promoting cell survival. In conclusion, our data indicate that anoikis in thyroid epithelial cells is p53-dependent but it is not induced by p53 modulation itself, whereas posttranslational modifications of apoptotic factors occur when cells are maintained in suspension, suggesting a role for phosphorylation events in anoikis.

Acknowledgements: We thank Dr. A. Levin for the plasmid pLTRp53cG. This work has been partly supported by the Ministero dell'Università e della Ricerca Scientifica.

References

[1] Frisch, S.M. and Ruoslahti, E. (1997) Curr. Opin. Cell Biol. 9, 701–706.
[2] Frisch, S.M. and Francis, H. (1994) J. Cell Biol. 4, 619–626.
[3] Ruoslahti, E. and Reed, J.C. (1994) Cell 77, 477–478.
[4] Nikiforov, M.A., Hagen, K., Ossovskaya, V.S., Connor, T.M., Lowe, S.W., Deichman, G.I. and Gudkov, A.V. (1996) Oncogene 13, 1709–1719.
[5] McGill, G., Shimamura, A., Bates, R.C., Savage, R.E. and Fisher, D.E. (1997) J. Cell Biol. 138, 901–911.
[6] Vogelstein, B. and Kinzler, K.W. (1994) Nature 370, 174–175.
[7] Vitale, M., Di Matola, T., Fenzi, G.F., Illario, M. and Rossi, G. (1998) J. Clin. Endocrinol. Metab. 83, 3673–3680.
[8] Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E. and Dixit, V.M. (1996) Cell 85, 817–827.
[9] Wyllie, A.H., Morris, R.G., Smith, A.L. and Dunlop, D. (1994) J. Pathol. 142, 67–77.
[10] Vitale, M., Di Matola, T., Rossi, G., Laezza, C., Fenzi, G.F. and Bifulco, M. (1999) Endocrinology 140, 698–704.
[11] Zhang, Z., Vuori, K., Reed, J.C. and Ruoslahti, E. (1995) Proc. Natl. Acad. Sci. USA 92, 6161–6165.
[12] Haldar, S., Jena, N. and Croce, C.M. (1995) Proc. Natl. Acad. Sci. USA 92, 4507–4511.
[13] May, W.S., Tyler, P.G., Ito, T., Armstrong, D.K., Qatsha, K.A. and Davidson, N.E. (1994) J. Biol. Chem. 269, 26865–26870.
[14] Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B. and Korsmeyer, S.J. (1995) Cell 80, 285–291.
[15] Hsu, S.Y., Kaipia, A., Zhu, L. and Hsueh, A.J. (1997) Mol. Endocrinol. 11, 1858–1867.
[16] Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P.H. and Downward, J. (1997) EMBO J. 16, 2783–2793.