Induction of apoptosis in thecal/interstitial cells: action of transforming growth factor (TGF) α plus TGFβ on bcl-2 and interleukin-1β-converting enzyme

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Abstract

Follicular atresia is characterized by the initial rapid loss of granulosa cells by apoptosis, followed by the loss of thecal cells at a slower rate. We have previously shown that treatment of subconfluent cultures of thecal/interstitial cells (T/I) with transforming growth factor (TGF) α plus TGFβ caused chromatin condensation and internucleosomal fragmentation characteristic of apoptosis, whereas in the presence of either TGFα or TGFβ alone the cells remained healthy. In this study we have examined the effect of TGFα and TGFβ alone and in combination on the levels of mRNA encoding bcl-2 and interleukin-1β-converting enzyme (ICE) in T/I cells using a semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) assay. Bcl-2, a cell survival gene, has been implicated in regulating the balance between cell proliferation and cell death in physiological processes. ICE, the homolog of the *C. elegans* cell death gene, ced-3, is also involved in apoptotic signal transduction. The levels of mRNA encoding specific PCR products for bcl-2 (430 bp) and ICE (453 bp) were amplified from T/I cell cDNA. Untreated T/I cells and TGFα- or TGFβ-treated cells contained comparable levels of bcl-2 mRNA. Treatment of T/I cells with TGFα plus TGFβ significantly decreased the levels of bcl-2 mRNA expression. TGFα plus TGFβ caused a significant decrease in bcl-2 mRNA levels within 3 h of treatment of T/I cells, followed by a progressive decline to 10% of control levels after 24 h of treatment. In contrast, in control T/I cells, the levels of ICE mRNA were low. TGFα plus TGFβ caused a progressive increase in ICE mRNA, reaching levels 2- and 3-fold higher than control cells after 5 and 7 h respectively. DNA analysis showed that DNA fragmentation, indicative of apoptosis, occurred after 10 h of treatment with TGFα plus TGFβ. These studies demonstrated that treatment of T/I cells with TGFα plus TGFβ influenced gene expression of bcl-2 and ICE prior to the time at which DNA fragmentation was observed. We propose that the gene products of bcl-2 and ICE are involved in the apoptotic signal transduction pathway induced by TGFα plus TGFβ in T/I cells.

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Introduction

Of the cohorts of follicles that develop throughout reproductive life, only a small proportion ovulate and the majority undergo atresia (Byskov 1978, Hirshfield 1991). Atresia is the process by which follicles that are not selected to ovulate are eliminated from the ovary. The balance that is established between the growth of healthy follicles and the loss of follicles by atresia maintains a constant cell mass and homeostasis of the ovary throughout reproductive life. Atresia is an active and strictly regulated process, characterized by the initial rapid loss of granulosa cells by apoptosis, followed by the loss of thecal cells at a slower rate (Hughes & Goespe 1991, Tilly *et al.* 1991,

Logothetopoulos *et al.* 1995). Evidence from studies on rat, porcine and avian ovaries indicates that apoptosis is the underlying mechanism by which not only granulosa cells but also thecal cells are eliminated from atretic follicles *in vivo* (Tilly *et al.* 1991, 1992, Palumbo & Yeh 1994). Little is known about the mechanism by which thecal cells are eliminated. In a recent study we have shown that subconfluent cultures of thecal/interstitial (T/I) cells treated with transforming growth factor (TGF) α plus TGFβ rounded up and detached from the culture dish, whereas, in the presence of either TGFα or TGFβ alone, the morphological appearances of the cells remained healthy (Foghi *et al.* 1996a). Chromatin condensation and internucleosomal fragmentation, indicative of cell loss by

apoptosis, were observed in TGFα- plus TGFβ-treated T/I cells by nucleic acid staining and fluorescence microscopy (Foghi *et al.* 1996*a*). The objective of this study was to identify molecular mechanisms involved in the induction of apoptosis in T/I cells. Bcl-2 is an anti-apoptotic proto-oncogene and is a homolog of the *C. elegans* gene, ced-9 (Barinaga 1994, Hengartner & Horvitz 1994, Schwartz & Osborne 1994, Li *et al.* 1995). Bcl-2 functions as a dominant suppressor of apoptosis in a wide variety of cell types and is negatively regulated by multiple and diverse apoptotic stimuli such as radiation, hyperthermia, growth factor withdrawal, glucocorticoids, and chemotherapeutic agents (Hockenberg *et al.* 1993, Knudson *et al.* 1995, Linette *et al.* 1995). Interleukin-1β-converting enzyme (ICE) is a mammalian cysteine protease and a homolog of the *C. elegans* cell death gene, ced-3. It plays a key role in a variety of physiological and pathological processes involving programmed cell death (Kumar 1995, Kumar & Harvey 1995). We have explored the possibility that TGFα plus TGFβ regulate gene expression of bcl-2 and ICE in T/I cells undergoing apoptosis.

Materials and Methods

Animals

Immature Wistar rats, 22 days of age, were obtained from Charles River Canada (Montreal, Canada) and maintained under conditions of controlled light and temperature.

Isolation and culture of T/I cells

T/I cells were isolated from 25–day-old rats by the method of Lobb *et al.* (1988) with modifications (Foghi *et al.* 1996*a*). Cells were plated at a density that generated subconfluent cultures after 24 h under control conditions. Two hours after plating, the medium was removed and the attached cells were gently washed twice in serum-free medium. Serum-free medium was added, and the cultures were treated immediately after washing with TGFα (10 ng/ml; Vector Biosystems, Toronto, Canada) and/or TGFβ (10 ng/ml; purified from porcine platelets, R&D Systems Inc., Minneapolis, MN, USA). After 24 h under control conditions the cultures contained approximately 3×10^5 cells/well. The T/I cell cultures prepared as described above have been characterized morphologically and biochemically in previous studies (Skinner *et al.* 1987, Bendell & Dorrington 1988, Lobb *et al.* 1988).

RNA extraction

The method used for RNA extraction was a modification of that of Chomczynski & Sacchi (1987). Briefly, cells were lysed in a solution containing 4 M guanidinium isothiocyanate (Gibco BRL, Burlington, Ontario, Canada), 0·5% sarcosyl (Fisher Scientific, Unionville, Ontario, Canada), 25 mM sodium citrate (Fisher Scientific) and 100 mM β-mercaptoethanol (200 μl per well). Lysates from three wells were pooled into microcentrifuge tubes, giving duplicate RNA samples for each treatment. RNA was extracted from the lysates by addition of 60 μl 2 M sodium acetate (pH 4·0), 600 μl water-saturated phenol (Gibco BRL) and 135 μl chloroform:isoamyl alcohol (24:1), with tubes vortexed between each addition. Samples were then kept on ice for 15 min, centrifuged at 13 000 *g* for 5 min, and the aqueous phases containing the RNA were then transferred to fresh microcentrifuge tubes. RNA was precipitated by addition of 600 μl isopropanol, and incubated at − 20 °C for at least 2 h. After collection of RNA by centrifugation at 13 000 *g* for 10 min, pellets were washed with 1 ml 70% ethanol, and allowed to dry. RNA was redissolved in 50 μl nuclease-free water (Promega Corporation, Madison, WI, USA), and RNA was reprecipitated by addition of 5 μl 3 M sodium acetate and 125 μl ethanol, followed by storage at − 20 °C for at least 2 h. RNA was again collected by centrifugation at 13 000 *g* for 10 min, pellets were washed with 1 ml 70% ethanol, and allowed to dry. Final pellets of RNA were dissolved in 50 μl nuclease-free water, and the concentration of RNA in each sample was carefully determined in duplicate by spectrophotometry.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

After determination of the linear range of the assay (unpublished data), cDNA was synthesized from each sample in duplicate, using 200 ng total RNA. PCR was carried out using cDNA equivalent to 60 ng total RNA. Each PCR run also included a blank, in which cDNA was replaced with water, and also a 'no–RT' control sample to allow detection of contamination of stock solutions. The reactions containing cDNA, specific primers and 1 μCi [α-^{32}P]dCTP (DuPont Canada Inc., Mississauga, Ontario, Canada) were performed under mineral oil, and PCR was carried out using a commercial thermocycler (Perkin Elmer Cetus, DNA Thermal Cycler). A hot start was employed to avoid primer-dependent PCR artifacts: tubes were heated to 97 °C for 5 min to denature DNA, and then held at 79 °C while *Taq* polymerase was added. Denaturing was carried out at 94 °C for 1 min, annealing at 58 °C and 60 °C for bcl-2 and ICE respectively for 1 min 20 s, and extension at 72 °C for 2 min. PCR was carried out for 35 cycles. An additional extension step (72 °C for 8 min) was added after the last cycle, and tubes were held at 6 °C at the end of the program.

Primers used for amplification of the bcl-2 cDNA were 5′-CCT GTG CCA CCA TGT GTC CAT-3′, and 5′-GCT GAG AAC AGG GTC TTC AGA GAC-3′. The specificity for the bcl-2 PCR product correlated with

that of Tao *et al.* (1994). The primers used for ICE PCR were 5′-AGG TCA ACA TCA GCT CCG AC-3′, corresponding to position 554 on bottom strand (20 out of 20 positions) and 5′-CCT GAA CCA GGA AGA GAT GG-3′, hybridized to position 102 on top strand (20 out of 20 positions). Complete cDNA sequence of ICE was retrieved from NCBI with accession code U14647 (1209 bp). Analysis for ICE PCR product was complemented by sequence analysis combined with restriction endonuclease digestion/mapping of the product. Restriction enzyme Bbs I (BioLabs, New England) was used to cleave the product at 235 bp restriction site generating two specific fragments (133 and 320 bp in length) visualized by ethidium bromide on 1% agarose gel under UV light. The housekeeping gene, G3PDH, was included with each individual PCR run. The primers used were: 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (Clontech Laboratories, Palo Alto, CA, USA).

PCR products were visualized by either electrophoresis in 8% polyacrylamide gels or 1·0% agarose gels containing 15 µl ethidium bromide at a voltage of 200 volts for 1·5 h. Polyacrylamide gels were dried and exposed to a phosphor-based computerized radioactive image analyzer (Molecular Imager, Bio-Rad Laboratories, Hercules, CA, USA). The intensity of radioactivity in bands of the predicted molecular weight (signal intensity) was measured, subtracting the background obtained using a control sample from which the reverse transcriptase had been omitted (to detect any contamination of samples and buffers with cDNA).

DNA purification and separation

DNA from T/I cells was prepared using the method of Tilly and Hsueh (1993) with some modifications. One hundred nanograms DNA were loaded in each well of the gel. Samples were run on a 1·5% agarose gel containing 15 µl ethidium bromide at a voltage of 200 volts for 1·5 h, and DNA fragments were visualized under UV light. A 1 kb DNA ladder marker (Gibco-BRL) was run simultaneously with the samples as an indicator of the band size of DNA fragments.

Data analysis

Due to the arbitrary units of signal intensity in which the raw data were obtained, data were expressed as a fold change in signal intensity relative to control. Results were plotted as the mean results (± S.E.M.) from a minimum of three independent experiments. These results were subjected to analysis of variance, and significant effects of test treatments were determined at the $P<0.05$ level by the least significant difference test.
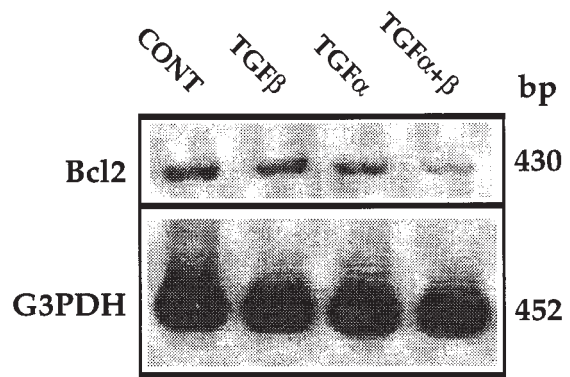

Figure 1 Regulation of bcl-2 mRNA levels in rat T/I cells by TGFα and TGFβ. T/I cells were treated with TGFα (10 ng/ml) and/or TGFβ (10 ng/ml) for 10 h. Bcl-2 and G3PDH PCR products were generated from cDNA prepared from total RNA. Aliquots of each PCR reaction were analyzed by polyacrylamide gel electrophoresis and autoradiography. CONT, control.

Results

The expression of bcl–2 in T/I cells cultured under control conditions was analyzed by PCR (Fig. 1). A specific PCR product for bcl-2 (430 bp) was amplified from cDNA isolated from T/I cells. Levels of mRNA for bcl-2 were unchanged following treatment of T/I cells with TGFα or TGFβ for 10 h. The level of bcl-2 mRNA was decreased significantly compared with the control level following treatment of cells with TGFα together with TGFβ (Fig. 1). TGFα plus TGFβ decreased bcl-2 mRNA levels in T/I cells within 3 h and this was followed by a progressive decline to 10% of control levels after 24 h of treatment (Fig. 2A,B). The expression of ICE in T/I cells was also analyzed by PCR (Fig. 3). A specific PCR product for ICE (453 bp) was amplified. Treatment of T/I cells with TGFα or TGFβ for 10 h did not influence the level of ICE mRNA appreciably. TGFα plus TGFβ caused a progressive increase in ICE mRNA reaching levels 2- and 3-fold higher than controls ($P<0.005$) after 5 and 7 h respectively (Fig. 4A,B). DNA was extracted from untreated T/I cells and from cells treated with TGFα, TGFβ, or TGFα plus TGFβ for 10 h, and analyzed by gel electrophoresis. DNA was analyzed for fragmentation at 2, 3, 5, 10 and 24 h of treatment with TGFα plus TGFβ. There was no evidence of fragmentation at 2 and 3 h. The earliest time point at which a DNA ladder was detected was 5 h; this was more pronounced at 10 h and 24 h. As shown in Fig. 5, the DNA in control cells and cells treated with TGFα or TGFβ remained intact. The DNA extracted from cells treated with TGFα plus TGFβ was cleaved into fragments that were multiples of 180–300 bp in length.

Discussion

In this study we have shown that TGFα plus TGFβ regulates bcl-2 and ICE apoptotic gene expression in T/I
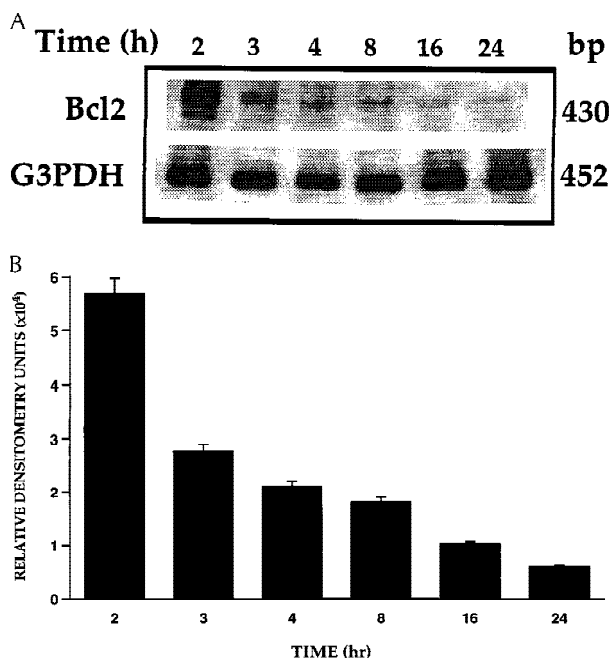
Figure 2 Time course (2–24 h) of effect of TGFα (10 ng/ml) plus TGFβ (10 ng/ml) on bcl-2 mRNA levels in T/I cells. Bcl-2 and G3PDH PCR products were obtained from cDNA prepared from total T/I cell RNA. Aliquots of each reaction were analyzed by polyacrylamide gel electrophoresis and radioautography (A). The intensity of the bands was measured using a phosphor-based computerized radioactive image analyzer (B).
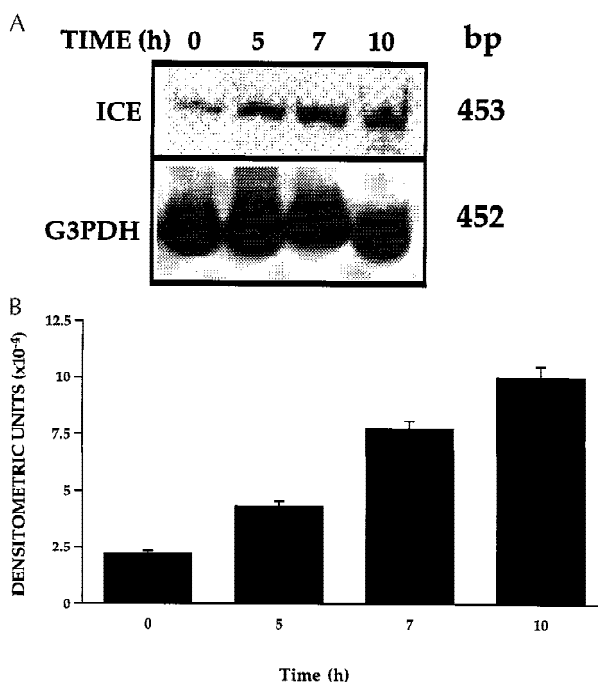

Figure 4 Time course (0–10 h) of the effect of TGFα (10 ng/ml) plus TGFβ (10 ng/ml) on ICE mRNA levels in T/I cells. ICE and G3PDH PCR products were obtained from cDNA prepared from total T/I cell RNA. Aliquots of each reaction were analyzed by polyacrylamide gel electrophoresis and radioautography (A). The intensity of the bands was measured using a phosphor-based computerized radioactive image analyzer (B).
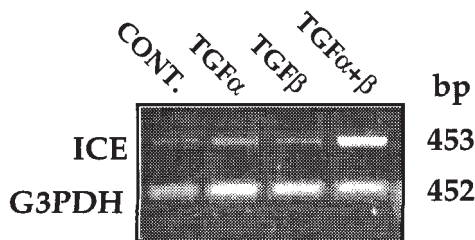

Figure 3 Regulation of ICE mRNA levels in rat T/I cells by TGFα and TGFβ. T/I cells were treated with TGFα (10 ng/ml) and/or TGFβ (10 ng/ml) for 10 h. ICE and G3PDH PCR products were obtained from cDNA prepared from total RNA. Aliquots of each PCR reaction were run on 1·5% agarose gels containing 15 µl ethidium bromide and were visualized under UV light. CONT., control.

Figure 5 Analysis of DNA fragmentation in T/I cells treated for 10 h with TGFα (10 ng/ml) and/or TGFβ (10 ng/ml). DNA was extracted and analyzed by gel electrophoresis. DNA fragmentation into pieces that were multiples of 180–300 bp in length was observed only in cells treated with TGFα plus TGFβ. 1 Kb, 1 kb DNA ladder marker; CONT, control.

cells. Treatment with TGFα plus TGFβ caused a decrease in the level of bcl–2 below that in control cultures. This down–regulation of the 'survival' gene was accompanied by the simultaneous up–regulation of the 'death' gene, ICE. Treatment of T/I cells with TGFα plus TGFβ, therefore, disturbed the balance between bcl-2 and ICE that existed in control cells and generated conditions that could cause their demise. TGFα plus TGFβ induced a significant reduction in bcl-2 mRNA levels after 3 h of treatment and a 2-fold increase in ICE mRNA after
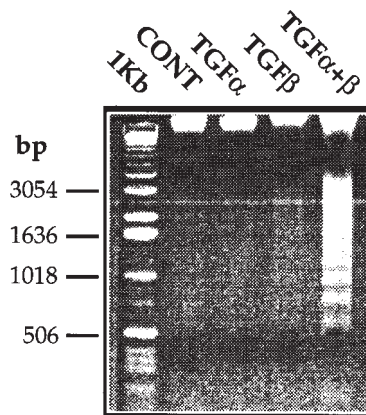
5 h. After 10 h of treatment with TGFα plus TGFβ, internucleosomal DNA cleavage into fragments that were multiples of 180–300 in length was observed.

Consequently the disruption of the balance between bcl-2 and ICE preceded the morphological manifestation of apoptosis, i.e. DNA fragmentation.

The expression of bcl-2 gene as well as other members of the bcl-2 family, bax and bcl-x, has been demonstrated in ovaries of immature rats and in isolated antral follicles (Tilly *et al.* 1995). In the latter study, immature rats were treated with equine chorionic gonadotropin to rescue follicles from atresia and promote the growth of a cohort of healthy follicles. Levels of bcl-2 and bcl-x in whole ovaries from treated animals were not significantly different from controls, whereas there was a significant reduction in the level of bax. Extensive DNA breakdown was detected *in situ* in granulosa cells of atretic antral follicles in ovaries of untreated animals, whereas thecal cells remained healthy (Tilly *et al.* 1995). On the basis of the data that we have presented, we anticipate that, during the later stages of atresia characterized by thecal cell elimination (Palumbo & Yeh 1994, Logothetopoulos *et al.* 1995), a reduction in the level of bcl-2 gene expression would be observed.

T/I cells are the only cells that have been identified in which there is a requirement for both TGFα plus TGFβ for regulation of bcl-2 and/or ICE and for the induction of apoptosis. Nevertheless, both of these growth factors have been implicated in the regulation of apoptosis. TGFβ has been identified as either an enhancer or an inhibitor of apoptosis in liver (Bayly *et al.* 1994), the immune system (Lomo *et al.* 1995) and mammary gland (Warri *et al.* 1993). Epidermal growth factor or TGFα protect rat granulosa cells from undergoing apoptosis (Tilly *et al.* 1992*b*) whereas they promote apoptosis in hepatocytes (Piacentini *et al.* 1991). Furthermore, TGFβ has been shown to down-regulate bcl-2 gene expression in M1 myeloid leukemia cells, a system in which it also induces apoptosis (Selvakumaran *et al.* 1994).

Apoptosis is a process that involves disruption of the cellular architecture that requires the involvement of proteases. ICE is a cysteine protease and its overexpression in various cell types has been implicated in cell death (Kumar 1995, Kumar & Harvey 1995). Gonadotropin-promoted follicular survival has been shown to be associated with reduced ovarian expression of two ced-3 family members (CPP32, Ich-1), whereas no changes were observed in the expression of ICE (Flaws *et al.* 1995).

All cells generate free radicals during normal metabolism and can cause cell damage by the peroxidation of membrane lipids (Hockenberg 1995, Korsmeyer *et al.* 1995). Recently, we have shown that the ability of TGFα plus TGFβ to cause death of T/I cells by an apoptotic mechanism was blocked by the reducing agent *N*-acetyl-L-cysteine, which is known to bind free radicals (Foghi *et al.* 1996*b*). This suggested that the action of free radicals was necessary for the induction of apoptosis in T/I cells. Bcl-2 acts as an antioxidant to protect cells from damage caused by free radicals. Therefore, we suggest that the combined action of TGFα and TGFβ on T/I cells that

results in a decrease in the levels of bcl-2 expression (and possibly other members of the bcl-2 family) increases oxidative stress within the cells and causes their demise. Tilly & Tilly (1995) have also shown that inhibitors of oxidative stress have the same effect as follicle-stimulating hormone in suppressing apoptosis in cultured rat follicles. On the basis of these studies, it was proposed that members of the bcl-2 gene family may be regulators of reduction–oxidation pathways in granulosa cells, and thereby determine the fate of developing follicles–i.e. growth to the preovulatory stage or atresia.

Studies on *C. elegans* have shown that ced-9/bcl-2 is only necessary for survival when the suicide gene ced-3/ICE is functional (Linette *et al.* 1995). This suggests that bcl-2 acts by preventing the actions of ICE. This hypothesis has been tested in different mammalian systems and, indeed, it has been shown that overexpression of bcl-2 prolongs the survival process by inhibiting ICE activity (Knudson *et al.* 1995, Kuida *et al.* 1995). Overexpression of ICE, on the other hand, causes programmed cell death in many cell types (Kuida *et al.* 1995), consistent with the notion that bcl-2 and ICE have opposing actions in regulating the balance between cell survival and cell death. The temporal changes in the levels of bcl-2 and ICE mRNA, induced by TGFα plus TGFβ in T/I cells *in vitro,* strongly suggest that these gene products are involved in the apoptotic pathway and the elimination of these cells.

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