

## Expression of Selected Apoptosis Related Genes, MIF, IGIF and TNF $\alpha$ , during Retinoic Acid-Induced Neural Differentiation in Murine Embryonic Stem Cells

Suparna A. Sarkar and Raghubir P. Sharma\*

Department of Physiology and Pharmacology, College of Veterinary Medicine, The University of Georgia, Athens, GA, 30602

**ABSTRACT.** Apoptosis plays an important role during embryonic development. Apoptotic cell death is executed by caspases and can be regulated by the Bcl-2 family of genes. Ribonuclease protection assay was used to investigate the expression of selected apoptosis-related genes of the Bcl-2 family, pro-apoptotic Bax, Bad and anti-apoptotic Bcl-2, during differentiation of murine embryonic stem cells (ES) mediated by all-*trans*-retinoic acid. The mRNA expression of caspase 3, caspase 6 and certain pro-inflammatory cytokines was also investigated simultaneously. ES cells exposed to 1  $\mu$ M all-*trans*-retinoic acid on day 8, 9 and 10 of differentiation revealed increased expression of Bax and Bad compared to the vehicle-treated cells. No effect on Bcl-2 mRNA was noted after all-*trans*-retinoic acid treatment. Increased mRNA expression of caspase 3 and caspase 6 in all-*trans*-retinoic acid-exposed ES cells suggested that caspases play an important role in retinoic acid-mediated apoptosis during ES differentiation. Increase in the expression of TNF $\alpha$  and macrophage migration inhibitory factor (MIF) was noted in retinoic acid-treated cells on day 14. Significant increase observed in interferon  $\gamma$  inducing factor (IGIF/IL-18) mRNA expression in all-*trans*-retinoic acid-treated cells on day 14 and 17 did not translate to increased INF $\gamma$  expression. No change in the expression of other pro-inflammatory cytokines was noted with all-*trans*-retinoic acid treatment. The function of TNF $\alpha$ , IGIF/IL-18 and MIF in all-*trans*-retinoic acid-treated cells during ES differentiation and apoptosis is still speculative. Results suggested that RA-mediated apoptosis during neural differentiation of ES cells involves up-regulation of caspase 3, caspase 6, Bad, and Bax.

**Key words:** apoptosis/embryonic stem cells/all-*trans*-retinoic acid/differentiation/MIF/IL-18

All-*trans*-retinoic acid (henceforth referred as retinoic acid), an important metabolite of retinol (Vitamin A), mediates apoptosis, differentiation, morphogenesis, reproductive, immune functions, epidermal, and bone growth. Retinoic acid plays an important physiological role in embryonic development and is teratogenic in large doses in all species (Lammer *et al.*, 1985; Alles and Sulik, 1989; De Luca *et al.*, 1991). A temporal and dose-dependant effect of retinoic acid on embryos has been reported (Armstrong *et al.*, 1994). Teratogenic response to excess dose of retinoic acid may be due to its ability to cause apoptosis (Dupe *et al.*, 1999; Jiang

and Kochhar, 1992). Retinoic acid exposure also causes apoptosis in many embryonic cell lines that have been used to mimic murine *in vivo* differentiation (Atencia *et al.*, 1994; Herget *et al.*, 1998; Glozak and Rogers, 1996). Apoptosis during differentiation of human embryonal carcinoma cells has been documented (Yamada *et al.*, 1996). Retinoic acid mediated apoptosis has also been noted in many tumor cell lines (Mangiarotti *et al.*, 1997; Guzely *et al.*, 1998).

Morphologically, apoptosis entails chromatin condensation, cytoplasmic shrinkage, blebbing and fragmentation that can be initiated by various physiological and pathological stimuli. Cysteine proteases, (caspases) play an important role during initiation and effector phase of apoptotic cells death (Kerr *et al.*, 1972). Caspases are secreted as inactive protease that can be activated by protein-protein interactions (Yamin *et al.*, 1996), autocatalytically (Thornberry, 1997), thereby unleashing the “caspase cascade” that can amplify the signals leading to apoptosis. Regulation of caspase activity occurs through increased gene transcription

\*To whom correspondence should be addressed: Raghubir P. Sharma, Department of Physiology and Pharmacology, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602-7389, U.S.A.

Tel: +1-706-542-2788, Fax: +1-706-542-3015

E-mail: rpsharma@vet.uga.edu

Abbreviations: ES, murine embryonic stem cell; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; IGIF/IL-18, interferon  $\gamma$  inducing factor; LIF, leukemia inhibitory factor; MIF, macrophage migration inhibitory factor; RA, all-*trans*-retinoic acid; RPA, ribonuclease protection assay.

and the members of Bcl-2 family of genes. The Bcl-2 family comprises of pro-apoptotic Bax, Bad, Bid and anti-apoptotic Bcl-2 and Bcl-xL. The anti-apoptotic Bcl-2 and Bcl-xL are located on the cytosolic part of the outer mitochondrial membrane and inhibit the mitochondrial release of cytochrome c (Zou *et al.*, 1997; Green and Reed, 1998). In addition, Bcl-2 and Bcl-xL can heterodimerize with the pro-apoptotic members of the family and sequester them, thereby preventing their apoptotic function in response to death signal (Oltavi *et al.*, 1993). The end process of apoptosis results in the engulfment of apoptotic cells by macrophages. The mechanism by which the phagocytic action is uncoupled to inflammation is poorly understood. Contrary to the general findings of lack of pro-inflammatory cytokines, release of death inducing cytokine CD95 ligand/Apo-1/Fas from macrophages has also been reported (Brown and Savill, 1999).

It has been established that retinoic acid or its metabolites mediate their action via nuclear receptors, named retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Leid *et al.*, 1992). These receptors with or without the ligand are capable of modulating transcription of a number of genes (Chambon, 1996). The present study was undertaken to investigate the changes in the expression of selected apoptosis related genes during retinoic acid-mediated differentiation of murine embryonic stem cells (ES). Embryonic cells derived from the inner cell mass of 4-day blastocysts can be maintained in an undifferentiated state *in vitro* by growing them on fibroblast feeder layers (Doetschman *et al.*, 1985). The ES cells respond to various internal and external signals of proliferation and differentiation and can thus mimic the *in vivo* differentiation process. We recently reported on the retinoic acid-mediated modulation of p53 and c-myc, Max, and Mad gene expression during neural differentiation of murine embryonic stem cells (Sarkar and Sharma, 2002a, 2002b). In the present report we hereby describe the expression of effector caspases (caspase 3 and caspase 6), Bcl-2 family of genes, pro-apoptotic (Bax and Bad) and anti-apoptotic, Bcl-2 genes expression compared simultaneously by the quantitative ribonuclease protection assay (RPA). Additionally, related changes in the pro-inflammatory cytokines production were studied concurrently by RPA. The results would contribute to greater understanding temporal modulation of effector caspases and Bcl-2 family during retinoic acid-mediated apoptosis in ES cells during neural differentiation.

## Material and Methods

### ES cell culture and retinoic acid treatment

Murine embryonic stem cells (ES-D3, ATCC # 1934-CRL) were procured from American Type Culture Collection (ATCC, Manassas, VA) and were maintained on mouse fibroblast feeder layers, (STO, ATCC # 1503-CRL) treated with 10 µg/ml mitomycin C, in

Dulbecco's modified Eagle's medium (Doetschman *et al.*, 1985). The media was supplemented with 15% Knock-Out™ serum replacement (Gibco, Life Technologies, Grand Island, NY, USA), 10-µM β-mercaptoethanol and 1000 IU/ml leukemia inhibitory factor (LIF) (Sigma, St. Louis, MO, USA). The medium was changed everyday. The ES cells were passaged every 2 days to maintain in an undifferentiated state. To induce differentiation, 2 × 10<sup>5</sup> ES cells were plated in a monolayer in the absence of feeder layers and LIF, in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA), and counted as day 1 of differentiation. It has been published previously that no difference had been noted in differentiation potential between ES cells grown in monolayer or in suspension form (Doetschman *et al.*, 1985). All-*trans*-retinoic acid (Sigma), dissolved in 85% ethanol at a concentration of 10<sup>-6</sup> M in yellow light (to prevent photo isomerization), was added to the media on days 8, 9 and 10 of differentiation. Our preliminary experiments indicated the optimal dose and duration of treatment with retinoic acid for neural differentiation in these cells. The control medium was treated with equal volume of 85% ethanol added to the medium. The total volume of 85% ethanol did not exceed 0.14% of the culture medium and did not influence spontaneous differentiation.

### RNA isolation and RNase Protection Assay (RPA)

RNase protection assay is a sensitive and quantitative way to measure expression level of several genes simultaneously and was performed with Pharmingen's Riboquant®, multi-probe RNase assay containing apoptotic signaling molecules (TNFα/c-Myc/caspase 3/caspase 6/Max/Mad/Bax/Bad/Bcl 2) and cytokine template set (mCK-2b) (Pharmingen, San Diego CA). ES cells were grown at a density of 2 × 10<sup>5</sup> cells in 6 well plates. To investigate level of apoptosis related genes in spontaneously differentiating cells, the cells were harvested at 0, 24, 48 h after differentiation in the absence of retinoic acid. To investigate retinoic acid-mediated differentiation, cells plated in similarly densities were concurrently treated with retinoic acid as mention before and harvested at days 11, 14, 17 and 21 of differentiation. One ml of TRI Reagent® LS (Molecular Research, Cincinnati, Ohio) was added to the cell suspension and total RNA was extracted according to the manufacturers protocol. The total RNA from three wells was pooled together and quantified in the spectrophotometer at Å260 and aliquoted in the amount of 40 µg. [α-<sup>32</sup>P]-UTP (ICN Biomedicals, Costa Mesa, CA) labeled anti-sense RNA probe was synthesized according to manufacturers protocol from DNA templates driven by T7 polymerase and quantified in a scintillating counter (Rackbeta, Pharmacia, Finland). The probe was then diluted with hybridizing buffer at strengths of 4 × 10<sup>5</sup> counts per minute/µl. Two µl of this was added to 40 µg of target RNA (dissolved in 8 µl of hybridization buffer) extracted from retinoic acid-treated and vehicle-treated ES cells and hybridized overnight at 56°C with overlaid mineral oil. Appropriate positive control in the form of mouse ribosomal RNA and a yeast negative t-RNA controls provided by the manufacturer were simultaneously hybridized. Subsequently, the free probe and single stranded RNA were treated with RNase A and RNase TI mix at

30°C for 45 min, followed by proteinase K digestion at 37°C for 15 min, thus destroying all single stranded RNA. The cRNA/mRNA complexes were purified according to manufacturers protocol and electrophoresed on denaturing polyacrylamide gels (5% acrylamide, 8 M urea) using IBI Base Runner™ 200 (Shelton Scientific, Shelton, CT). The labeling efficiency and integrity was established by running a non-hybridized probe simultaneously. The gels were disassembled and adsorbed onto filter papers and dried under vacuum at 80°C for 1 h in a gel dryer (Labconco, Kansas City, MO) and quantified by autoradiography on X-ray films after appropriate time (16 h) of exposure. During the duration of developing, the gels were stored at -70°C. With the undigested probe serving as a size marker, a standard curve was plotted on semi-log paper with migration distance versus log nucleotide length to identify the RNase protected bands. The undigested probes contain a polylinker sequence and thus migrate slower than the protected species in the gels. The gene expression was quantified by using densitometer imaging UN-SCAN-IT software (Silk Scientific INC., Orem UT). The gene expression was normalized against house-keeping ribosomal gene L32 and glyceraldehyde-3

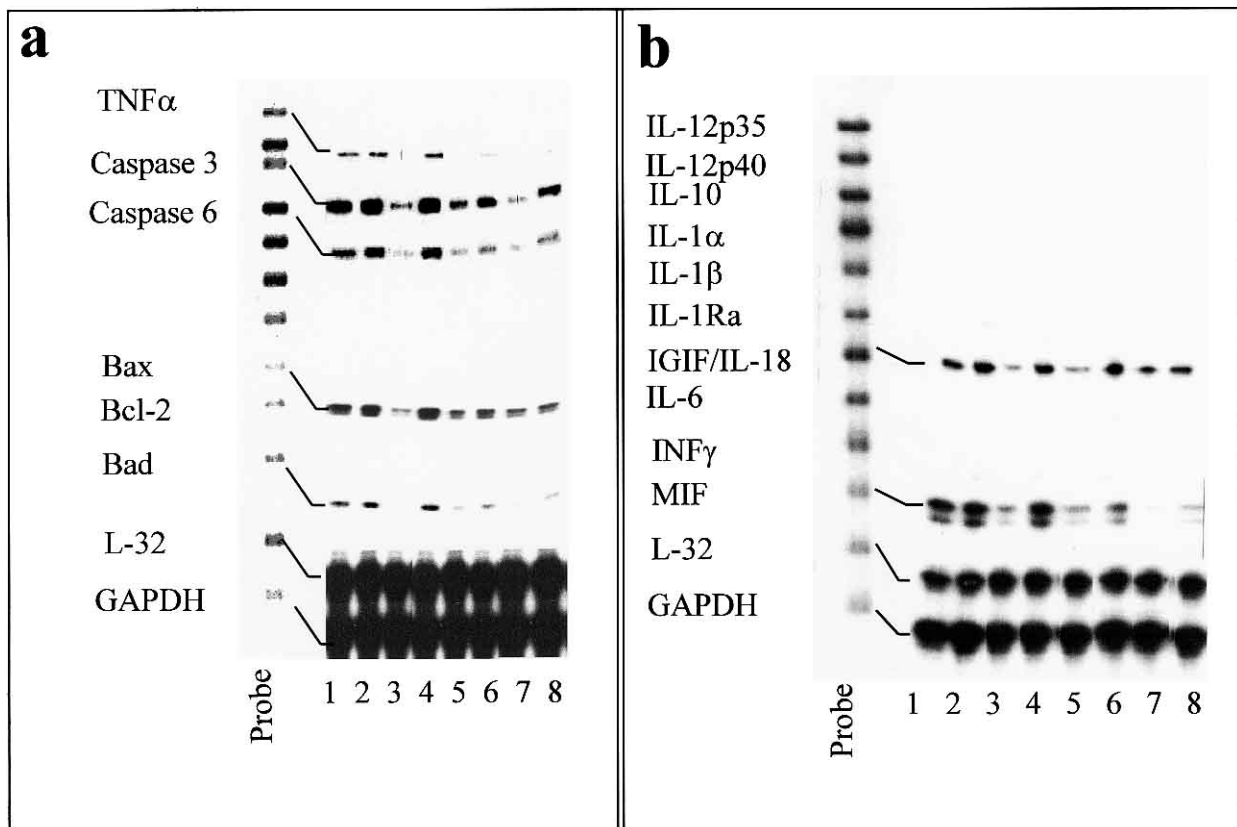
phosphate dehydrogenase (GAPDH). For appropriate normalization the gels were exposed for 3 h for house keeping genes.

### Statistical analysis and replications

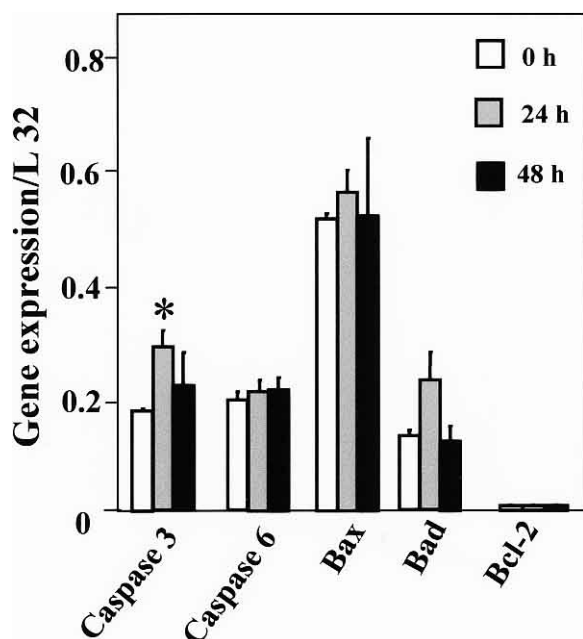
Pooled RNA from three wells was used for RPA. The RPA was repeated twice for consistency. Mean  $\pm$  standard error (s. e. m) of a representative experiment (n=2) are presented in the results. The difference between vehicle- and retinoic acid-treated samples was analyzed using Student's *t* test assuming equal variances. Probability (*p* value) of  $\leq 0.05$  was considered significant.

### Results

Results showed differential expression of apoptosis-related genes during retinoic acid-induced ES differentiation. Representative gels from the two RPA templates are shown (Fig. 1). Measurable quantities of caspase-3 and caspase-6 mRNA, normalized to ribosomal gene L32, (housekeeping gene) were detected in undifferentiated (0), 24 and 48 h of



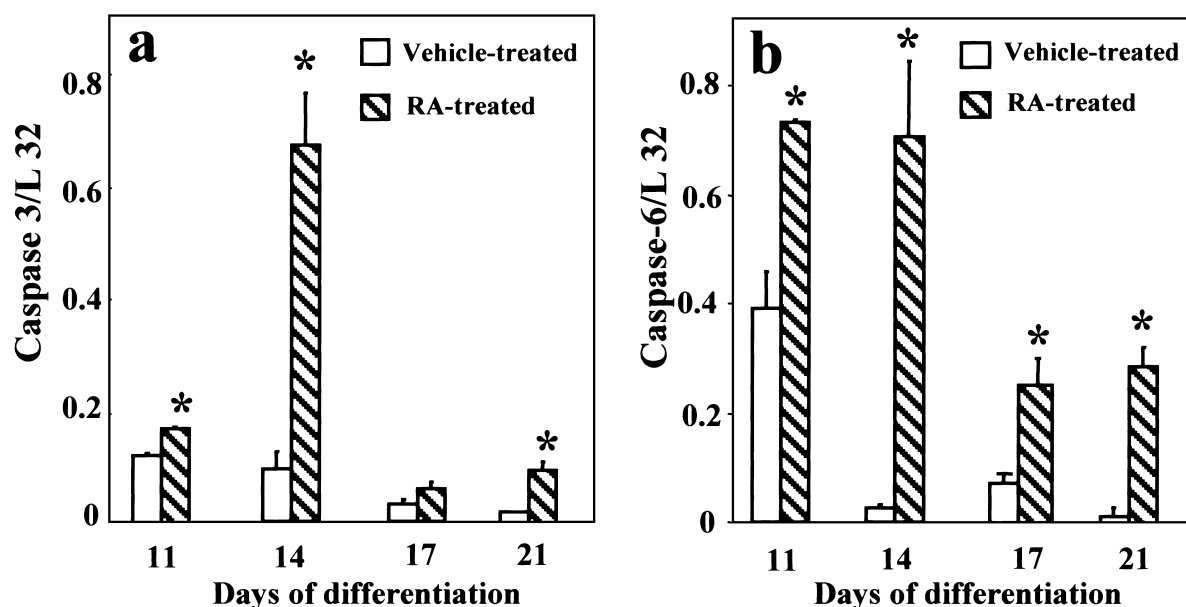
**Fig. 1.** Alteration in TNF $\alpha$ , caspases and Bcl-2 family expression during RA-mediated ES differentiation using Pharmingen Riboquant® templates. Panel (a): Representative gel of RNase protection assay using apoptosis signaling molecule probe. A non-hybridized probe was run as a size marker. The names of the genes are listed on the left. L32 and GAPDH represent the housekeeping genes. Treatment groups are indicated as; (1) 11 day vehicle-treated, (2) 11 day RA-treated, (3) 14 day vehicle-treated, (4) 14 day RA-treated, (5) 17 day vehicle-treated, (6) 17 day RA-treated, (7) 21 day vehicle-treated and (8) 21 day RA-treated. Panel (b): Changes in cytokine expression using Riboquant mCK-2b probe. The relative mRNA expression of cytokines interferon  $\gamma$  inducing factor (IGIF/IL 18) and macrophage migration inhibitory factor (MIF) are indicated against L32 and GAPDH. Lane indications are same as panel a.



**Fig. 2.** Differential expression of caspase 3, caspase-6, Bax, Bad and Bcl-2 expression by RNase protection assay (RPA) during early spontaneous differentiation. The relative mRNA expression was normalized to house keeping gene L32 at 0=undifferentiated, 24 and 48 h after differentiation. Pooled RNA from three wells was used for the assay, and the results are expressed as mean  $\pm$  standard error ( $n=2$ ). \* Indicates significantly different from concurrent vehicle-treated cultures at  $p \leq 0.05$ .

early spontaneous differentiation. Increase in caspase 3 was detected at 24 h of differentiation. However, no difference in caspase 6 was detected between undifferentiated phenotype and spontaneously differentiated ES cells at 24 and 48 h (Fig. 2).

Relative changes in Bcl-2 family were also detected following RPA analysis. Expression of pro-apoptotic gene Bax remained steady at 0, 24 and 48 h after spontaneous differentiation. No difference in the expression of Bad was noted at 24 h after differentiation (Fig. 2). Low levels of Bcl-2 were detected at 0, 24 and 48 h. In retinoic acid-treated cells, significant increase in caspase-3 mRNA was detected at 11, 14 and 21 days as compared to the vehicle-treated cultures (Fig. 3a). Maximum expression of caspase 3 was noted on day 14. Caspase-3 mRNA expression declined from day 11 onwards in vehicle-treated cells and day 14 from retinoic acid-exposed cells. However, increased caspase 3 mRNA continued to be detected in retinoic acid-treated cells as compared to vehicle-treated cells even during this declining phase. Significant increase in caspase-6 was observed in retinoic acid-treated cells on days 11, 14, 17 and 21 compared to the vehicle-treated cells (Fig. 3b). A gradual decline in the expression of caspase 6 was seen in both retinoic acid- and vehicle-treated ES cell, as the cells reached terminal differentiation. Similar to the expression of caspase 3 mRNA, higher expression of caspase 6 mRNA was detected in retinoic acid-treated cells compared to vehicle-treated cells as differentiation proceeded to the termi-



**Fig. 3.** Alterations in caspase mRNA expression during RA-mediated ES differentiation using apoptosis signaling molecule template. The cells were treated on day 8, 9 and 10 of differentiation with 1  $\mu$ M all-*trans*-retinoic acid. (a) The relative mRNA expression of caspase 3 is indicated against L32, gene encoding for ribonuclear protein. (b) The relative mRNA expression of caspase 6 is indicated against L32 gene encoding for ribonuclear protein. The results are expressed as mean  $\pm$  standard error ( $n=2$ ). \* Indicates significantly different from concurrent vehicle-treated cultures at  $p \leq 0.05$ .

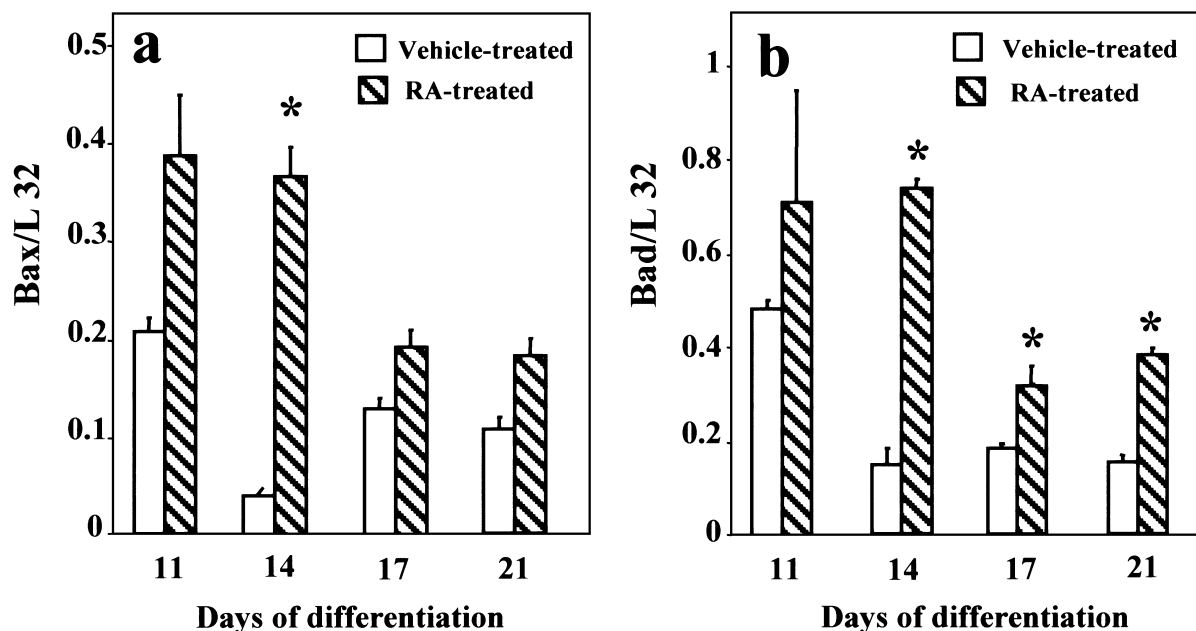
nal phase.

Relative changes in Bcl-2 family were also detected by RPA following retinoic acid treatment. When the ES cells were exposed to 1  $\mu$ M retinoic acid on day 8, 9 and 10, Bax mRNA expression significantly increased in retinoic acid-treated cells on day 14 compared to the vehicle-treated cells (Fig. 4a). Increase in Bad mRNA levels was also noted in retinoic acid-exposed ES cells on days 11, 14, 17 and 21, compared to the vehicle-treated cultures (Fig. 4b). The expression of Bax and Bad declined from days 17 to 21, in both retinoic acid- and vehicle-treated cultures, although the level of mRNA expression of both proteins were relatively higher in the retinoic acid-treated cells from the vehicle-treated cells, even as the cells reached terminal differentiation. The anti-apoptotic Bcl-2 mRNA was expressed in extremely low quantities as compared to Bax and Bad, in both retinoic acid- and vehicle-treated cells (data not shown). Ribonuclease protection analysis of TNF $\alpha$  from apoptosis signaling molecule template revealed relative absence of TNF $\alpha$  gene expression at 0, 24 and 48 h. After the ES cells were exposed to retinoic acid and/or treated with equal volume of 85% ethanol, TNF $\alpha$  gene expression was observed in both retinoic acid-treated and vehicle-treated cells on day 11 of differentiation (Fig. 5a). Expression of very low quantities of TNF $\alpha$  (normalized to L32, house-keeping gene) was noted only in retinoic acid-treated cells on day 14. Cytokine expression in ES cell with mCK-2b template was investigated after retinoic acid exposure and

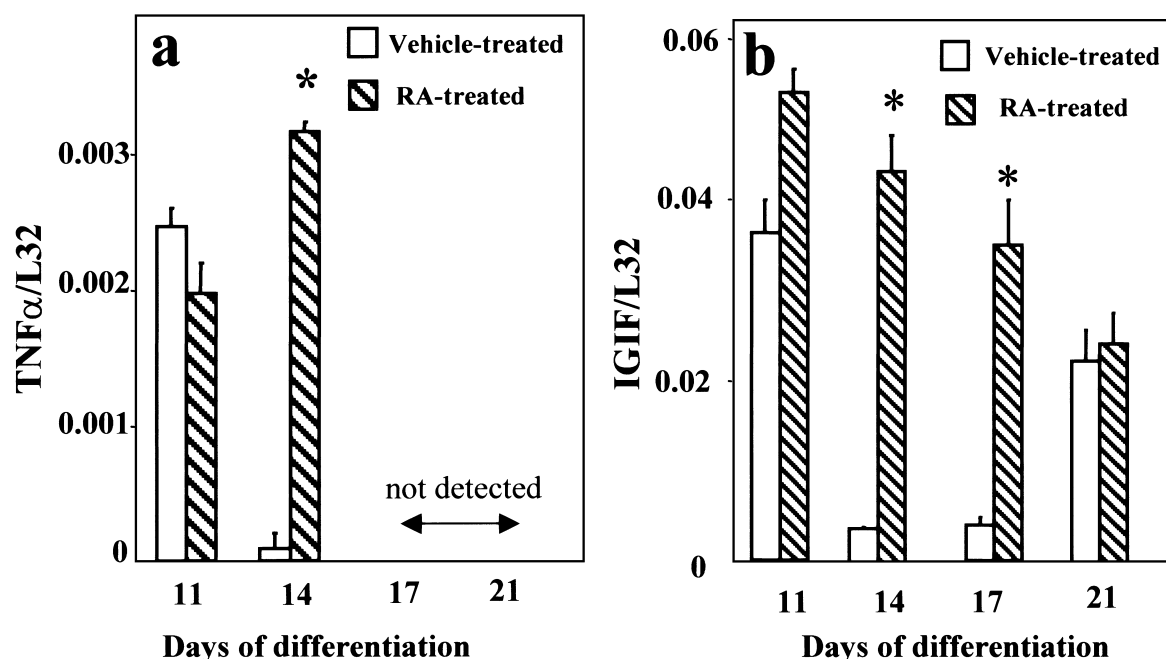
compared to vehicle-treated ES cells. Very low to undetectable levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-1Ra, IL-6, IL-10, IL-12p35, IL-12-p40, and INF $\gamma$  were seen in both retinoic acid- and vehicle-treated cells (representative gel shown, Fig. 1B). Significant increase in the expression of interferon gamma inducing factor (IGIF/IL-18) was seen on days 14 and 17 of differentiation in retinoic acid-treated cells compared to the vehicle-treated cells (Fig. 5b). Changes in mRNA expression of INF $\gamma$  were not detected concurrent to increased IGIF/IL-18 in retinoic acid-treated cells. Significant increase in the expression of macrophage migration inhibitory factor (MIF) normalized to L32 was noted on day 14 in retinoic acid-treated cells as compared to the vehicle-treated cells (Fig. 6). No difference in MIF gene expression between retinoic acid-treated and vehicle-treated ES cells was detected on subsequent days of differentiation.

### Discussion

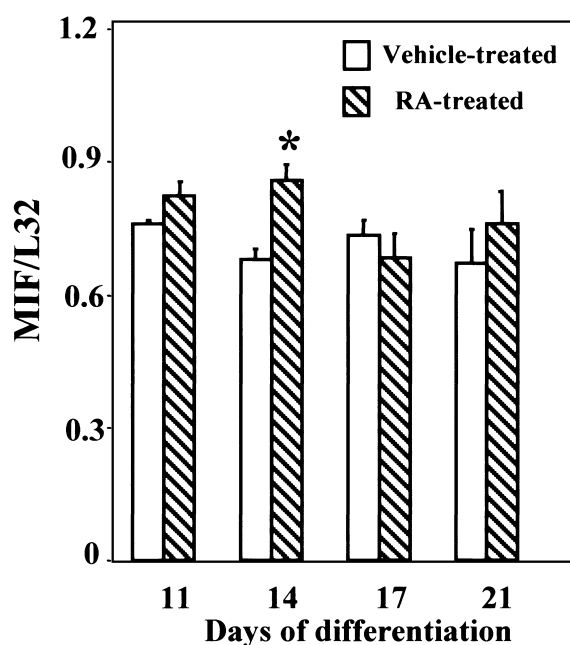
The results of this study show that retinoic acid can effectively modulate caspase 3, caspase 6 and Bcl-2 related family gene expression during apoptosis occurring in ES neural differentiation. Although, increased TNF $\alpha$ , MIF and IGIF mRNA expression was detected in retinoic acid-treated cells during differentiation by RPA, an overwhelming lack of other proinflammatory cytokine expression suggests apoptosis may not be a secondary phenomenon due to changes in proinflammatory cytokines. To our knowledge,



**Fig. 4.** Alterations in proapoptotic members of Bcl 2 family; Bax and Bad mRNA expression during RA-mediated ES differentiation using apoptosis signaling molecule template. (a) The relative mRNA expression of Bax is indicated against L 32 gene encoding for ribonuclear protein. (b) The relative mRNA expression of Bad is indicated against L 32, gene encoding for ribonuclear protein. The results are expressed as mean  $\pm$  standard error (n=2). \* Indicates significantly different from concurrent vehicle-treated cultures at  $p \leq 0.05$ .



**Fig. 5.** Alterations in selected proinflammatory cytokine expression during RA-mediated ES differentiation using apoptosis signaling molecule template. (a) The relative mRNA expression of TNF $\alpha$  is indicated against L 32, gene encoding for ribonuclear protein. (b) The relative mRNA expression of IGIF/IL 18 is indicated against L32, gene encoding for ribonuclear protein.



**Fig. 6.** Alterations in macrophage migration inhibitory factor (MIF) expression during RA-mediated ES differentiation using apoptosis signaling molecule template. The relative mRNA expression of MIF is indicated against L32 gene encoding for ribonuclear protein. The results are expressed as mean  $\pm$  standard error ( $n=2$ ). \* Indicates significantly different from concurrent control  $p \leq 0.05$ .

it is the first report of temporal expression of these selected genes during retinoic acid-mediated murine ES-D3 cell differentiation. Our results are consistent with previously published literature that retinoic acid promotes neural differentiation of ES cells and induces early apoptosis (Sarkar and Sharma, 2002a). In vitro models of neural differentiation with high concentration of retinoic acid ( $10^{-6}$  to  $10^{-7}$ ) have been developed from ES cells (Bain *et al.*, 1995; Fraichard *et al.*, 1995; Strubing *et al.* 1995; van Inzen *et al.*, 1996; Guan *et al.* 2001). Retinoic acid promotes neural and represses mesodermal gene expression in ES cells (Bain *et al.*, 1996).

Apoptosis or programmed cell death, a highly conserved evolutionary process, plays a vital role in development by removing unwanted cells (Uren and Vaux, 1996). Throughout the evolutionary systems, apoptosis remains a highly conserved process required for normal development (Raff, 1992). Apoptosis has been known to occur simultaneously with retinoic acid-mediated differentiation in P 19 cell (Ninomiya *et al.*, 1997). Among the various molecules that take part in the apoptotic process, caspases are the final executors of apoptotic death. The short pro-domain caspases 3, 6 and 7 are known to act at the final stage of cell death. Increase in caspase 3 at 24 h during spontaneous differentiation suggests that apoptotic cell death is an integral part of ES differentiation and is mediated by caspase 3. We have noted that apoptosis during retinoic acid-induced ES differ-

entiation was accompanied by increased expression of effector caspases 3 and 6. It has been reported previously that retinoic acid can induce activation of caspase-3 and cause apoptosis during neural differentiation of P19 embryonal carcinoma cells and can be prevented by bFGF (Miho *et al.*, 1999). We have found significant increased in caspase 6, as well as caspase 3 during ES neural differentiation.

Alteration or abolition of caspase activity by the use of physiological or pharmacological agents has been known to rescue the cells, or decrease apoptosis (Earnshaw *et al.*, 1999). Among the physiological agonists and antagonists is the Bcl-2 family that act as physiological modulators of caspase activity. The pro-apoptotic members; Bax, Bad, and anti-apoptotic Ced 9/Bcl-2 can homodimerize and heterodimerizes and the relative expression of pro-and anti-apoptotic fraction of the family is suggested to take part in the cellular fate (Reed, 1997). Increased expression of Bad and Bax in retinoic acid-exposed cells on days 14, 17 and 21 suggests that Bax and Bad play an important pro-apoptotic role in retinoic acid-mediated neural differentiation.

Among the important anti-apoptotic molecules of Bcl-2 family, Bcl-2 has been known to rescue neurons from apoptotic cell death during development (Garcia *et al.*, 1992). Low levels of Bcl-2 mRNA was detected in undifferentiated, 24 and 48 h after spontaneous differentiation suggesting that Bcl-2 may provide survival signal to the population of cell not undergoing apoptosis. Lower levels to complete absence of Bcl-2 mRNA detected in both retinoic acid- and vehicle-treated cells suggests that Bcl-2 may not play an active role in retinoic acid-mediated differentiation and apoptosis. The ratio of Bcl-2 to Bax has been generally thought to provide survival signal during apoptotic insult. Increased expression of Bcl-2 by retroviral vectors has been reported to reduce the number of apoptotic cells during retinoic acid exposure in P19 embryonal carcinoma cells (Okazawa *et al.*, 1996). It may be possible that Bcl-xL is the far more important anti-apoptotic protein during ES differentiation and elucidation of mechanism that mediate apoptosis during retinoic acid-induced differentiation need further investigation.

The culmination of apoptotic death is the rapid removal of dead cells by phagocytosis by macrophages. The role of macrophages in apoptosis and immunological defense is crucial. In contrast to the host defense mounted during bacterial and viral infection, the release of pro-inflammatory cytokines is remarkably absent during apoptotic death (Meagher *et al.*, 1992). It has been reported that inhibition of pro-inflammatory activity occurs in macrophages ingesting apoptotic cells (Voll *et al.*, 1997; Fadok *et al.*, 1998). Our data is consistent with the biochemical phenomenon of uncoupling of inflammatory cytokine release from apoptotic cell death, as we did not find an increase in mRNA expression of several pro-inflammatory cytokines during apoptosis in ES cells undergoing neural differentiation. However, we have noted an increase in the expression of TNF $\alpha$

in retinoic acid-treated cell on day 14. Increased differentiation and reduced proliferation of ES cells with membrane bound and free TNF $\alpha$  has been noted earlier (Kohchi *et al.*, 1996). Contrary to the above report, it has also been reported that TNF $\alpha$  decreased the formation of embryoid bodies in ES cells (Wuu *et al.*, 1998). These reports suggest the temporal importance of TNF $\alpha$  signaling during differentiation.

Increased expression of IGIF/IL-18 was also seen in retinoic acid-treated cultures. IGIF or IL-18 is a relatively new cytokine possessing many structural similarities with IL-1 family (Okamura *et al.*, 1995). It has been demonstrated that IL-18 can induce apoptosis of KG-1 myelomonocytic cells in a dose dependant manner (Ohtsuki *et al.*, 1997). Macrophage migration inhibitory factor (MIF) is yet another powerful proinflammatory cytokine and has been attributed neuroendocrine properties (Fingerle-Rowson and Bucala, 2001). We have noted an increased expression of MIF on day 14 of differentiation as compared to ethanol-treated cells, suggesting that MIF could possibly play an important role during neural differentiation. MIF has been linked to chick lens differentiation (Wistow *et al.*, 1993) and has been speculated to serve as an intercellular messenger or a part of the differentiation pathway. At this time we do not know the source or the function of TNF $\alpha$ , IGIF/IL-18 or MIF during ES differentiation and apoptosis, increased TNF $\alpha$ , IGIF/IL-18 and MIF mRNA in retinoic acid-treated ES cultures could signify a crucial role in neural differentiation of ES cells, as no neural differentiation is seen in the absence of retinoic acid. Retinoic acid has been long known to play an important role in immunomodulation and in this study, we add IGIF/IL-18 and MIF to the growing list of cytokines that can be directly or indirectly modulated by retinoic acid.

The results of this study has demonstrated that apoptosis during retinoic acid-mediated neural differentiation of ES cells is executed by an increased transcription of effector caspases, caspase3 and caspase 6. Gene transcription of pro-apoptotic members of the Bcl-2 family, Bax and Bad is also upregulated for effective modulation of death signal mediated by retinoic acid. The presence of Bcl-2 mRNA during early phase of spontaneous differentiation and undetectable levels of expression in both retinoic acid-differentiated and vehicle-treated cells suggests that Bcl-2 may not be the anti-apoptotic protein functional during ES differentiation. Research to elucidate the role of IL-18, MIF and TNF $\alpha$  during differentiation and apoptosis of ES cells will be attempted in our laboratory in future.

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