SUPARNA AJOY SARKAR
Involvement of p53 and selected proto-oncogenes in all-*trans*-Retinoic Acid-mediated murine embryonic development.
(Under Direction of RAGHUBIR PRASAD SHARMA)

Tumor-suppressor p53 regulates cell cycle, differentiation and apoptosis. The effect of all-*trans*-retinoic acid (RA) on p53, other protooncogenes and downstream signaling molecules were investigated during murine development in the following studies.

Temporal modulation of p53 in murine embryonic stem cells (ES) was investigated after exposure to 1 μM RA on day 8, 9 and 10 of differentiation. ES cells are pluripotent cells derived from the inner cell mass of blastocysts and can spontaneously differentiate into cells of various lineages. ES cells also respond to various internal and external signals of proliferation and differentiation and can thus mimic the *in vivo* differentiation process.

Undifferentiated ES cells expressed high levels of *p53* mRNA and protein that declined as differentiation proceeded. Increased apoptosis was observed after RA treatment. In RA-treated cells, a transient increase in the expression of *p53* and *c-myc* mRNA and protein was correlated with neural differentiation. Increased expression of *Max* and *Mad* genes along with elevated c-*myc* in RA-treated ES cells suggested that RA could modulate Myc family for effective signaling.

Increased expression of *caspase 3, caspase 6, Bax,* and *Bad* was seen during RA-mediated ES cell differentiation. Increased expression of tumor necrosis factor α, macrophage migration inhibitory factor and *IL-18* were also noted in RA-treated cells.

To investigate the effects of RA during murine organogenesis, assessment of p53 and Myc family was undertaken in Swiss Webster mice fetuses after maternal treatment

with 100 mg/kg of oral RA. *In utero* RA-exposure resulted in decreased expression of *p53 m*RNA and protein in a temporal and spatial manner in the fetuses. Temporal modulation of Myc family and other pro-apoptotic genes were also noted after *in utero* RA-exposure. Increased apoptosis was noted in RA-treated fetuses at 24-48 h, which declined by 72 h.

These studies demonstrated that RA can temporally modulate the expression of p53 and the Myc gene family during *in vitro* ES cell differentiation and *in vivo* murine development. A basic understanding of embryonic development requires an appreciation of the complexity involved in proto-oncogene and tumor-suppressor signaling that, in turn, can facilitate understanding the loss of orderly control during oncogenic transformation.

INDEX WORDS: p53, c-Myc, All-*trans*-Retinoic acid, Murine embryonic stem cells, Differentiation.

INVOLVEMENT OF P53 AND SELECTED PROTO-ONCOGENES IN ALL-*TRANS*-RETINOIC ACID-MEDIATED MURINE EMBRYONIC DEVELOPMENT

by

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CHAPTER 1

INTRODUCTION

Differentiation, growth arrest and apoptosis are highly controlled and regulated processes during development and embryogenesis. Complex proliferative signals mediated by various transcription factors, cyclins and cyclin-dependant kinases, are suppressed for undergoing cellular differentiation. Any alteration in the interactions between genes controlling these critical events during the cell cycle leads to a departure from homeostasis.

Protein 53 (p53), aptly described as puzzle and a paradigm (Ko and Prives, 1996), in addition to its role as tumor-suppressor, regulates cell cycle and differentiation (Almog and Rotter, 1997; Gottlieb and Oren, 1996). Previous studies have examined the role played by p53 during embryonic development. In-*vivo* studies on *p53* null mouse have suggested the dispensability of p53, however deregulation of p53 function leads to detrimental effects on development (Donehower et al., 1992). Other groups have reported exencephaly in *p53* null embryos (Armstrong et al., 1995; Sah et al., 1995).

Apoptosis or programmed cell death, a highly conserved evolutionary process, plays a vital role in development by removing unwanted cells (Uren and Vaux, 1996). It is now well accepted that p53 plays an important role in programmed cell death or apoptosis (Younish-Rouach, 1996). Over-expression of

p53 causes growth arrest and apoptosis (Younish-Rouach et al., 1991). However, it has been reported that high levels of p53 induced apoptosis and lower levels caused cell cycle arrest (Chen et al., 1996). P53 has been shown to simultaneously mediate apoptosis and differentiation in neurons and oligodendrocytes. It is not known what specific functions of p53 are required during early embryogenesis. However, a highly coordinated and tightly coupled relationship can be speculated between the two p53-associated functions, with the level of p53 and cell type, as major factors effecting embryogenesis and differentiation.

The physiological role of all-*trans*-Retinoic Acid (RA) in development has been studied extensively. Previous studies have indicated the involvement of p53 with RA on cancer cells and reported that the level of p53 declines during RA-mediated differentiation in neuroblastoma cell lines (Davidoff et al., 1992). Later, an upregulation of p53 by RA was reported (Joikim and Chopra, 1993, Ren et al., 1996). It has also been shown that RA confers resistance to p53-mediated apoptosis in SH-SY5Y neuroblastoma cells (Ronca et al., 1999). Studies in F9 embryonal carcinoma cells have shown that p53 becomes functionally active when undergoing differentiation with RA (Lutzker and Levine, 1996).

The above studies suggest the biological importance of p53 during RA-mediated differentiation and apoptosis. In this project, the effect of RA on p53 expression was investigated during murine development, along with its downstream signaling, and various interactions were produced due to p53 induction.

Murine embryonic stem cells (ES) from a non-transformed cell line were chosen to study the influence of RA on p53 expression during differentiation and

apoptosis in *vitro*. Embryonic cells are derived from the inner cell mass of 4-day blastocysts and can be maintained in an undifferentiated state *in vitro* by growing them on fibroblast feeder layers (Evans and Kaufman, 1981; Doetchman et al., 1985). They differentiate into cell types of varied lineage in the absence of a feeder layer (Smith et al., 1988; Keller, 1995). The ES cells respond to various internal and external signals of proliferation and differentiation and can thus mimic the *in vivo* differentiation process.

The hypothesis tested was: **"Retinoid–mediated differentiation and apoptosis involve the induction of tumor suppressor gene p53 and other proto-oncogenes with its downstream–effectors, cell type specificity, and p53 levels responsible for the responses observed".** The following specific aims were investigated to accomplish the objectives.

1. **To investigate the effect of RA on p53 in murine embryonic stem cells (ES).**
2. **To determine the involvement of downstream effectors of p53 function (p^{21} /Waf_1 / Cip 1), caspases, other proto-oncogenes namely Myc and Bcl-2 family during RA mediated differentiation and apoptosis.**
3. **To verify the effect of RA on p53 in mouse embryos.**
4. **To examine gene alteration in Myc and Bcl-2 family in mouse embryos during retinoid teratogenesis.**

CHAPTER 2

LITERATURE REVIEW

Retinoic Acid: A pleotropic morphogen

Retinol (Vitamin A) has been recognized for its role in vision for a long time. Retinol, along with naturally occurring carotinoids has no biological activity in itself, but forms an important substrate for the biosynthesis of (a) 11-*cis*–retinal, linked covalently to opsin to form rhodopsin, and (b) all-*trans*-retinoic acid (RA), 9-cis-retinoic acid (9-cRA) and 3,4 didehydroretinol (Vit A_2), that regulate gene expression during homeostasis, differentiation and apoptosis. RA and 9-cRA bind to the ligand activated receptors named retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Chambon, 1995). Retinoid receptors belong to a broad class of steroid nuclear receptors that have a characteristic modular structure. It is now known that both RAR and RXR genes have three isoforms (α, β, and γ) due to differential splicing (Leroy, 1991). RA binds to both RAR and RXR, while 9-cis RA binds exclusively to RXR (Mangelesdorf and Evans, 1995; Levin et al., 1992; De Luca et al., 1997). Several *in situ* hybridization and histochemical experiments have shown that different isoforms of RAR and RXR are expressed in different spatio-temporal pattern during vertebrate embryogenesis (Sharma and Kim, 1995; Kim and Sharma, 1995; Durston et al., 1997).

Pleotropic effects mediated by retinoids are generated by the complexity in the retinoid signaling pathway from an array of humoral ligands interacting with cellular retinoid binding proteins (CRBP I, CRBP II, CRABP I, CRABP II), retinoid receptors,

and retinoic acid response elements (Durand et al, 1992; Leid et al., 1992a). A further range of diversity is created by heterodimerization of retinoid receptors and their interaction with other members of the nuclear receptor superfamily (Kliewer et al., 1992; Marks et al., 1992). RARα and RXRβ isoforms are expressed ubiquitously during murine embryogenesis, whereas the expression of RARβ, RARγ, RXRα and RXRγ is limited (Kastner, et al 1990; Ruberte et al., 1991; Mendelsohn et al., 1994). It is inconceivable how a group of simple lipids convey and communicate such a wide array of biologically important responses. However, the complexity and the diversity of the genes containing retinoic acid response elements (RARE) in their promoter region make the phenomena plausible (Leid, et al. 1992b). Some of the genes with functional RAREs and retinoid X response elements (RXREs) are listed in Table 2.1. The RAR and RXR homo-and heterodimers respond to changes in retinoid concentration by binding to the RARE located in the promoter region of the target genes as a part of early response to the presence of the ligand. The availability of ligands for vitamin D receptor (VDR), thyroid receptor (TR) and 9-cis-RA activate the second order response by binding to RARE sequences in target genes (Yu et al, 1991). The ability of RA to modulate the expression of retinoic acid receptors during hamster organogenesis has been previously investigated (Colon-Teicher et al., 1996). Additionally, some genes are also repressed as a result of secondary activation and binding of AP1 complexes.

Retinoids are known to mediate proliferation, differentiation and cell death, by regulating gene expression (Lotan, 1980; Napoli, 1996).

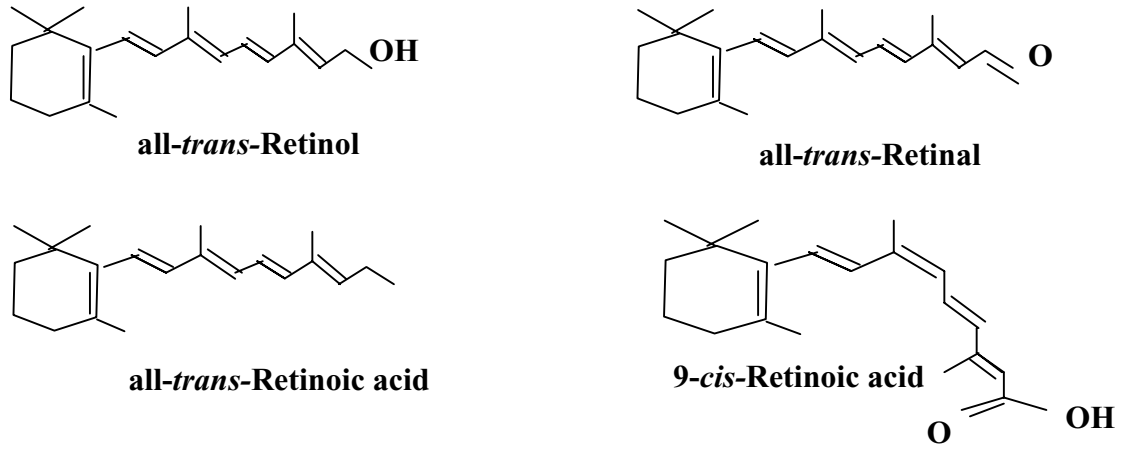

Figure 2. 1. Structure of Retinoids

Table 2.1. Genes with functional RAREs and RXREs.

Target genes	Nucleotide sequence (5'-3')	Reference
mouse RARα2	**AGTTCA**GCAAG**AGTTCA**	Leroy et al., 1991
human RARα2	**AGTTCA**GCGAG**AGTTCA**	Mattei et al., 1988
mouse RARβ2	**GGTTCA**CCGAA**AGTTCA**	Sucov et al., 1990
human RARβ2	**GGTTCA**CCGAA**AGTTCA**	Hoffmann et al., 1990
human RARγ2	**GGGTCA**GGAGG**AGGTGA**	Mattei et al., 1988
mouse CRBP1	**AGGTCA**AA**AGGTCA**	Smith et al., 1991
mouse CRABP11	RARE 1 **AGTTCA**CC**AGGTCA**	Durand et al., 1992
	RARE 2 **TGACCT**C**TGCCCT**	
human (ADH3)	**GGGTCA**TTCAG**AGTTCA**	Duester et al., 1991

Human alcohol dehydrogenase gene (ADH3)

RA plays an important physiological role in embryonic development and is teratogenic in large doses in all species (Kochhar, 1975; Lammer et al., 1985; Alles and Sulik, 1989; De Luca et al., 1995). A temporal and dose dependant effect has been reported (Armstrong et al., 1994). Teratogenic response to excess dose of RA may be due to its ability to cause apoptosis (Dupe et al., 1988; Jiang and Kochhar, 1992). RA-exposure also causes apoptosis in many embryonic cell lines that have been used to mimic murine differentiation *in vivo* (Atencia et al., 1994; Herget et al., 1998, Glozak and Rogers, 2001).

RA and Embryonic Stem Cells

Pluripotent, murine embryonic stem cells (ES) represent a non-transformed cell line that is derived from the inner cell mass of 4-day blastocysts. Throughout the past decade, intense research on ES cells has established that these pluripotent cells hold the key to understanding the elementary but intriguing question of morphogenesis and differentiation. They provide an invaluable in vitro tool to unravel the complex puzzle of embryonic development. The federal government approval for the application of ES technology in humans (Shamblott et al, 1998; Thomson et al 1998), this year, opened up exciting new areas of research as well as some serious ethical and legal questions.

ES cells can be maintained in an undifferentiated state *in vitro* by growing them on fibroblast feeder layers (Evans and Kaufman, 1981; Doetchman et al., 1985). Irradiation or Mitomycin C, mitotically inhibits the feeder layers used to support the ES cells. Feeder layers secrete leukemia inhibitory factor (LIF) that maintains the ES cells in an undifferentiated state (Williams et al., 1988). The ES cells respond to various internal

and external signals of proliferation and differentiation and can thus mimic the *in vivo* differentiation process. They spontaneously differentiate into cystic embryoid bodies that have the potential to form cell types of varied lineage in the absence of a feeder layer (Smith et al., 1988; Keller, 1995). It has been observed that genes expressed after RA-mediated differentiation of embryoid bodies from ES cells correlated to *in vivo* gene expression during embryo development (Gajovic et al., 1998). Similarly, 8 day embryoid bodies express early neurogenic genes corresponding to approximately 8.5 days post coitum mouse embryos (Leahy et al., 1999).

The *in-vitro* models of hemopoetic differentiation (Schmitt et al., 1991), cardio-muscular differentiation (Wobus et al., 1988), glial cell and functional neurons (Fraichard et al., 1995; Bain et al.; 1995; van Inzen et al., 1996) have subsequently been developed from ES. During differentiation expression of several genes are modulated by RA. RA promotes neural and represses mesodermal gene expression in ES cells (Bain et al., 1996). These cell with unlimited differentiating potential open up new approaches to investigate embryonic development.

Tumor suppressor p53

P53 was first described in 1979 as a cellular protein bound to simian virus 40 large T antigen (SV40LT) (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). It was initially labeled a proto-oncogene as its overexpression led to malignant transformation of cells. Paradoxically, the normal function of p53 was found to be that of tumor-suppressor. Wild type p53 or p53 is expressed in normal cells while mutated p53 is found in more than 50% of human malignancies. Tumor-suppressor p53,

functions as a sequence-specific transcription activator by binding to DNA sequences located in specific genes and promotes transcription (Almog and Rotter, 1997).

P53 has been the focus of intense research in the last decade often providing intriguing and complex answers (Ko and Prives, 1996). P53 was featured as the "Molecule of the Year" in *Science* (Cullota and Koshland, 1993). Latent p53 can be activated by several mechanisms, mainly post-translational modification, phosphorylation at C or N- terminals and covalent/non covalent modifiers. Particularly in DNA damaged cells, active p53 has been shown to either cause cell cycle arrest or apoptosis (Yonish-Rouach, 1996). This has been shown to prevent further genomic damage by ensuring that potentially dangerous cells are not allowed to propagate (Kastan et al., 1991a; Fritsche et al., 1993; Nelson and Kastan, 1994). In addition, p53 monitors DNA damage, activates genes required for DNA repair, and DNA repair quality control (Lane, 1992). It has also been shown that p53 possesses 3'-5' exonuclease activity (Mummenbrauer, 1996).

Downstream effectors of p53

Many genes like p21, MDM-2, GADD 45, cyclin G and bax have p53 binding sites and serve as potential target genes. MDM-2 gene serves as a direct target for sequence-specific p53 trans-activation and can regulate intracellular activity of p53 by acting as a physiological antagonist. MDM-2 negatively regulates p53 by preventing its function as a transcriptional activator (Momand et al., 1992; Oliner, 1993). It also promotes p53 degradation through ubiquitin-proteosome pathway.

P21/waf-1/Cip 1, another direct target for p53, encodes for a protein that inhibits cyclin-dependant kinase (cdk) (El-Deiry et al., 1993; Harper et al., 1993). Irradiated cells

accumulate cdk/cyclin E that are inactivated by $p21^{waf-1}$ association (Dulic et al., 1994). The inhibition of G_1, phase-specific kinase results in a hypophosphorylated, retinoblastoma gene product pRb, blocks the E2-F specific transcription of genes required for S phase entry (Xiong et al., 1993). Thus p53 mediated cell cycle block is seen mainly in the G_1 or G_2 phase. P21 also binds to and blocks proliferating-cell nuclear antigen (PCNA), thus interfering in the DNA replication (Waga et al., 1994). The signaling pathway of p53 is shown in Figure 2.1.

P53 and Differentiation

Initial experiments with p53 null mice yielded surprising results, as they appeared to develop normally. However, it was later discovered that they were prone to increased frequency of tumors in adult life (Armstrong et al., 1995) and also exhibited exencephaly (Sah et al., 1995). In addition to its role as a tumor-suppressor protein, it has been established that p53 is an important protein in embryonic development (Choi and Donehower, 1999). P53 plays an important role in hemopoetic differentiation. Induction on ML-1 myeloblastic human leukemia cells showed increased p53 during differentiation (Kastan et al., 1991b), whereas a decrease in the level of p53 has been reported in erythroleukemic cells (Khochbin, 1988). P53 up-regulation was reported in the differentiation of mouse myogenic cell line, C2 and chicken satellite cells (Halevy, 1993). P53 has been shown to be involved during epithelial cell differentiation, where a down regulation of p53 mRNA and protein was accompanied by increased ability of p53 to trans-activate transcription during differentiation (Weinberg et al., 1995). The expression of p53 correlates with specific stages of embryonic development. Previous

studies indicate a tissue specific expression pattern of p53 with terminally differentiated tissues exhibiting very low p53 levels. High expression of p53 during early embryogenesis, and a decline in tissues undergoing terminal differentiation have been reported (Schmid et al., 1991).

In situ hybridization studies (Mora et al., 1980) reported p53 expression in primary cell cultures of 12-14 days mouse embryo, and reduction in the p53 mRNA in embryo tissues from day 11 was reported (Rogel et al., 1985). The decrease in p53 protein and mRNA was also seen during chick development and is controlled at the post-transcriptional level (Louis et al., 1988). Differentiating embryonal carcinoma cells express decreased levels of p53 (Oren et al., 1982). Regulation of RA mediated early differentiation by functional and conformational modulation of p53 in ES cell has been reported (Sabapathy et al., 1997). It has been reported that p53 is necessary in protecting embryos from chemical or radiation-induced damage. The incidence of 2-chloro-2'-deoxyadenosine-induced eye defect was higher in p53 wild type fetuses as compared to heterozygous and null mutants (Wubah et al., 1996). Contrary to the above findings, a teratological suppressor function for p53 in p53 deficient mice exposed to benzo[a]pyrene has also been reported, where increased resorption of p53-/- fetuses has been observed suggesting that p53 protects the developing embryo from DNA damaging agents (Nicol et al., 1995). Additionally, p53-dependant apoptosis has been reported to suppress radiation-induced teratogenesis (Norimura et al., 1996).

C-Myc protooncogenes: Involvement of c-Myc in cell cycle regulation, differentiation and apoptosis.

Human c-*myc* gene, discovered about 20 years ago as a homologue to the retroviral gene *v-myc is* activated in several human cancers (Ayoma et al., 1998, Berns, 1996; Campisi, 1984; Escot, et al., 1986, Little, et al., 1983). Elevated expression of c-Myc protein was found in breast (Bieche et al., 1999; Escot et al., 1993) and colon cancers (Erisman et al., 1988; Erisman et al., 1989) but has not been associated with gene amplification or rearrangement of the gene. The myc family of genes includes c-*myc*, B-*myc*, L-*myc*, N-*myc* and s-*myc* (Henricksson and Luscher, 1996). Of these, only c-Myc, L-Myc and N-Myc can oncogenically transform cells.

The confirmation that c-Myc regulates the cell cycle comes from the data relating to the increase in the doubling time of nullizygous *c-myc* cells (Hanson et al., 1994). C-Myc regulates the cell cycle primarily in G_1 phase or G_1/S transition and has been known to repress differentiation and cell adhesion while actively promoting cellular proliferation (Kato and Dang, 1992; Amati et al., 1998, Lemaitre, et al., 1996). It is speculated that growth arrest genes like gadd45 and gas1 are repressed by c-Myc, disrupting the exit from the cell cycle to initiate differentiation (Marhin et al., 1997; Amundson et al., 1998). Several cell cycle proteins play an important role in the downstream signaling. Activation of cyclin D1 is complex and dependent on the cell system and stimulus (Roussel, 1997; Roussel, 1998). Deregulated expression of c-Myc has also been shown to increase cyclin E and A expression (Hanson et al., 1994; Jasen–Durr et al., 1993).

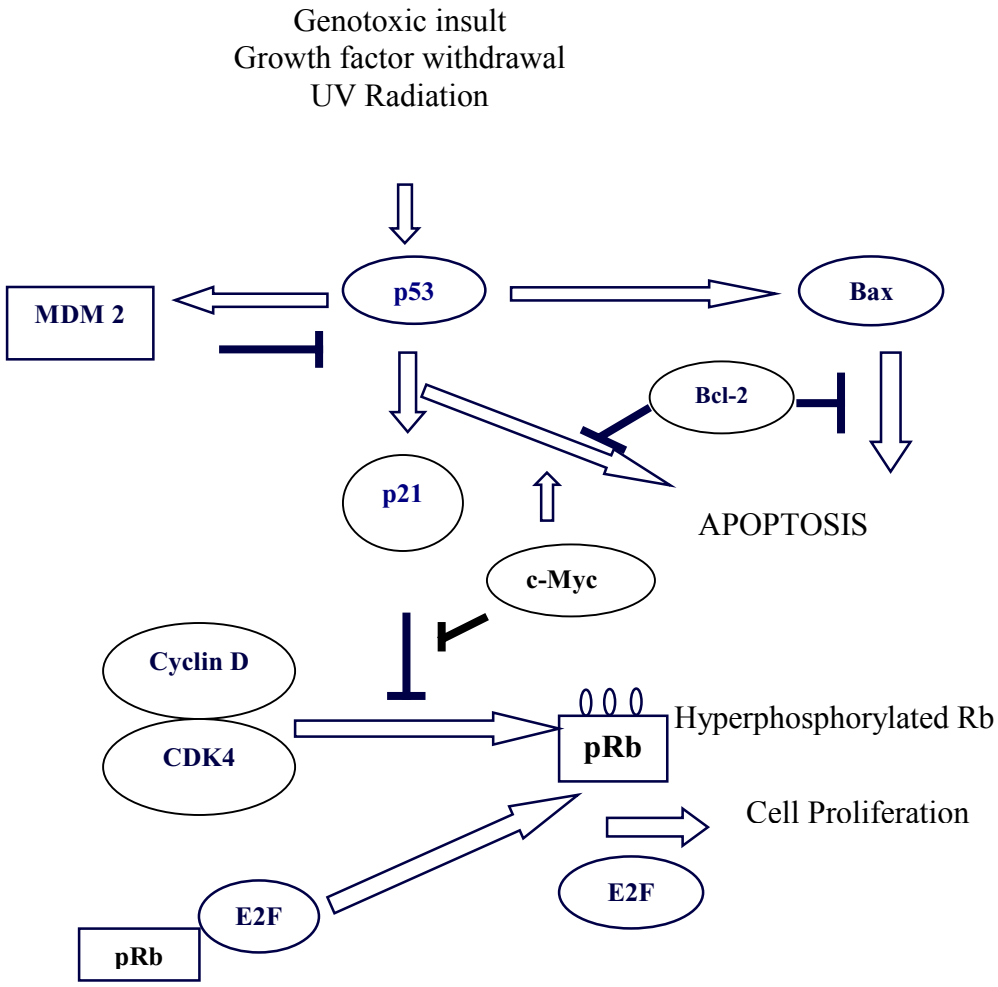

Figure 2. 2. p53 signaling pathway

C-Myc initiated decrease in the level of cyclin dependant kinase inhibitor p27 has been reported (Perez-Rogers et al., 1997). However, the exact mechanism of interaction with p27 is not known. All of these events prime the cell for entry into the S phase and actively promote cellular proliferation.

In addition to its role as an oncogene, overexpression of *c*-Myc causes marked apoptosis in serum depleted and IL 2 deficient cells (Askew et al., 1991; Evan et al., 1992; Wagner et al., 1993). It has also been shown that expression of wild-type p53 was required for Myc-mediated apoptosis and did not require the induction of p21, the trans-activation target of p53 (Wagner et al., 1994)

Several studies have reported both p53 dependant and independent pathways for c-Myc mediated apoptosis (Shim et al., 1998; Hermeking and Eick, 1994). Bcl 2 expression has been noted to protect the cells from undergoing c-Myc induced apoptosis (Wagner et al., 1993; Papas et al., 1999). Over-expression of c-Myc leads to non-regulation and consequently a reduced requirement for growth factors, accelerated cell division, and increased cell size has been reported (Sorrentino et al., 1986; Stern et al., 1986; Karn et al., 1989). Consequently, elevated expression of c-Myc can impede differentiation in many cell types (Coppola and Cole, 1986, Miner and Wold, 1991). In addition, exposure of antisense oligonucleotide to c-*myc* leads to growth arrest and terminal differentiation in Hl-60 and MEL cells (Griep and Westphal, 1988; Holt et al., 1988). It has been reported that c-*myc* can inhibit myogenic differentiation by Myo-D or myogenin (Miner and Wold, 1991).

Proliferation and differentiation exhibit the opposite ends of the spectrum of cellular fate. A direct interaction of c-Myc with the differentiation machinery has not yet been elucidated. It would be extremely valuable to find a direct interaction between c-Myc expression and the differentiation pathway. It has been reported that retinoic acid induces persistent RARα-mediated anti-proliferative responses in Epstein-Barr virus-immortalized B lymphoblasts carrying an activated c-Myc oncogene (Cariati et al., 2000,

Lee et al., 2000). Thus altered expression of retinoid receptors may lead to differential modulation of Myc family and could possibly influence the cellular fate and affect normal embryonic development. It is highly likely that RA might variably modulate c-Myc expression and the amount of c-Myc expression may influence the fate of the cell towards differentiation or proliferation and play a key role in the cellular decision-making process.

Myc/Max/Mad interaction

C-Myc binds to the DNA in association with its dimerizing partner Max (Blackwood et al., 1992). Max-Max homodimer and the Max-Myc heterodimers can effectively bind to specific DNA sequences to promote transcription while c-Myc alone is unable to bind to DNA (Kato et al., 1992). Recruitment of histone acetylators is the cornerstone of Myc-Max transcription activation. The transcriptional properties of c-Myc are also influenced by its binding with Mad family of proteins that recruit sin3 and other co-repressors that compete for c-Myc binding sites, and preclude c-Myc-mediated gene regulation (Amati and Land, 1994; Ayer et al., 1993). Consequently, increased expression of Mad proteins has been associated with cellular differentiation and growth arrest (Ayer et al., 1995). It can be envisioned that Mad and c-Myc can inversely regulates differentiation mediated by RA by Max interaction (Figure 2. 2). In neuroblastoma cells lines, Max over expression can enhance RA mediated growth arrest and differentiation in association with Max (Peverali et al., 1996). It is highly likely that Mad acts as a switch between proliferation and differentiation and retinoids may act as a master controller of the cellular fate.

Caspases: The final executioners

Throughout the evolutionary systems, apoptosis remains a highly conserved process required for normal development (Raff, 1992). Morphologically, apoptosis entails chromatin condensation, cytoplasmic shrinkage, blebbing and fragmentation. Initially, the cell membrane integrity is maintained leading to rapid phagocytosis of the dead cells and absence of inflammation. Several chemicals have been known to induce apoptosis. Cysteine proteases (c-asp-ases), play an important role during initiation and effector phase of apoptotic cells death (Kerr et al., 1972).

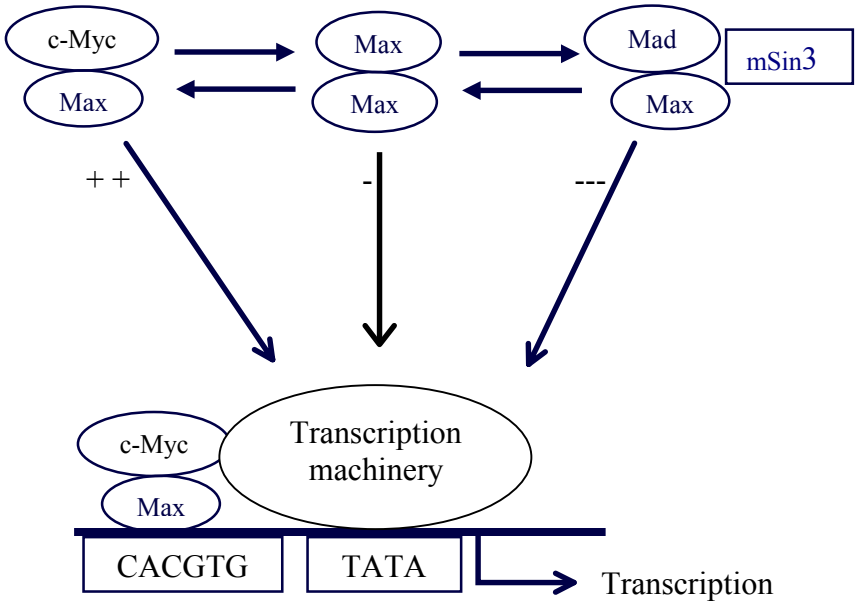

Figure 2. 3. Myc family interaction and influence on gene transcription

Caspases are secreted as inactive protease that can be activated by protein-protein interactions (Yamin et al., 1996), auto-catalytically (Thornberry, 1997), thereby unleashing the "caspase cascade" that can amplify the signals leading to apoptosis. Caspases cause the activation of DNA fragmentation factor, which is responsible for internucleosomal DNA damage (Liu et al., 1997). In addition the caspases also cleave and disable structural components of the cells like poly ADP ribose polymerase (PARP). The initiator caspases (caspases 8, 9 and 10) activate the down steam executioner caspases. These short pro-domain caspases 3, 6 and 7 act at the final stage of cell death. It has been reported previously that RA 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).

Bcl-2 family: Physiological regulators of caspase activity

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 are Bcl-2 family that act as physiological modulators of caspase activity. The Bcl-2 family comprises of pro-apoptotic Bax, Bad, Bid and anti-apoptotic Bcl-2 and Bcl-xL (Larson, 1994). 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). Thus the relative

expression of Bcl-2 members function as a rheostat between cell survival and death signal.

Cytokines during apoptosis

The end process of apoptosis results in the engulfment of apoptotic cells by macrophages. The mechanism by which the phagocytic action in 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). 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 proinflammatory cytokines is remarkably absent during apoptotic death (Meagher et al., 1992). It has been reported that inhibition of proinflammatory activity occurs in macrophages ingesting apoptotic cells (Voll et al., 1997; Fadok et al., 1998). The immuno-modulatory role of RA and its regulation of TNFα has been previously investigated in our laboratory in murine macrophage cell lines (Mathew and Sharma, 2000). Increased differentiation and reduced proliferation of ES cells with membrane bound and free TNFα has been noted earlier (Kohchi et al., 1996). Contrary to the above report, it has also been reported that TNFα decreased the formation of embryoid bodies in ES cells (Wuu et al., 1998). These reports suggest the temporal importance of TNFα signaling during differentiation. Interferon γ inducing factor 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 pro-inflammatory cytokine and has been attributed neuroendocrine properties (Fingerle-Rowson and Bucala, 2001). MIF has been linked to chick lens differentiation (Wistow et al., 1993) and has been speculated to serve as an intercellular messenger as a part of the differentiation pathway.

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CHAPTER 3

ALL-*TRANS*-RETINOIC ACID-MEDIATED MODULATION OF P53 DURING NEURAL DIFFERENTIATION IN MURINE EMBRYONIC STEM CELLS[1]

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Abstract

All-*trans*-retinoic acid (RA) plays an important physiological role in embryonic development and is teratogenic in large doses in all tested species. Tumor suppressor gene *p53* encodes phosphoproteins, which regulate cellular proliferation, differentiation, and apoptosis. Temporal modulation of p53 by RA was investigated in murine embryonic stem cells during differentiation and apoptosis. Undifferentiated embryonic stem cells express a high level of p53 mRNA and protein followed by a decrease in p53 levels as differentiation proceeds. The addition of RA during 8-10 days of differentiation increased the levels of p53 mRNA and protein, accompanied by accelerated neural differentiation and apoptosis. Marked increase in apoptosis was observed at 10 h to 20 h after RA treatment when compared with vehicle-treated controls. Retinoic acid-induced morphological differentiation resulted in predominantly neural-type cells. Maximum increase in p53 mRNA in RA-treated cells occurred on day 17, whereas maximum protein synthesis occurred on days 14-17 that coincided with increased neural differentiation and proliferation. Increased p53 levels did not induce p21 transactivation, and a decrease in p21 was observed on day 17 of RA exposure. The level of p53 declined by day 21 of differentiation. The results demonstrated that RA-mediated apoptosis preceded the changes in p53 expression, suggesting that p53 induction does not initiate RA-induced apoptosis during development. However, RA accelerated neural differentiation and increased the expression of p53 in proliferating neural cells, accompanied by decreased p21 levels, indicating the importance of cell type and stage specificity of p53 function.

Keywords: all-*trans*-retinoic acid, murine embryonic stem cells, neural differentiation, p21 induction, p53

Introduction

Embryonic development is a field that has been characterized by intense research for a long time. However, not all the clues are available to decipher the complex puzzle of development. Embryonic growth and development entails differentiation, growth, apoptosis and morphogenesis in a highly coordinated and controlled environment. Numerous complex proliferative signals mediated by various transcription factors, cyclins and cyclin-dependant kinases are suppressed for cell differentiation. Consequently, any alteration in the interactions between genes controlling these critical events during the cell cycle leads to departure from homeostasis and growth.

P53 poses a great enigma to researchers (Ko and Prives, 1996). In addition to its role as a tumor-suppressor it regulates cell cycle and differentiation (Almog and Rotter, 1997; Gottlieb and Oren 1996). Initial experiments with *p53* null mice yielded surprising results, as they appeared to develop normally and have suggested the dispensability of p53. However, deregulation of *p53* function leads to spontaneous tumor formation at an early age of six months (Donehower et al., 1992). Recent studies have implicated the role of p53 in the development of the central nervous system (CNS) and indicated defects like non-closure of neural tubes leading to exencephaly in *p53* null embryos (Armstrong et al, 1995; Sah et al., 1995). The development of the CNS is governed by the temporal spatial expression of various homeobox genes (Scott et al., 1989) and trophic factors responsible for changes in local cellular *melieu*. Cellular proliferation and cell

differentiation are balanced by growth arrest and apoptosis. The key aspect of embryonic development lies in cell cycle control and p53 is intricately involved in the cellular decision making process. It is now known that p53 is an important mediator of cell cycle checkpoint at the G1- S phase transition (Kastan et al., 1982). Induction of p53 is generally characterized by increased transcription of p21, a cyclin dependant kinase inhibitor (CDKI) (el-Deiry et al., 1993; Harper et al., 1993). They interact with the cyclin–cyclin dependant complexes and regulate the cell cycle. However, it is becoming increasingly clear that p21 can be induced in a p53 independent manner (Gartel and Tyner, 1999).

In addition, p53 also induces cell death. Apoptosis or programmed cell death, a highly conserved evolutionary process, plays a vital role in development by removing unwanted cells (Uren and Vaux, 1996). It is now well accepted that p53 plays an important role in apoptosis (Yonish-Rouach, 1996). Over-expression of p53 causes growth arrest and apoptosis (Yonish-Rouach et al., 1991). It has also been reported that high levels of p53 induced apoptosis, whereas lower levels cause cell cycle arrest (Chen et al., 1996). P53 has been shown simultaneously to mediate apoptosis and differentiation in neurons and oligodendrocytes. It is not known what specific role p53 plays during early embryogenesis. However, a synchronized and tightly coupled liaison can be envisioned between the two p53-related roles, with the level of p53 and cell type important in influencing embryogenesis and differentiation.

The physiological role of all-*trans*-retinoic acid (RA) in development has been studied extensively. Previous studies have indicated the involvement of p53 with RA on cancer cells, and it was reported that the level of p53 declines during RA-mediated

differentiation in neuroblastoma cell lines (Davidoff et al., 1992). Later, an increase of p53 with RA administration was reported (Joiakim and Chopra, 1993). It has also been shown that RA confers resistance to p53-mediated apoptosis in SH-SY5Y neuroblastoma cells (Ronca et al., 1999). Induction of differentiation by RA in F9 embryonal carcinoma cells has shown that p53 becomes functionally active (Lutzker and Levine, 1996). These results have suggested that p53 plays an important role during RA-mediated differentiation and apoptosis.

Murine embryonic stem cells (ES), a non-transformed cell line was chosen to study the influence of RA on p53 expression during differentiation and apoptosis. 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 (Evans and Kaufman, 1981; Doetchman 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. They differentiate into cell types of varied lineage in the absence of a feeder layer (Smith et al., 1988; Keller, 1995).

To answer questions regarding the influence of RA on the temporal relationship between p53 induction during differentiation and apoptosis of ES-D3 stem cells, here we report that p53 is differentially expressed during early and late differentiation of ES cells. Undifferentiated ES cells express high levels of p53 and differentiation leads to a dramatic decrease in p53 mRNA and protein expression. Retinoic acid accelerated apoptosis in ES cells and induced cellular differentiation, which resulted in predominantly neural type cells, accompanied by increased p53. Although RA both accelerated differentiation and apoptosis and caused an increase in p53 mRNA and

protein, the latter cellular effects preceded those on p53, suggesting that p53 induction does not initiate RA-induced apoptosis during development. However, p53 induction on day 14-17 of differentiation was marked by increased neural cell proliferation and a possible role in early neural differentiation and proliferation can be envisioned.

Materials and methods

ES cell culture and retinoic acid treatment.

Murine embryonic stem cells (ES-D3, ATCC # 1934-CRL) were maintained on mitotically inactivated mouse fibroblast feeder layer, (STO, ATCC # 1503-CRL) treated with 10 µg/ml Mitomycin-C in Dulbecco's modified Eagle's medium (Doetchman et al., 1985). The medium was supplemented with retinol free 15% Knock-Out^{TM} Serum Replacement (Life Technologies, Grand Island, NY), 10 µM ß-mercaptoethanol (Sigma, St. Louis, MO) and 1000 units/ml of Leukemia Inhibitory Factor (LIF) (Sigma). The medium was changed everyday. The ES cells were passaged every 2 days onto fresh feeder layers to maintain an undifferentiated state. To induce differentiation, 1 X 10^5 ES cells/well were plated as monolayers in 6 well plates, in the absence of feeder layers and LIF and was counted as the first day of differentiation. All-*trans*-retinoic acid (RA) (Sigma) dissolved in 85% ethanol to provide a concentration of 10^{-6} M was added to the media on the eighth, ninth, and tenth day of differentiation. In our preliminary trials, we have noted that RA exposure for three consecutive days; beginning on day 8 increases neural differentiation. An equal volume of vehicle (85% ethanol) was added to the control medium. The final concentration of 2.75 X 10^{-2} M (1.5 µl/ml) of ethanol in the culture media did not influence differentiation. Formulation and addition of RA was

done under yellow light to reduce isomerization. All experiments were repeated three times; data from a representative experiment are presented in this report.

Morphological scoring of ES cells.

ES cells aggregate into cystic embryoid bodies (EB) containing complex embryonic structures. Embryoid body formation, with and without RA, was scored for morphological differentiation on a scale of 0 to 5 for a period of 28 days under light microscopy. The cells were scored as: 0 = no differentiation and 5 = maximal differentiation. The morphological criteria for differentiation are listed in Table 1 and included size, thickness, formation of inner and outer membrane structures of EBs, and cell type.

Cell viability and estimation of cell death.

Undifferentiated ES cells were plated in triplicate in six-well plates (Costar, Corning Incorporated, Corning, NY) at a density of 1 X 10^5 cells/well and treated with 10^{-6} M RA on the eighth day of differentiation. The media was changed daily. To study normal ES cell viability in the absence of RA, undifferentiated cells were treated with an equal volume of 85% ethanol. The cells were collected with the culture media by gentle scraping and repeated pipeting at 8, 11, 14, 17 and 21 days of differentiation. The use of trypsin to dissociate cells was avoided to prevent cellular damage due to action of trypsin. After staining with 0.4% trypan blue dye, the numbers of stained and total cells were counted on a hemocytometer to determine viability (by exclusion of the stain) within 5 min.

Detection of apoptosis.

For detecting apoptosis by changes in nuclear morphology, 1 X 10^4 ES cells were grown on 4-chambered glass slides. After RA treatment on the eighth day for 10 and 20 h, the culture medium was discarded and replaced with 4% paraformaldehyde in phosphate buffer saline (PBS) for 10 min. The fixed cells were rinsed in PBS and stained with 2.5 µg/ml Hoechst-33258 (Sigma) for 5 min. Subsequently the cells were washed in PBS and glass cover slips mounted with glycerol: PBS in (9:1) ratio (Gshwind and Huber, 1997). The excitation and emission maxima of the fluorescent dye were 346 nm and 460 nm, respectively; visualization of the apoptotic nuclei was accomplished by fluorescent microscopy using appropriate filters.

P53 mRNA expression by reverse transcriptase- polymerase chain reaction (RT-PCR).

ES cells were grown in triplicate in the absence of fibroblasts layers in 6 well plates at a density of 1 X 10^5 and treated with RA in the absence of feeder layer and LIF (as described above). Total cellular RNA was extracted from ES cells by TRI Reagent® LS (Molecular Research, Cincinnati, Ohio) according to the manufacturer's protocol and 1 µg of total RNA was used to synthesize first strand c-DNA by using Superscript (Life Technologies, Grand Island, NY). The sense and antisense primers for *β-actin* and *p53* were chosen by Primer3 program (Whitehead Institute, Cambridge, MA); these were 5'-GTACCTTATGAGCCACCCGA-3' and 5'-CTTCTGTACGGCGGTCTCTC -3' for *p53*, and 5'-ATGGATGACGATATC GCT -3' and 5'-ATGAGGTAGTCTGTCAGGT-3' for *β-actin*, respectively. Samples equivalent to 1 µl of the first strand reaction cDNA were

then used as a template for amplification in the exponential phase of PCR reaction for 35 cycles at an annealing temperature of 48°C (β-*actin*) and 55°C (*p53*), respectively. Preliminary trials indicated that DNA amplification was in the exponential phase upto 35 cycles (Figure 5 inset). The RT-PCR products were mixed with 10X DNA dye [5 mg/ml bromophenol blue, 50% glycerol, 100 mM Tris, 20 mM NaCl, 1 mM ethylenediaminetetraaceticacid, (EDTA)] in 10:1 ratio and run on 2% agarose gel for electrophoretic separation. The gels were photographed in a backlighted UV transilluminator. Photographs were scanned and quantified on UN-SCAN-IT™ automated digitizing system, (Silk Scientific, Inc. Orem Utah). The total pixel count for *p53* was normalized to β-*actin*, a housekeeping gene.

Determination of p53 and p21 protein by western blot.

ES cells grown in triplicate in 6-well plates at a density of 2 X 10^5, (without fibroblast feeder layers or LIF) in the presence and absence of RA were lysed on ice for 30 min in lysis buffer (Wali and Strayer, 1996). Cell lysates were centrifuged at 14,000 rpm at 4°C for 15 min to remove cell debris. The aliquots of supernatant were stored at –80°C and assayed using Bradford reagent (Sigma). An aliquot of 30 µg of the supernatant protein from each sample was heated with 4X Sodium dodecyl sulphate (SDS), sample buffer at 95°C for 5 min, and separated electrophoretically on a 10% SDS–polyacyramide gel. Subsequently, the proteins were transferred onto 0.45 µm pore size nitrocellulose membranes for 90 min and blocked overnight with 5% milk Tween-Tris buffer saline (TBS). Nitrocellulose membranes were then exposed to p53 primary monoclonal antibody for 1 h in blocking buffer at 1:10 dilution. p53 primary antibody was generated

by immunizing mice with p53 protein and fusing with NS-1 mouse melanoma cells. It was characterized as IgG_{2a} isotype, epitope localized within the carboxyl terminal region and cross-reactive to human, mouse and rat. A dilution of 1:100 was used for p21 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, the membranes were incubated for 1 h with horseradish peroxide-conjugated polyclonal anti-mouse IgG at 1:5000 dilution. Between each exposure, the nitrocellulose membranes were washed for 30 min with Tween–TBS. The proteins were visualized autoradiographically by enhanced chemiluminescent substrate (ECL® Amersham Pharmacia, Piscataway, NJ), after 60 min of exposure that was optimized by initial trials. The radiographs were scanned and digitized using UN-SCAN-IT™ automated digitizing system as described for PCR gels above.

Statistics.

All values are given as mean ± standard error. Data were analyzed by one way analysis of variance (ANOVA) followed by Student's *t*-test. Statistical analysis system (SAS) was employed for all calculations (SAS Institute, Cary, NC). The level of $P \leq 0.05$ was considered statistically significant for difference.

Results

Retinoic acid promotes morphological differentiation of ES cells

To study the process of spontaneous differentiation, 1×10^5 ES cells were grown in monolayers 6-well plates in triplicate in the absence of feeder layers and LIF, with and without RA. It has been previously reported that no difference in ES cell development

potential was found in suspension, micromass or monolayer cultures (Doetschman et al., 1989). The cells were scored for morphological differentiation under light microscopy for 28 days. ES cells treated with 1 μM RA showed an increased degree of differentiation and predominantly differentiated into neural type, seen at the base of the attaching embryoid body (Figure 3. 1). It was observed that the maximal differentiation into neural cells occurred on day 17 of differentiation with RA treatment. Additionally, increased proliferation was also noted on day 17 of differentiation in the presence of RA. No further changes in morphological structures could be detected beyond days 21 to 28 (Figure 3. 2). Cells undergoing normal differentiation in the absence of RA showed slower differentiation. Cystic EB formation was seen, with distinct inner and outer structures. Some EBs were optically dense and resembled 5-day embryo structure. Interestingly, in the absence of RA, the differentiating cells were of mixed lineage with very few neural cells detected. Maximal differentiation was seen around days 17-21, and no further differentiation was observed beyond day 28. The above findings indicated that RA could regulate the differentiation and proliferation of neural cells in ES cells (Table 3. 1).

Retinoic acid-induced cell death and apoptosis in ES cells

During embryonic development, growth arrest, differentiation, and apoptosis occur side by side. It was noted that the addition of 1 μM RA for three consecutive days to the medium resulted in increased cell death (Figure 3. 3). On day 11 of differentiation, about 40% of the ES cells stained blue with trypan blue in RA-treated cultures compared with 26% cell stained blue in ES cultures undergoing spontaneous differentiation in the

absence of RA. In spontaneously differentiating cell cultures, the number of dead cells averaged from 25% to 27%, whereas in RA-treated cultures the value ranged from 39% to 54% ± standard error on the day 11 of differentiation, as determined by three independent experiments with three replicates for each group (Table 3. 2). To determine if the process was apoptosis, ES cells were stained with Hoechst 33258, a fluorescent DNA dye. Control cells treated with equal volume of 85% ethanol showed a background apoptosis of 47% at 10 h. Retinoic acid increased apoptosis in ES cells within 10 to 20 h of exposure. After 10 h of RA exposure, about 64% cells showed typical morphological signs of apoptosis including chromatin condensation, DNA cleavage, nuclear fragmentation, and cytoplasmic vacuolation. At 20 h, about 27% apoptotic cells were noted in control cultures, whereas RA treatment resulted in 56% apoptosis (Figure 3. 4). Results suggested that RA mediated cell death in ES cell is characterized by chromatin condensation, cytoplasmic blebbing and the formation of apoptotic bodies that are the hallmark of apoptosis.

Expression of p53 mRNA in ES cells

To elucidate a detailed pattern of gene expression during differentiation in ES cells, p53 mRNA expression was studied. Undifferentiated ES cells expressed high levels of *p53*. Even in the absence of any differentiation-inducing agent, the level of *p53* is dramatically decreased from 12 to 48 h of plating the ES cells in the absence of feeder layers and LIF (Figure 3. 5). In spontaneously dividing cells, no change in *p53* was observed up to day 21 of differentiation. However, in ES cells treated with 1 μM RA on the day 8, 9 and 10 of differentiation, increase in *p53* levels was noted on day 17 of differentiation, followed

by a decline on day 21 (Figure 3. 6). Expression of p53 mRNA was also examined after 10 h and 20 h of RA exposure where typical apoptotic changes in nuclear morphology were noted. No change in the *p53* level was detected at these times (Figure 4 inset). Thus a complete profile of p53 expression in spontaneously and RA–mediated differentiation was noted. Furthermore, increased expression of p53 coincided with the increased number of neural cells observed in ES cell cultures exposed to RA.

Expression of P53 protein

Kinetics of protein expression was studied for undifferentiated ES cells and after 12, 24, 36 and 48 h of differentiation. Undifferentiated ES cells expressed high levels of p53 that declined during differentiation (Figure 3. 7) and the transcribed gene correlated to the translated product. The protein was barely detectable from day 4 to 8 of differentiation. The level of p53 protein expression increased in RA-treated cells with maximal protein expression seen on days 14 and 17 of differentiation (Figure 3. 8). The protein expression during initial and RA-mediated differentiation paralleled the mRNA expression. In RA-treated cells, the level of p53 protein expression on day 17 of differentiation, increased as compared with the control for the concurrent day. At terminal differentiation, protein levels decreased (Figure 3. 8). No change in the amount of p53 was noted at 10 and 20 h of exposure to RA (data not presented). The level of p53 during this period remained very low to undetectable.

Expression of p21

Induction of p53 has been correlated to transactivation of p21. P21 has been shown to inhibit proliferation both in vivo and in vitro. We examined the expression of p21 during

the appearance of neural cells in RA-treated cultures, on days 14, 17 and 21 of differentiation (Figure 3. 9). In ES cells treated with RA no change in the expression of p21 was noted on day 14 and 21 days of differentiation. Interestingly, a decrease in p21 level was noted on day 17 of differentiation with RA-exposure. This decrease coincided with increased p53 seen on days 14-17 of differentiation. For terminal differentiation to proceed, induction of p21 is a necessity. However, a decline in p21 would favor the proliferation of newly formed neural cells as noted in our experiment. At terminal differentiation, p21 levels were similar in control and treated cells. This may indicate that p21 can function independent of p53 induction during differentiation.

Discussion

Results indicate differential expression of p53 in embryonic stem cells. Undifferentiated ES cells express a high level of p53 mRNA and protein. As ES cells begin to differentiate, in the absence of feeder layers and any differentiation-inducing agent, p53 mRNA and protein levels rapidly decrease (Sabapathy et al., 1997). Retinoic acid-mediated differentiation is preceded by apoptosis and leads to up-regulation of p53 mRNA and protein, accompanied by increased neural differentiation noted on day 17 of differentiation. We observed that when 1 μM RA is added to the culture medium on the eighth, ninth and tenth day consecutively, the level of expression of p53 mRNA and protein on days 14-17 of differentiation is increased, accompanied by increased population of differentiated neural cell. The gene expression was found to parallel protein expression, during RA-mediated differentiation.

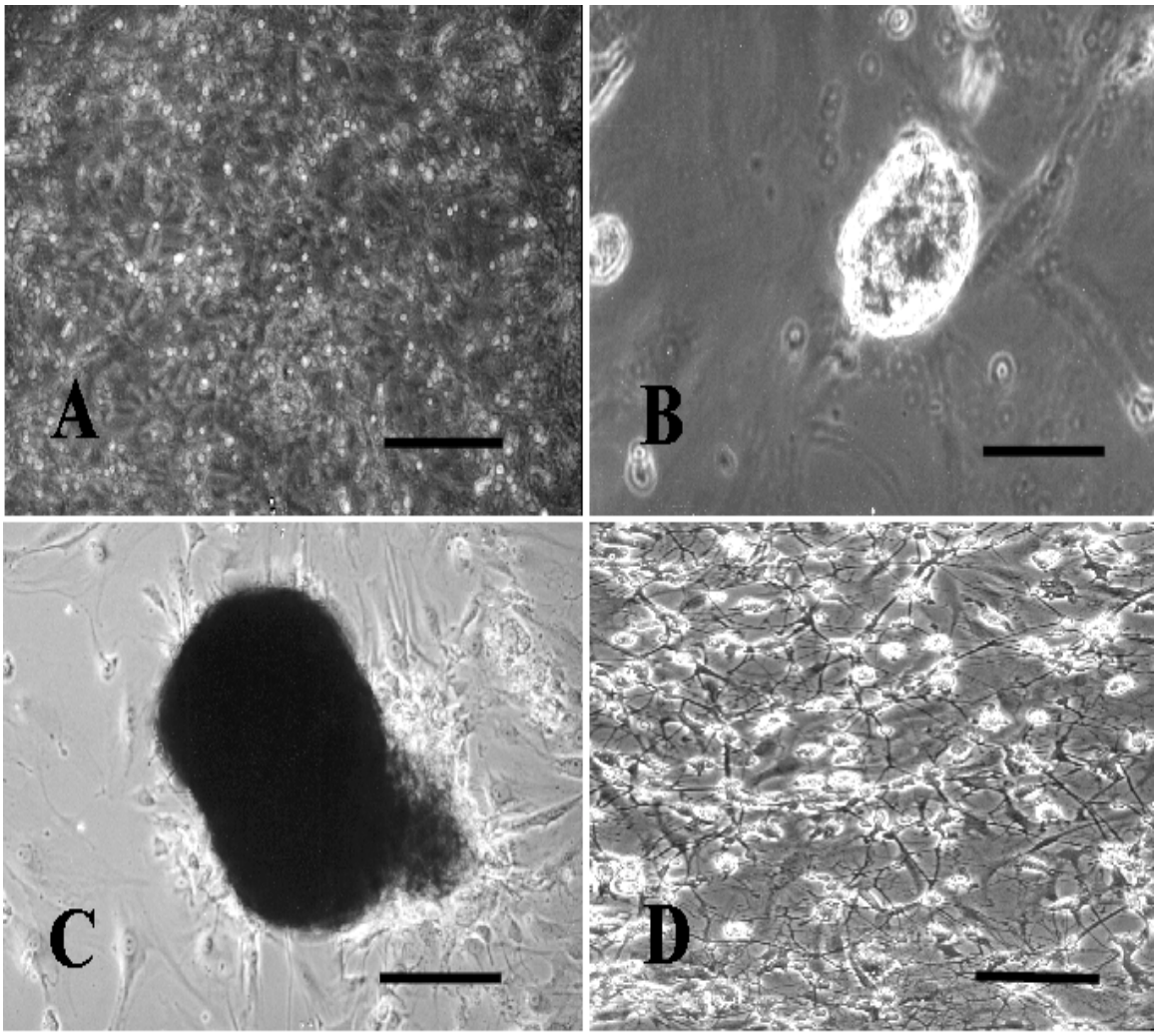

Figure 3. 1. Morphological differentiation of ES cells. ES cells were grown in 6-well plates and allowed to differentiate in the presence and absence of Retinoic acid (RA). A, undifferentiated ES cells; B, embryoid body morphology after 3 days of 1 μM RA exposure (11 day of differentiation); C, an attaching cystic embryoid body with differentiating cells at the base with RA treatment (14 days of differentiation). D shows neural differentiation with RA (21 days of differentiation). Magnification on fig A, B, D is 200X and fig. C is 400X and bar represents 100 μm for fig. A, B, D and 50 μm for fig. C.

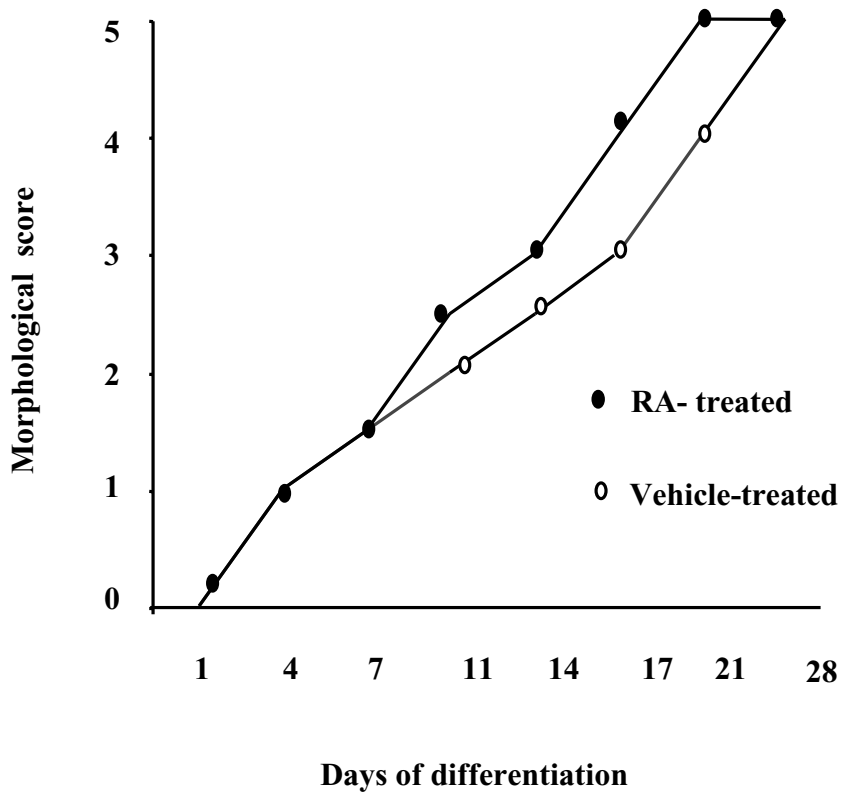

Figure 3. 2. Morphological Scoring of ES cells during differentiation in the presence and absence of 1 μM RA. The cells were scored by light microscopy on a scale of 0 - 5 for a period of 28 days. 0 = no differentiation vs. 5 = maximal differentiation.

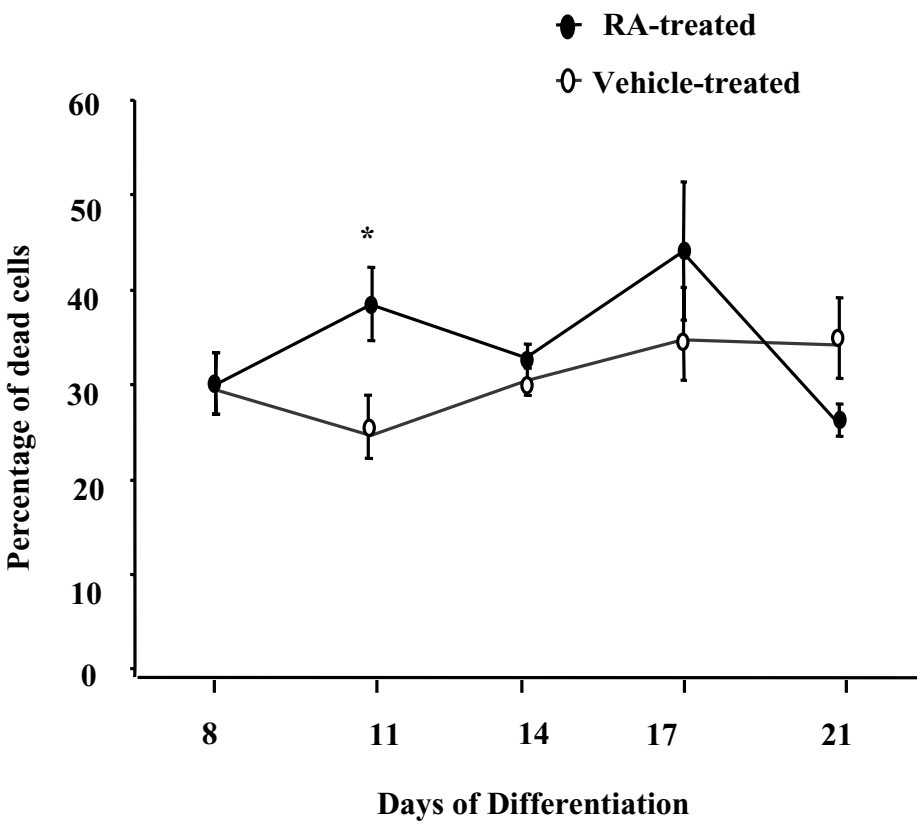

Figure 3. 3. Viability of ES cells during differentiation with and without RA. The cells were plated in 6-well plates in triplicate and treated with 1 μM RA on the eighth, ninth and tenth day of differentiation and the number of viable and dead cells was determined using 0.4% trypan blue dye uptake. Cells showed increased cell death after treatment with RA on day 11 of differentiation ($P< 0.05$). Data represents mean ± standard error of mean (s.e.m) of 3 independent wells of a representative experiment, n=3. ∗ Indicates significant difference ($P<0.05$) compared to the concurrent vehicle control on that day.

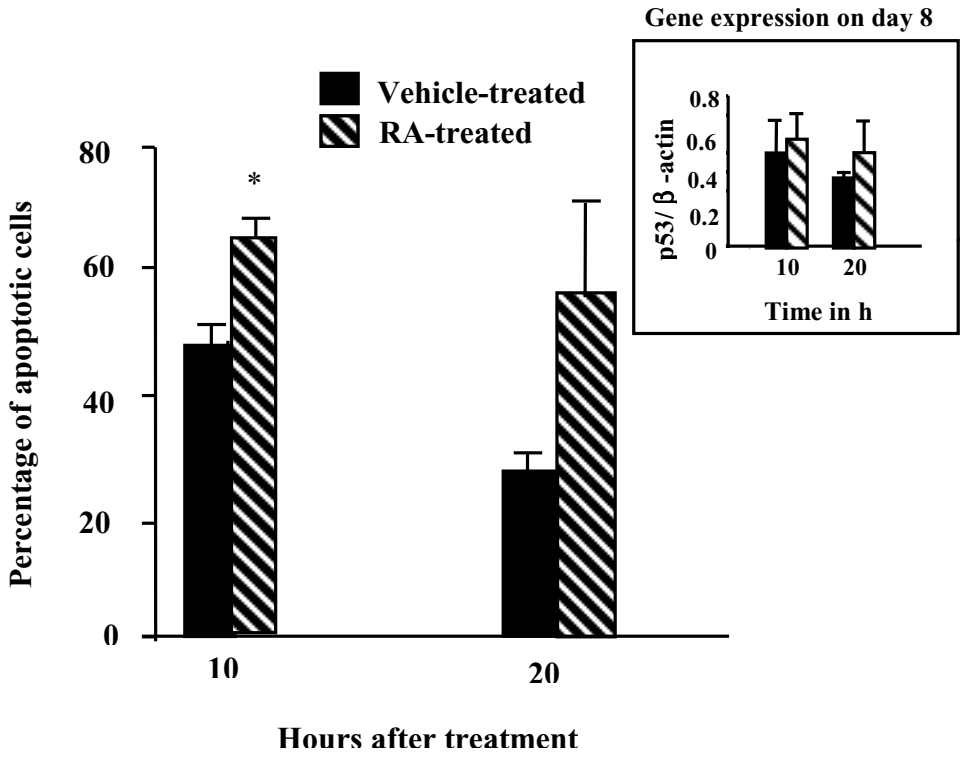

Figure 3. 4. Retinoic acid-mediated apoptosis by changes in nuclear morphology. The cells treated with RA were stained with Hoechst 33258 dye showing apoptosis characterized by nuclear fragmentation and cytoplasmic blebbing in 10-and 20-hr. Data represent mean ± s.e.m. of 3 independent wells of a representative experiment, n=3. ∗ Indicates significant difference (P<0.05) compared with the concurrent vehicle control on that day. Inset: expression of p53 mRNA normalized to *β-actin* during 10 to 20 h of RA exposure. Three μg of total RNA was used to synthesize cDNA. No change in p53 expression was noted during this time.

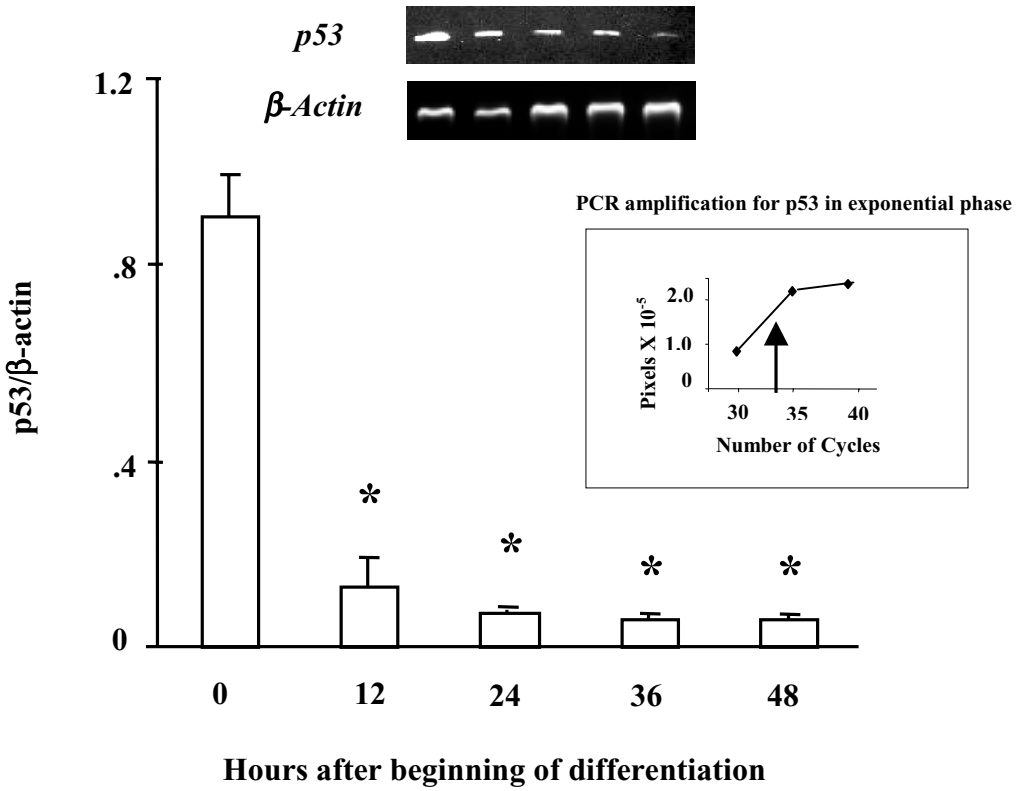

Figure 3. 5. Expression of p53 mRNA in spontaneously differentiating ES cells for a period of 48 h. Undifferentiated ES cells express high levels of *p53* and a decline was noted during differentiation. The mRNA was subjected to reverse transcriptase polymerase chain reaction (RT-PCR) in the exponential phase and normalized to a similar expression of β-actin (reference) in the sample. Data represent mean ± s.e.m. of 3 independent wells of a representative experiment, n=3. ∗ Indicates significant difference (P<0.05) compared with the concurrent vehicle control on that day. 0 h = undifferentiated ES cells. Graph on the right shows the validity of 33 cycles for a semi-quantitative measurement of p53, within the exponential phase of increase in product size.

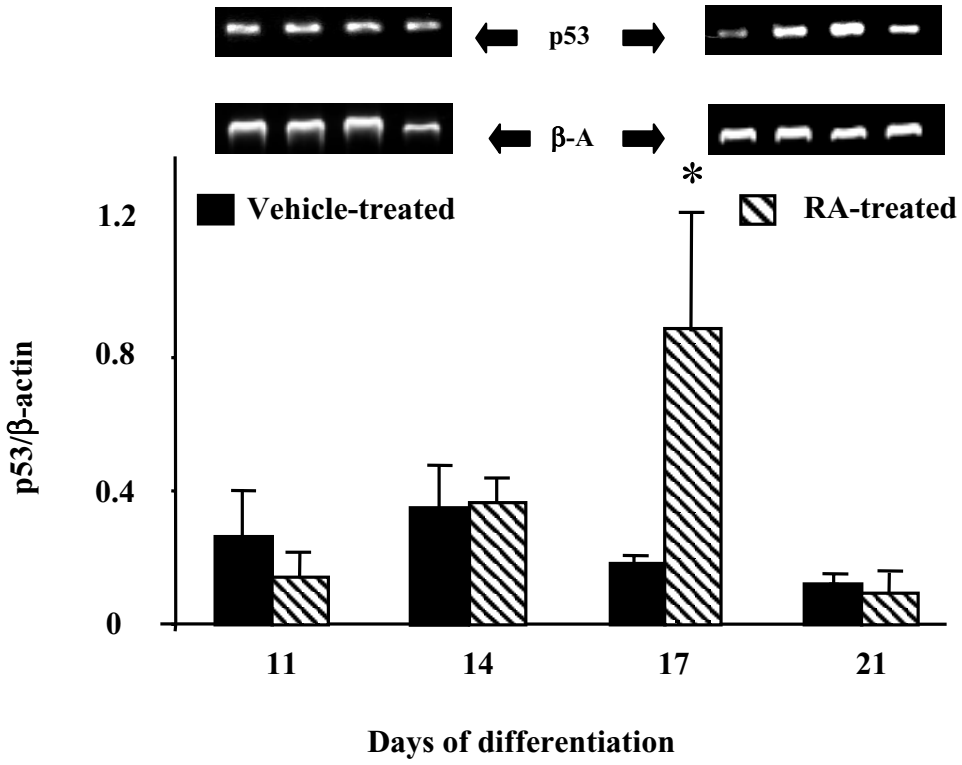

Figure 3. 6. Expression of p53 mRNA in vehicle and retinoic acid-treated ES cells. Increased expression of p53 gene was noted on day 17 of differentiation in RA-treated cells. The mRNA was subjected to RT-PCR and normalized to the expression of β-actin (reference) in the sample. Data represent mean ± s.e.m. of 3 independent wells of a representative experiment, n=3. ∗ Indicates significant difference (P<0.05) compared with the concurrent vehicle control on that day.

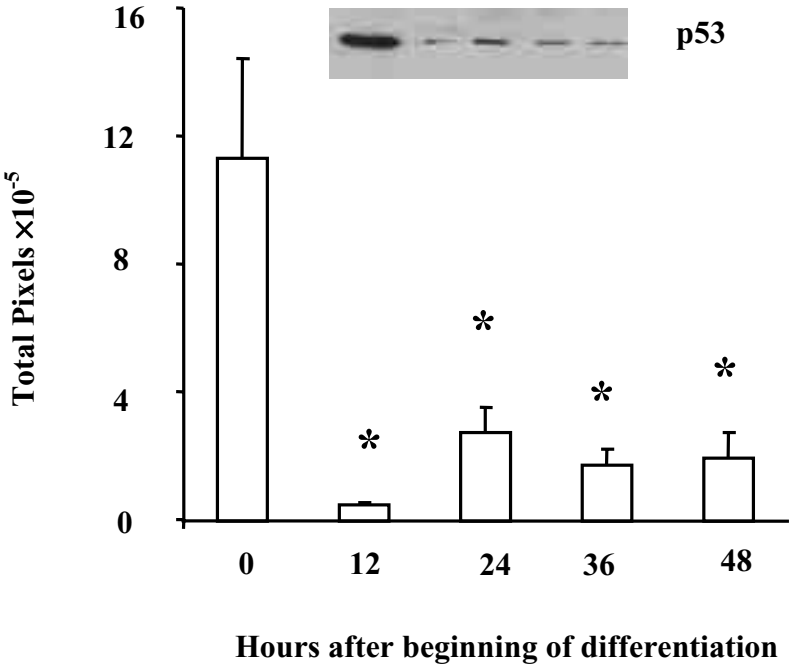

Figure 3. 7. Expression of p53 protein in spontaneously differentiating ES cells determined by western blot. Increased expression of p53 was noted in undifferentiated ES cells and a decline was noted with progression of differentiation. An aliquot of 30 µg of the supernatant protein from each sample was analyzed on 10% SDS–PAGE and assayed using a chemiluminescence probe; the resulting X ray films were subsequently scanned and data are represented as pixels. 0 h = undifferentiated ES cells. Data represent mean ± s.e.m. of 3 independent wells of a representative experiment, n=3. ∗ Indicates significant difference (P<0.05) compared with the concurrent vehicle control on that day.

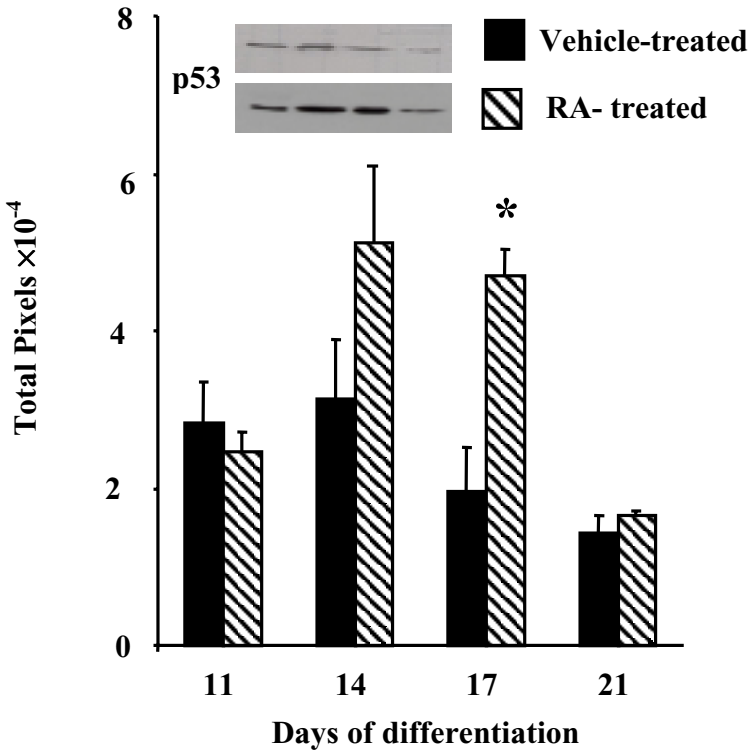

Figure 3. 8. Expression of p53 protein in vehicle and RA-treated cells analyzed by western blot. Increased expression of p53 in RA-treated cells was noted on day 14 and 17 of differentiation. An aliquot of 30 µg of the supernatant protein was separated electrophoretically and chemiluminescently probed. Subsequently the X ray films were scanned and the data presented as pixels. Data represent mean ± s.e.m. of 3 independent wells of a representative experiment, n=3. ∗ Indicates significant difference (P<0.05) compared with the concurrent vehicle control on that day.

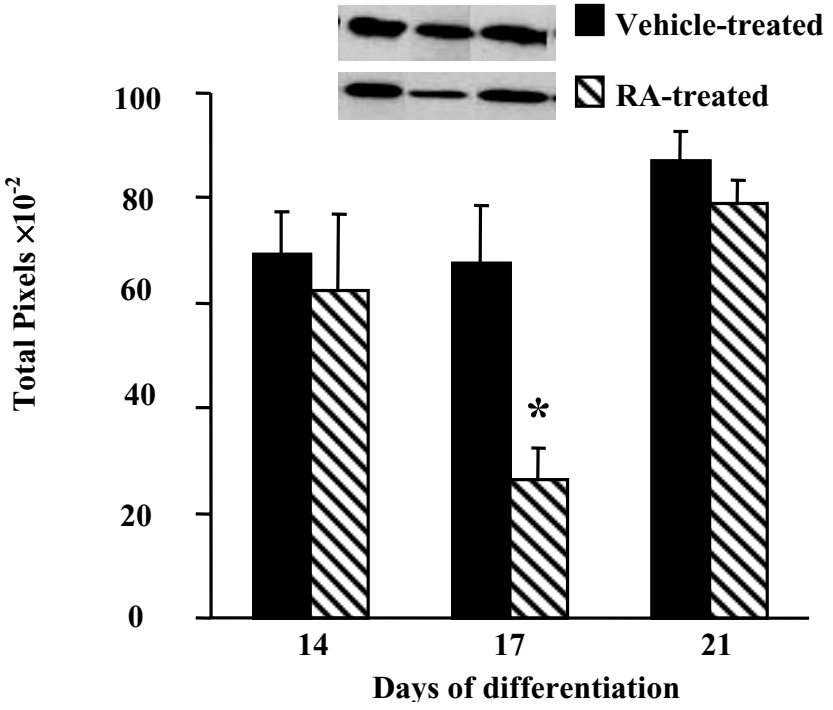

Figure 3. 9. Expression of p21 protein in vehicle and RA-treated cells analyzed by western blot on day 14, 17 and 21 of differentiation. P21 was decreased in RA treated cell on day 17 of differentiation. An aliquot of 30 µg of the supernatant protein was separated electrophoretically and chemiluminescently probed. Subsequently the X ray films were scanned and the data presented as pixels. Data represent mean ± s.e.m. of 3 independent wells of a representative experiment, n=3. ∗ Indicates significant difference (P<0.05) compared with the concurrent vehicle control on that day.

Table 3. 1. Stem Cell Scoring Index for Morphological Differentiation in Days

Morphology	Score	Day
Cells dispersed	0	1
4-6 cell clumped	1	3
Small embryoid body (EB) formation starts	1.5	7
8-12 cell cluster, few EB seen	2	11 C
EBs with thin outer membrane	2.5	11 RA/ 14 C
Well defined EBs with definite outer membrane, cells seen differentiating into neural cells.	3	14 RA
EB looked like 5-day embryos and inner structures seen.	3.5	17 C
Well defined EBs, thick outer membrane and most cells differentiating into neural cells with RA, mixed lineage of cells seen in vehicle-control.	4.0	17 RA/21 C
Mostly neural cells seen with RA, mixed cells in vehicle control.	5.0	21-28 RA 28 C

ES cells were scored for morphological differentiation under light microscopy. Score = 0: no differentiation and 5 = maximal differentiation in the presence and absence of all-*trans*-retinoic acid (RA). Cells treated with equal volume of 85% ethanol were used as vehicle control (C). Embryoid body (EB) differentiation into neural cells was noted after 3 days of RA exposure.

Table 3. 2. Percentage of dead ES cells on day 11 of differentiation after 1 μM RA treatment. Mean ± s. e. m. of three observations

Experiment #	Vehicle-treated	RA-induced	*P* value
1	25.0 ± 0.0	40.6 ± 0.1	0.03
2	27.3 ± 0.1	53.8 ± 0.0	0.02
3	26.0 ± 0.0	39.3 ± 0.1	0.03

Maximal mRNA expression was noted on day 17 of differentiation, whereas maximal protein expression was seen on day 14. The increase in p53 protein levels on day 17 of differentiation was found to be significantly different from the concurrent ethanol control. A decline in p53 mRNA and protein level on day 21 was followed by no further morphological differentiation. Two conclusions can be drawn from the present analysis. Firstly, we believe that increase in p53 seen during RA-mediated neural differentiation could possibly be a function of proliferation of newly formed neural cells. We have noted an absence of p53 induction with retinoic acid-mediated non-neural differentiation. Interestingly, it is only during retinoic acid –mediated neural differentiation that increase in p53 mRNA and protein level was observed. It is increasingly becoming clear that that function of p53 during neural development is

spatially and temporally regulated. The expression of p53 in proliferating areas and newly generated post-mitotic neurons has been reported in developing and adult rat brain and the role of p53 has been envisioned to control cell division and early differentiation rather than apoptotic events (van Lookeren Campagne and Gill, 1998). It has also been reported that p53 is expressed in neuroblasts and is down regulated when migrating neurons reach their destination (Ferreira and Kosik, 1996). Similarly, upon terminal differentiation of neurons in *vivo* and in a neuronal precursor cell line, ST15A down regulation of p53 mRNA has also been reported (Hayes et al., 1991) corresponding to the decline in p53 mRNA and protein in terminally differentiated ES cells in our study. Furthermore, the important role of p53 in the development of the nervous system is illustrated by studies in mice in which disruption of p53 gene affected the development of normal nervous system (Armstrong et al., 1995; Fulci and Meir, 1999).

Regulation of RA-mediated early differentiation by functional and conformational modulation of p53 in ES cell has also been reported (Sabapathy et al., 1997). Undifferentiated teratocarcinoma cells have been reported to become transcriptionally active when undergoing differentiation with RA (Lutzker and Levine, 1996). It can be envisioned that high level of p53 mRNA can encode high level of protein. However in the absence of DNA damage and the inherently labile nature of p53, transactivation of p53-responsive genes is not evoked. We have observed that the increased expression of p53 is not correlated with p21 induction. The level of p21 declined at day 17 in ES cells exposed to RA. Moreover, a repression of p21 would be desirable for the proliferation of newly formed neural cells. Although, p21 is transcriptionally activated by p53, however a detailed analysis of proliferating areas of rat brain has revealed that mRNA of p53 and

p21 are not co-localized (van Lookeren Campagne and Gill, 1998). Interestingly, studies in p53 null mice have also shown that p21 can function during differentiation independent of p53 (Parker et al., 1995). P53 expression has been found to be tissue specific during late organogenesis stages and a decline in p53 levels have been noted in terminally differentiated tissues. High expression of p53 during early embryogenesis, and a decline in tissues undergoing terminal differentiation have been reported (Schmid et al., 1991). In situ hybridization studies (Mora et al., 1980) revealed p53 expression in primary cell cultures of 12 to 14 day mouse embryos. Subsequently, a reduction in the p53 mRNA in embryo tissues from day 11 was reported (Rogel et al., 1985). The decrease in p53 protein and mRNA was also seen during chick development and is controlled at the post- transcriptional level (Louis et al., 1988). In addition to its role as a tumor-suppressor protein, p53 has been established as an important protein in embryonic development (Choi and Donehower et al, 1999). P53 also plays a significant role in hemopoetic differentiation. Induction of differentiation on ML-1 myeloblastic human leukemia cells showed increased p53 during differentiation (Kastan et al., 1991), whereas a decrease in the level of p53 has been reported in erythroleukemic cells (Khochbin et al., 1988). Increase in p53 was reported in differentiation of mouse myogenic cell line, C2C12, and chicken satellite cells (Halvey, 1993). P53 has also been co-related during epithelial cell differentiation, in which a decrease in p53 mRNA and protein has been reported (Weinberg et al., 1995). The decrease in p53 mRNA observed during early spontaneous differentiation of ES cells in the absence of any inducing agents, was accompanied by a decrease in p53 protein suggesting a regulatory mechanism that is not influenced by protein stability but a decrease in translatable level of mRNA.

Differentiating embryonal carcinoma cells also showed decreased levels of p53 (Oren et al., 1982).

Latent p53 can be activated by several mechanisms, mainly post-translational modification, phosphorylation at C or N terminals, and covalent/noncovalent modifiers. Active p53 has been shown to either cause cell cycle arrest or apoptosis. This is particularly apparent in DNA damaged cells where p53 arrests further genomic damage by preventing potentially dangerous cells from propagating (Kastan et al. 1991b; Fritsche et al., 1993; Nelson and Kastan, 1994). Throughout the evolutionary systems, apoptosis remains a highly conserved process required for normal development (Raff, 1992). Morphologically, apoptosis entails chromatin condensation, cytoplasmic shrinkage, blebbing, and DNA fragmentation. Initially, the cell membrane integrity is maintained leading to rapid phagocytosis of the dead cells and absence of inflammation. The multifarious relationship between apoptotic cell death and p53 expression is mediated by several factors including the transactivation and posttranslational modifications. It is becoming increasingly clear that apoptosis in ES cells can be mediated by p53-independent pathways (Schmidt-Kastner et al., 1998; Aladjem et al., 1998). However, it has been reported that induction of apoptosis in P19 ES cells by RA during neuronal development was inhibited by Bcl-2 (Okazawa et al., 1996). In this study, increased ES cell death was noted with RA exposure in the culture medium. Specific induction of apoptosis by RA has been reported in P19 embryonal carcinoma cells (Glozak and Rogers, 1996) and F9 embryonal carcinoma cells (Atencia et al., 1994). Increased cell death was observed following 3 days of 1 μM RA addition to the media. Retinoic acid induced apoptosis was noted after 10 and 20 h of exposure. Apoptotic events mediated

by RA occurred earlier than p53 induction. The level of p53 noted was very low during the addition of RA, and no change in p53 mRNA and protein was noted during this period (Fig. 3, inset) indicating that p53 may not play a direct role in apoptosis during development.

Retinol (vitamin A) has been recognized for its role in vision. Retinol, along with naturally occurring carotinoids has no biological activity in itself. However it forms an important substrate for the biosynthesis of (a) 11-*cis*–retinal, linked covalently to opsin to form rhodopsin, and (b) RA, 9-cis-retinoic acid (9-cRA) and 3,4 didehydroretinol (vitamin A_2) that regulates gene expression during homeostasis, differentiation, and apoptosis. RA and 9-cRA bind to the ligand activated transcription factors RA receptors, RAR and RXR (Chambon, 1995). Retinoic acid plays an important physiological role in embryonic development and is teratogenic in large doses in all species (Kochhar, 1975; De Luca et al., 1995; Alles and Sulik, 1989). A temporal and dose dependant effect of RA on embryos has been reported (Armstrong, 1984). Retinoids mediate proliferation, differentiation, and cell death by regulating gene expression (Lotan, 1980; Napoli, 1996). In vitro models of hemopoetic differentiation (Schmitt et al., 1991) cardio-muscular differentiation (Wobus et al., 1997), and glial cell and functional neurons (Bain et al., 1996; Fraichard et al., 1995; van Inzen et al., 1996) have been developed from ES cells. During differentiation, RA modulates expression of several genes. Retinoic acid promotes neuronal and represses mesodermal gene expression in ES cells (Bain et al., 1995). It has been reported that 8 day EBs express early neurogenic genes corresponding to approximately 8.5 day post coitum mouse embryos (Leahy et al., 1999). It has also been observed that genes expressed after RA-mediated differentiation of embryoid

bodies, correlated to in vivo gene expression during embryo development (Gajovic et al., 1998). The expressions of several proto-oncogenes (*erB, src and myc*) are altered during RA-mediated neuronal differentiation in P19 embryonal carcinoma cells (Nakamura and Hart, 1989). Throughout the past decade, intense research on ES cells has established that these pluripotent cells hold the key to understanding the elementary but intriguing question of morphogenesis and differentiation. They provide an invaluable in vitro tool to unravel the complex puzzle of embryonic development.

p53 plays a complex role during development, however the exact function of p53 is not yet known. The importance of p53 in affecting normal development of nervous system has been demonstrated by targeted disruption of *p53* in mice (Armstrong et al., 1995). In recent years emerging area of consensus is that p53 plays a significant part in neural development and its distribution and functions is cell specific and may depend on the stage of development. In conclusion, present results indicate that RA-treatment increased p53 mRNA and protein expression in ES cells during neural differentiation. The induction of p53 coincides with the number of the newly formed neurons rather than apoptotic events during ES development. The expression of p21 indicates that it can function independently of p53 and may have separate role during development. Although p53 induction does not appear to initiate apoptosis during development, it may play a significant role during early neurogenesis. Given the importance of p53 during embryonic development where it functions in cell cycle regulation, differentiation, growth arrest, and apoptosis, its response to RA treatment merits further study.

Acknowledgements

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CHAPTER 4

MODULATION OF C-*MYC*, *MAX* AND *MAD* GENE EXPRESSION DURING NEURAL DIFFERENTIATION OF EMBRYONIC STEM CELLS BY ALL -*TRANS*-RETINOIC ACID[1]

[1]Sarkar, S. A. and R. P. Sharma. 2001. To be submitted to *Cell Growth and Differentiation*

Abstract

c-Myc regulates cellular proliferation, differentiation, and apoptosis. Temporal expression of c-Myc during all-*trans*-retinoic acid (RA)-mediated neural differentiation in murine embryonic stem cells was investigated. Correlation to the modulation of dimerizing partners Max and Mad that may influence c-Myc signaling and transcription regulation was investigated for the first time in these cells. In RA-treated cells, increase in c-*myc* mRNA was detected by reverse-transcriptase polymerase chain reaction on day 11 and 14 of differentiation as compared to the vehicle-treated controls. The results were further corroborated by ribonuclease protection assay. Western blots revealed an increase in c-Myc protein only on day 14 in RA-treated cells. Increases in *Max* and *Mad* gene transcription at times of elevated c-Myc in RA-treated embryonic stem cells suggest that a transient increase in c-Myc protein expression may influence differential dimerization of Myc partners needed for signaling in the neural differentiation of embryonic stem cells.

Key words: Myc family/ Murine embryonic Stem Cells/Differentiation/RA

Introduction

Oncogenic transcription factor, c-Myc, regulates cellular proliferation, differentiation, cellular adhesion, metabolism, and apoptosis (1). Activation of c-Myc by different pathways has been noted in oncogenic transformation of cells. Normally expressed c-Myc is extensively regulated. However, deregulation is the cardinal feature in malignant transformations of cells. The confirmation that c-Myc regulates the cell cycle comes from the data relating to the increase in the doubling time of nullizygous c-*myc* cells (2).

Several studies have suggested that c-Myc plays an important role during embryonic development and growth (3). Homozygous deletion of the c-*myc* gene leads to embryonic lethality at 10.5 days post coitum (4). It has been noted that c-*myc* expression in mice is highest in the proliferating tissues of mesodermal origin. Conversely, ectodermal and endodermal tissues express little or no c-*myc* (5-7). Several studies have reported a decrease in c-*myc* mRNA with the initiation of differentiation and growth arrest (8-10). However, in some cell types a decrease in c-*myc* expression is noted on terminal differentiation (11-13). Over-expression of c-*myc* leads to non-regulation and consequently a reduced requirement for growth factors, accelerated cell division and increased cell size (14-16). Consequently, elevated expression of c-*myc* can impede differentiation in many cell types (17). In addition, exposure of anti-sence olinucleotide to c-*myc* leads to growth arrest and terminal differentiation in erythroleukemic HL-60 and MEL cells (18, 19). Contrary to the above findings, c-*myc* RNA has been reported to undergo a transient increase in differentiating lens cells (20). Lens maturation studies in transgenic mice have also shown that differentiation can proceed in the presence of elevated levels of c-*myc* (21).

The mechanism by which c-Myc influences differentiation has not been fully elucidated. It has been speculated that c-Myc in conjunction with the Max family of proteins, promotes the activation of genes that control proliferation (22, 23) and that the process is inhibited by Mad (24). Max/Max and Max/Mad heterodimers has been thought to repress c-Myc targets and promote differentiation (25, 26).

All-*trans*-retinoic acid (RA) is an important metabolite of retinal (vitamin A) that mediates epidermal as well as bone growth, differentiation, reproductive and immune

functions (27) by its interaction with retinoid alpha receptor (RAR) and retinoid X receptor (RXR) (28). The retinoid-receptor complex influences gene regulation by inducing or repressing gene transcription. Murine embryonic stem cells (ES) were chosen to examine the influence of RA on c-Myc expression during differentiation and apoptosis. Embryonic cells derived from the inner cell mass of 4-day blastocysts can be maintained in an undifferentiated state by growing them on fibroblast feeder layers (29). The ES cells respond to various internal and external signals of proliferation and differentiation thus mimicking the *in vivo* differentiation process. All-*trans*-RA has been used extensively as a differentiating agent in ES neuronal models (30, 31) because of its ability to mediate proliferation, differentiation, and cell death by regulating gene expression (32).

Because retinoids play an important role in proliferation, differentiation, and morphogenesis, the aim of this study was to investigate the modulation of RA-mediated modulation of the Myc family of genes, which appear to be the key players in the cellular decision making-process between differentiation and proliferation during ES differentiation. Carcinogenesis entails de-differentiation and reverting back to embryonic stages. A basic understanding of embryonic development requires an appreciation of the complexity involved in Myc signaling and facilitate understanding the loss of orderly control during oncogenic transformation. Our study demonstrates the temporal pattern of c-*myc*, *max,* and *mad* expression during RA–mediated differentiation in murine embryonic stem cells.

Results

Expression of c-Myc Gene by Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR) and Ribonuclease Protection Assay (RPA). Mouse ES cells can be induced to differentiate into cells exhibiting neural morphology with RA, whereas absence of RA leads to differentiation into mesodermal tissue. To answer the question of temporal pattern of c-Myc expression during ES neural differentiation, c-*myc* gene and protein expression was investigated by RT-PCR, RPA, and Western blot. However, because Max and Mad essentially regulates c-Myc signaling, it was important to elucidate the pattern of gene expression of the dimerizing partners simultaneously, the subsequent experiments were therefore performed by RPA. Undifferentiated ES cells grown in the presence of fibroblast feeder layers and leukemia inhibitory factor (LIF), express basal quantities of c-*myc* gene. Changes in the level of c-*myc* were noted upon induction of spontaneous differentiation in the absence of feeder layers, LIF or RA. Figure 1A shows expression of c-*myc* gene normalized to ß-*actin* (house keeping gene) at undifferentiated= 0, 12, 24, 36, and 48 h of differentiation. A transient increase in the level of c-*myc* mRNA was noted at 12 and 24 h after initiation of differentiation and subsequently a decline was observed by 48 h as detected by RT-PCR (Figure 4. 1). The results obtained by RT-PCR were corroborated by RPA. Total RNA was extracted from the ES cells, hybridized to template RNA, and treated with RNase A, as explained in the materials and methods. The assay revealed an increased expression of c-*myc* RNA after 24 h of initiation of differentiation, which was also found to be statistically significant ($P \leq 0.05$) as compared with the undifferentiated controls harvested at 0 h. Similar to the observations of RT-PCR reactions, after 48 h of initiation of differentiation, c-*myc*

mRNA levels returned to basal values of their undifferentiated phenotype as noted by RPA (Figure 4. 2A and B).

When ES cells were allowed to differentiate in the presence of vehicle only, they differentiated into fibroblasts, endodermal, and mesodermal cell types. No neural differentiation was seen in the absence of RA. During spontaneous differentiation, both RPA and RT-PCR showed a transient increase in c-*myc*, similar to the 24 h peak on day 11 and 14, respectively, followed by a decline noted from days 14 to 21, when cells reached terminal differentiation (Figure 4. 3, 4. 4A, and B).

Modulation of c-*myc* gene expression during RA-mediated differentiation was also examined by RT-PCR and RPA. The ES cells were exposed to 1 μM RA on day 8, 9, and 10 of differentiation. Differentiation in the presence of RA leads to neural cell types. Interestingly, a distinct response was observed upon RA-exposure by RT-PCR. The ratio of c-*myc* RNA, normalized to *β–actin,* was elevated in RA-treated cells relative to the ethanol-treated controls on all the days. However, the increased gene expression on day 11 was significant ($P \leq 0.05$) (Figure 4. 3).

Following RA-exposure, an increase in c-*myc* mRNA expression was also detected by RPA, in RA-treated cells as compared with the concurrent control for the respective days (11, 14, 17, and 21) (Figure 4. 4A and B). The difference in gene expression between RA-and vehicle-treated cells was found to be statistically significant on days 11 and 14. Maximal amount of RNA expression was seen on day 14 in RA-treated cells, and a subsequent decline was seen from day 17 to 21. Additionally, even during the declining phase of c-*myc* expression, about two-to three-fold higher level of c-*myc* RNA was detected in RA-treated cells from concurrent controls on days 17 and 21.

Expression of c-Myc Protein. Upon initial differentiation, c-Myc protein expression paralleled the gene expression and an increased amount of translated product was detected 36 h after differentiation, relative to the undifferentiated controls (Figure 4. 5A). Additionally, differentiation in the presence of RA, on days 8, 9 and 10, which predominantly promotes the formation of cells resembling neural morphology, revealed a significant increase in the level of c-Myc protein on day 14 of differentiation, compared with the vehicle-treated controls. Thereafter, a gradual decline was noted in the expressed protein. No difference in the level of protein expression was noted in the presence or absence of RA on subsequent days (Figure 4. 5B).

Expression of Related Myc Family of Genes Encoding for Dimerizing Partners Max and Mad by RPA. After having established that RA-treatment during ES differentiation could modulate c-*myc* gene and protein levels, concurrent expression of the dimerizing partners of c-Myc was investigated to understand the implications of up-regulation of c-Myc. No difference in *max* mRNA was detected by RPA upon initial differentiation at 24 and 48 h (Figure 4. 2). In vehicle-treated ES cells, not in RA-treated cells, *max* RNA expression declined two-fold on day 14 from day 11 mRNA levels and subsequently remained steady throughout the period of differentiation. Increased expression of *max* mRNA was noted on all days in RA-treated ES cells compared with the concurrent vehicle-treated cells, and the increase was statistically significant ($P \leq 0.05$) on days 11, 14, and 21 of differentiation (Figure 4. 4A, 4. 6). About a three-fold induction of *max* was noted on day 14 as compared with concurrent vehicle-treated controls.

Contrary to *max* expression a significant decline in the level of *mad* mRNA was noted at 24 h compared with the undifferentiated cells (Figure 4. 2A, B). However, increased

mad mRNA was detected at 11 and 14 days upon RA-treatment, as compared with the concurrent vehicle-treated cells. In addition, the level of *mad* mRNA expression was found to be significantly elevated from concurrent vehicle-treated cells on day 14 of differentiation (Figure 4. 4 A and 4. 7). Furthermore, non-quantifiable to very low levels of *mad* transcripts were detected on subsequent days of differentiation in both RA-and vehicle-treated cells.

Materials and Methods

ES Cell Culture and RA Treatment. Murine embryonic stem cells (ES-D3, ATCC # 1934-CRL) 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 (29). The media was supplemented with 15% Knock-Out^{TM} Serum Replacement (Gibco, Life Technologies, Grand Island, NY), 10 µM ß-mercaptoethanol and 1000 IU/ml leukemia inhibitory factor (Sigma, St. Louis, MO). The medium was changed everyday. The ES cells were passaged every 2 days to maintain an undifferentiated state. To induce differentiation, 2 x 10^5 ES cells were plated in a monolayer in the absence of feeder layers and LIF on 6 well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ), which was 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 suspension form (29). All-*trans*-retinoic acid (Sigma) dissolved in 85% ethanol at a concentration of 10^{-6} M was added to the media on days 8, 9, and 10 of differentiation. Our preliminary experiments have indicated the optimal dose and duration of treatment with RA for neural differentiation of these cells. The control

medium was treated with equal volume of 85% ethanol that was added to the experimental medium. The total volume of ethanol in the culture media did not exceed 0.15% and did not influence the differentiation process.

C-Myc mRNA Expression by RT/PCR. ES cells were grown in the absence of fibroblasts and LIF in 6 well plates at a density of 2 x 10^5. The cells were harvested at 0 (undifferentiated), 12, 24, 36, and 48 h after differentiation to look at early events during differentiation in the absence of RA. To look at cellular response in the presence of RA, ES cells seeded at similar density were treated on day 8, 9 and 10 of differentiation with 10^{-6} M RA and harvested on days 11, 14, 17, and 21 of differentiation to investigate modulation of c-Myc genes in response to RA. Control cells were treated with equal volume of 85% ethanol. One ml of TRI Reagent® LS (Molecular Research, Cincinnati, Ohio) was added to the cell suspension, and total RNA was extracted according to the manufacturer's protocol. Three μg of total RNA was used to synthesize first strand c-DNA using Superscript II reverse transcriptase (Life Technologies, Grand Island, NY). Samples equivalent to 1 μl of the first strand reaction cDNA was then used as a template for amplification in 50 μl PCR reaction. Two μl of dimethyl sulphoxide (DMSO) (Fisher, Fair Lawn, NJ) was added to c-Myc reaction to enhance PCR specificity. The sense and antisense primers for β-actin and c-Myc was chosen by Primer3 program (Whitehead Institute, Cambridge, MA). These were 5'-ATGGATGACGATATCGCT-3' and 5'-ATGAGGTAGTCTGTCAGGT-3' 5'-ATCTGCGACGAGGAAGAGAA-3' and 5'-ATCGCAGATGAAGCTCTGGT 3' for β-*actin* and c-*myc,* respectively. The DNA was initially denatured for 5 min at 95°C. The following parameters were used for amplification: 95°C for 30 sec, annealing temperature of 48°C (β-*actin*) and 55°C (c-

myc) for 30 sec, 72°C for 1 min and run for 25 cycles and 32 cycles for β-*actin* and c-*myc,* respectively. Initial trials were run to ensure that the genes are amplified in the exponential phase (inset: Fig. 1). β-*actin* was used as a positive control and a negative control with respective primers, and no cDNA in the PCR reaction was used. After amplification, 10 µl of PCR products were mixed with 10X DNA dye [5 mg/ml bromophenol blue, 50% glycerol, 100 mM Tris, 20 mM NaCl, 1 mM ethylenediaminetetraaceticacid, (EDTA)] in 10:1 ratio, and run on 2.0 % agarose gel stained with ethidium bromide for electrophoretic separation. The gels were photographed in a backlighted UV transilluminator. Photographs were scanned and quantified on UN-SCAN-IT™ automated digitizing system, (Silk Scientific, Inc. Orem Utah). Total pixel count for c-*myc* was normalized to β-*actin*. All experiments were repeated several times with the number of independent replicates (n=3) for each investigated time point.

Determination of c-Myc Protein by Western Blot. The control and RA treated ES cells were lysed on ice for 30 min in lysis buffer. Cell lysates were centrifuged at 14,000 rpm at 4°C for 15 min to remove cell debris. The aliquots of supernatants were stored at –80°C and assayed using Bradford reagent (Sigma) for detection of protein. Fifteen µg of the supernatant protein from each sample was heated with 4X sodium dodecyl sulphate (SDS) sample buffer at 95°C for 5 min and separated on 10% SDS–polyacyramide gel electrophoretically. Subsequently, the proteins were transferred onto 0.45 µm pore size nitrocellulose membranes for 90 min and blocked overnight with 5% milk in Tween-Tris buffer saline (TBS). Nitrocellulose membranes were then exposed to c-Myc primary monoclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). The membranes

were incubated for 1 h with horseradish peroxide-conjugated polyclonal goat anti-mouse IgG at 1:5000 dilutions. Between each exposure, nitrocellulose membranes were washed for 30 min with Tween–TBS. c-Myc was visualized autoradiographically by enhanced chemiluminescent substrate (ECL® Amersham Pharmacia, Piscataway, NJ) after 60 min of exposure. The radiographs were scanned and digitized using UN-SCAN-IT™ automated digitizing system as mentioned for PCR gels above. All experiments were repeated several times with the number of replicates, (n=3) for each investigated time point.

Interaction of Max, Mad and c-Myc During RA-Mediated Cellular Differentiation by RNase Protection Assay. RNase Protection Assay (RPA) for c-Myc-Max/Mad family was undertaken to simultaneously analyze their gene expression during ES differentiation. RNase protection assay is a sensitive and quantitative method of measuring the expression level of several genes simultaneously. The assay was performed with Pharmingen's riboquant, multi-probe RNase assay custom kit (Pharmingen, San Diego, CA). ES cells were grown at a density of 2 X 10^5 cells in 6 well plates. The cells were harvested at undifferentiated = 0, 24, and 48 h after differentiation in the absence of RA. To investigate RA-mediated differentiation, cells plated in similarly densities were treated with RA as mention before and harvested at days 11, 14, 17, and 21 of differentiation. One ml of TRI Reagent® LS (Molecular Research, Cincinnati, OH) was added to the cell suspension and total RNA was extracted according to the manufacturer's protocol. Total RNA from three wells was pooled together and quantified in the spectrophotometer at A260 and 40 μg aliquoted and stored at –80°C. [α-^{32}P]-labeled anti-sense RNA probe was synthesized according to

manufacturer's protocol from custom DNA templates and quantified in a liquid scintillating counter (1214 Rackbeta, Pharmacia, Finland). The probe was diluted with hybridizing buffer at strengths of 4 X 10^5/μL counts per minute and hybridized overnight to the target RNA previously extracted from RA-and vehicle-treated ES cells. Appropriate positive and yeast t-RNA negative controls, provided by the manufacturer were simultaneously hybridized. Subsequently the free probe and single-stranded RNA were treated with RNAse A to destroy single-stranded RNA. The cRNA/mRNA complexes were purified and electrophoresed on denaturing polyacrylamide gels. The gels were transferred onto filter papers and dried at 80°C for 1 h and quantified by autoradiography after 16 h of exposure. During the duration of exposure, the gels were stores at –80°C.

Statistical Analysis and Replications. All cell culture experiments were repeated several times using at least three independent replicates. Pooled RNA from three wells was used for RPA. The RPA was repeated twice for consistency. Mean ± standard error (s.e.m) of a representative experiment are presented in the results. The difference between control and treated samples was analyzed using Student's-t test assuming equal variances. The error bars represent the s.e.m. Probability (*P* value) of ≤ 0.05 was considered significant.

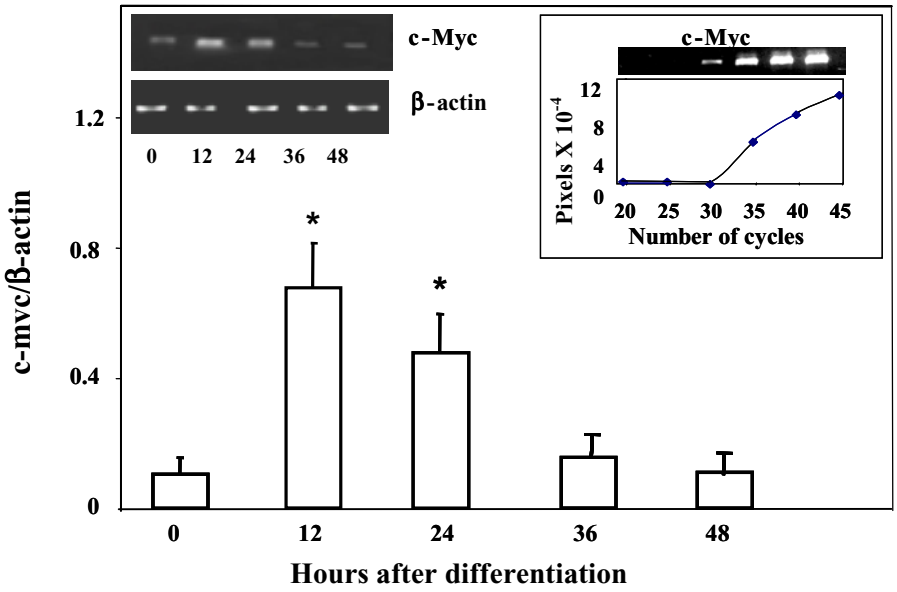

Figure 4. 1**.** Increased c-*myc* mRNA expression in spontaneously dividing ES cells at 12 and 24 h as detected by RT-PCR. The mRNA expression was normalized to house keeping gene ß-actin at, 0= undifferentiated, 12, 24, 36, and 48 h after initiation of differentiation. The primers were amplified at 33 cycles in the exponential phase of PCR reaction and the gel representative of exponential amplification of c-*myc* is shown (inset). The results are expressed as mean ± standard error of mean (n=3). The photograph of representative gel shows ß-actin and c-*myc* mRNA at respective times. * Indicates significantly different from undifferentiated group at $P \leq 0.05$.

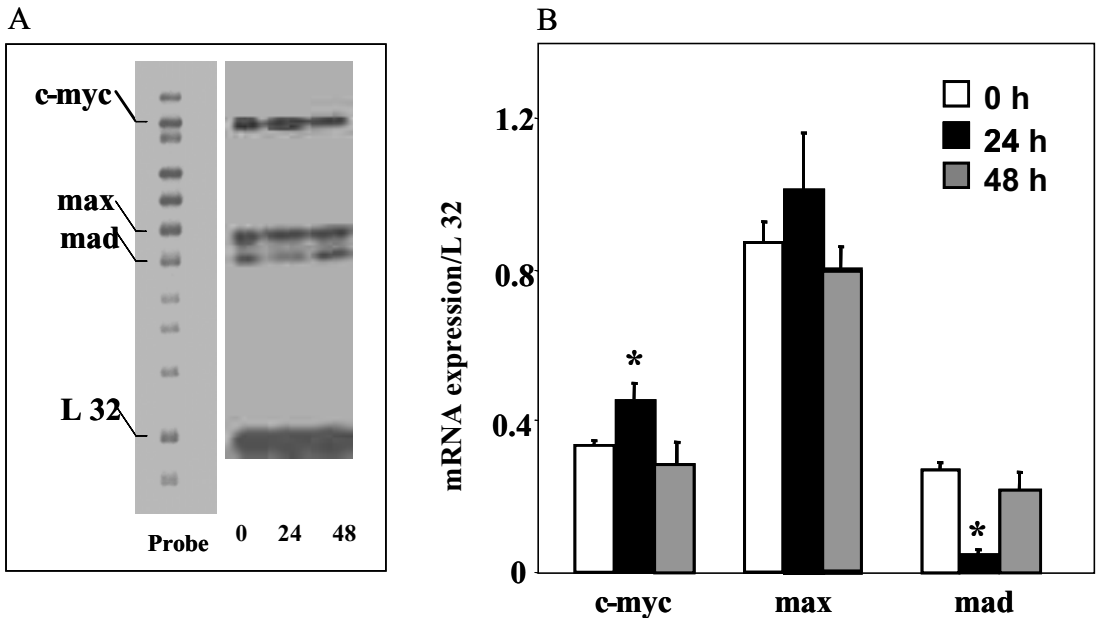

Figure 4. 2**.** Differential expression of c-*myc*, *max,* and *mad* mRNA by RNase protection assay (RPA). Panel A: Representative gel of RPA showing only c-*myc*, *max* and *mad* (indicated on the left) mRNA expression at 0=undifferentiated, 24 and 48 h. L32 represents the housekeeping gene. A non-hybridized probe set was run as a size marker. Panel B: The relative mRNA expression normalized to house keeping gene L32 at 0=undifferentiated, 24 and 48 hrs after differentiation. The results are expressed as mean ± standard error of mean (n=2). * Indicates significantly different from undifferentiated group at $P \leq 0.05$.

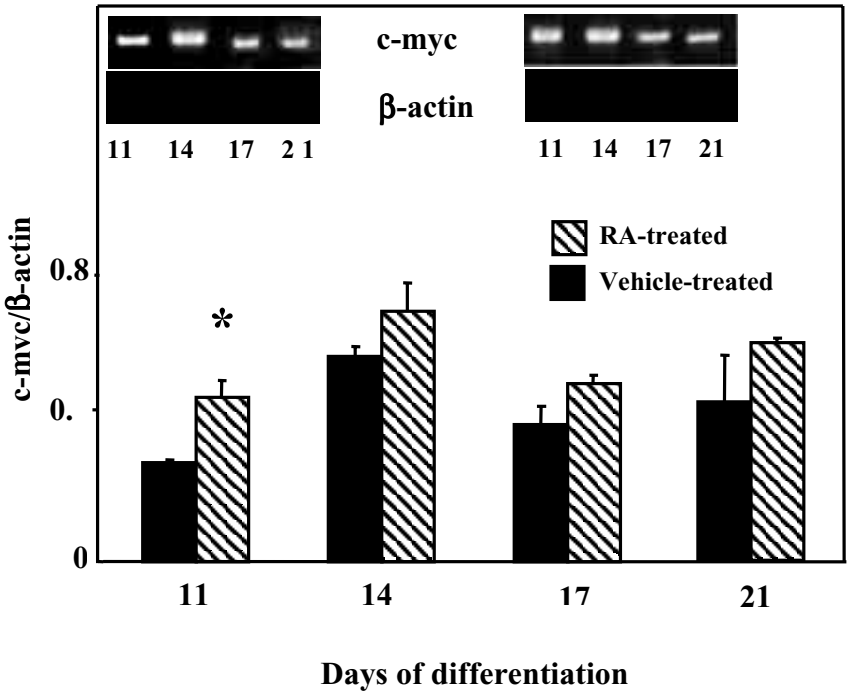

Figure 4. 3. Modulation of c-*myc* mRNA expression by RT-PCR during RA-mediated ES neural differentiation. The cells were treated with RA on day 8, 9, and 10 of differentiation. The relative c-Myc gene expression was normalized to ß-*actin*. The results are expressed as mean ±standard error of mean (n=3). The photograph of representative gel shows ß-*actin* and c-*myc* mRNA on days 11, 14, 17, and 21 of differentiation. * Indicates significantly different from concurrent vehicle-control $P \leq 0.05$.

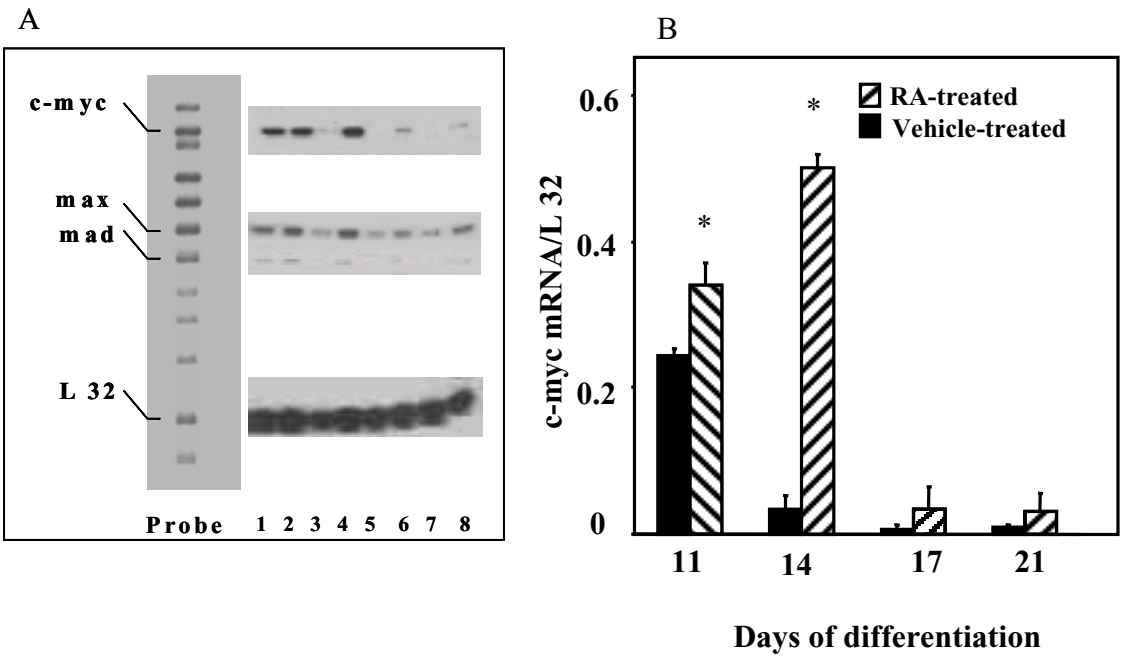

Figure 4. 4. Alteration in Myc family expression during RA-mediated ES differentiation. Panel A: Representative gel of RNase protection assay. A non-hybridized probe was run as a size marker. The names of the genes are listed on the left. L32 represents the housekeeping gene. Treatment groups are indicated as control: (1), 11 day vehicle-treated, (3), 14 day vehicle-treated, (5), 17 day vehicle-treated, and (7), 21 day vehicle-treated. RA-exposed: (2), 11 day RA-treated, (4), 14 day RA-treated, (6), 17 day RA-treated, and (8), 21 day RA-treated. Panel B: The relative mRNA expression is indicated against L 32. Pooled RNA from three independent wells was used for the assay, and the results are expressed as mean ± standard error of mean (n=2). * Indicates significantly different from concurrent vehicle-control $P \leq 0.05$.

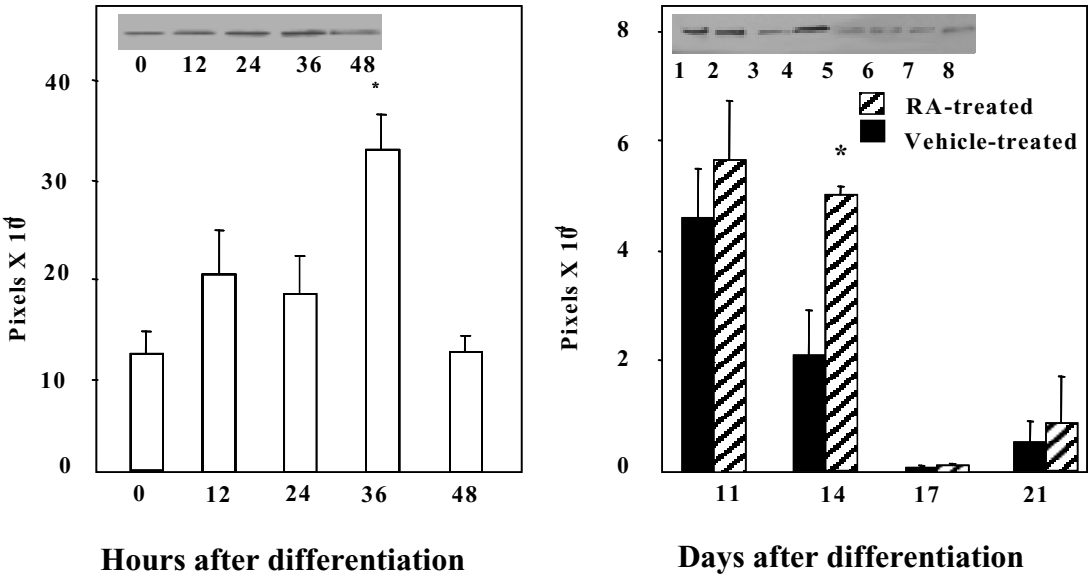

Figure 4. 5. Alteration in c-Myc protein expression during differentiation by Western blot. Panel A: Fifteen μg of cytosolic cell lysate was electrophoretically separated and transferred onto nitrocellulose membranes. After incubation with primary and secondary antibody, the proteins were visualized autoradiographically with chemiluminiscent reagents and quantified. Results expressed are mean ± standard error of mean (n=3). Representative gel shows c-Myc expression at 0=undifferentiated, 12, 24, 36, and 48 h after initiation of differentiation. * Indicates significantly different from undifferentiated group at $P < 0.05$. Panel B: Alteration in c-Myc protein expression during RA-mediated ES differentiation on 11, 14, 17 and 21 days of differentiation. The results are expressed as mean ± standard error of mean (n=3). * Indicates significantly different from concurrent control $P \leq 0.05$. Representative photograph of gel is shown. Treatment groups are indicated as control: (1), 11 day vehicle-treated, (3), 14 day vehicle-treated, (5), 17 day vehicle-treated, and (7), 21 day vehicle-treated. RA-treated: (2), 11 day RA-treated, (4), 14 day RA-treated, (6), 17 day RA-treated, and (8), 21 day RA-treated.

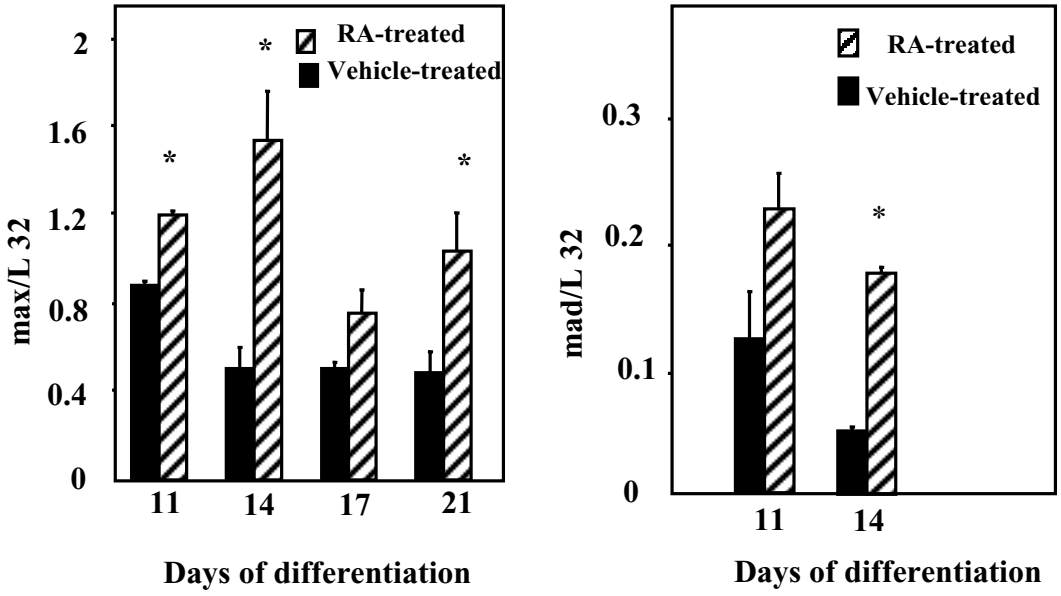

Figure 4. 6. Alterations in *max and mad* expression during RA-mediated ES differentiation by RPA. The relative mRNA expression is indicated against L32. Pooled RNA from three independent wells was used for the assay, and the results of a representative experiment are expressed as mean ± standard error (n=2). * Indicates significantly different from concurrent control $P \leq 0.05$.

Discussion

The results of this study show that RA can effectively modulate Myc family gene expression during ES neural differentiation. Undifferentiated ES cells express Myc and its dimerizing partners, Max and Mad. Upon spontaneous differentiation in the absence of feeder layer and LIF, an increase in c-*myc* mRNA and a decline in *mad* mRNA was observed at 24 h. No change in the level of *max* was noted. Exposure to RA at days 8, 9, and 10 during ES neural differentiation causes distinct changes in the Myc family of genes. The most prominent effect in response to RA-exposure was an up-regulation of c-*myc* gene and protein expression on day 14 of differentiation and a gradual decrease in their levels. That RA-exposure can effectively modulate Myc family at specific stages of ES neural differentiation is suggested by increased presence of c-*myc*, *max,* and *mad* mRNA at specific times during ES neural differentiation.

A biphasic response was observed during differentiation of ES cells. Upon initiation of differentiation even in the absence of differentiating-inducing agents, c-*myc* mRNA showed a transient increase in 12 to 24 h as detected by RT-PCR. This finding was also corroborated by RPA where an increase in c-*myc* mRNA levels was observed at 24 h and returned to basal levels of undifferentiated state at 48 h. This initial peak of increased expression of c-*myc* has been correlated to commitment to differentiate rather than RA-exposure in P19 cells (33, 34). Modulation of c-*myc* expression was also noted during RA-mediated differentiation, where the ES cells were exposed to 1 μM of RA on days 8, 9, and 10 of differentiation. A distinct temporal response to RA-exposure was seen on days 11 and 14 as compared with the ethanol-treated controls in the form of increased c-*myc* mRNA expression as detected by RT-PCR and RPA. A similar biphasic

response to RA-treatment has been previously elicited in mouse embryonal carcinoma cells that were induced to differentiate into neurons, astrocytes by RA (35). Increased c-*myc* mRNA has also been noted during mouse brain development (5). Increased biphasic expression of c-*myc* mRNA has been also reported in erythroleukemic cells with DMSO and hexamethylene bisacetamide (36, 37). Although, the overwhelming body of evidence suggests that over expression of c-Myc causes cellular proliferation and repression of differentiation, contrary to the common findings of decreased c-Myc expression during differentiation, several studies have reported high levels of c-Myc during differentiation in a variety of cell lines (38, 39, 40).

There was a gradual decline in the overall message from days 17 to 21 when the ES cells were morphologically differentiated in control and RA-treated cells. However, the level of mRNA was elevated in RA-treated cells only, suggesting increased c-*myc* mRNA in neural cells. There is mounting evidence that c-Myc may not be required for terminal differentiation and that its expression is insufficient by itself to suppress the differentiated phenotype (41). Increased c-*myc* noted in RA-treated cells may be required for maintenance and metabolism of newly formed neural cells.

An increased level of c-*myc* transcription was paralleled by increased protein observed at 36 h after initiation of differentiation, in the absence of feeder layers, LIF or RA. However, upon RA exposure, increased levels of c-Myc protein was observed only on day 14 of differentiation and not on all the times of elevated c-*myc* mRNA. Increased c-*myc* gene expression is not followed by increased detection of protein by Western blot at similar times. This observation may be caused by increased c-Myc degradation as c-Myc is a short-lived nuclear protein. However, increased availability of c-Myc could be

indicative of increased stability or translation control (42). On day 14 in RA-treated cells, increased availability of c-Myc protein may provide the necessary switching signal during ES cell neural differentiation in conjunction with other associated proteins.

Investigation of c-*myc* gene and protein expression alone can only partially be correlated to ES proliferation and differentiation because Myc family is a group of transcription factors that act together to influence apoptosis, differentiation and growth arrest. Contrasting its expression with other interacting partners can better elucidate the implications of c-Myc up-regulation during RA-mediated neural differentiation. The c-Myc signaling effectively requires dimerizing partners Max and Mad as c-Myc by itself is unable to bind to DNA. In contrast to c-Myc, Max is more stable and exhibits a longer half-life (43). The role of Max in differentiation is controversial. It is generally agreed that Max expression does not change during cell cycle or differentiation (44). However down regulation of *max* mRNA and protein in erythroid differentiation (45, 46) has been noted. Over expression of Max may lead to reduced growth and delayed differentiation (47). Interestingly, Max over expression in neuroblastoma cells lines can enhance RA-mediated growth arrest and differentiation (48). No change in *max* mRNA was noted between undifferentiated ES cells and initial differentiation at 24 and 48 hrs. However, increased expression of *max* mRNA levels was noted by RPA on RA-exposure on days 11, 14, 17, and 21. About a three-fold increase in Max transcripts was seen on day 14 of differentiation upon RA-exposure, coinciding with increased c-Myc mRNA expression detected by RPA. Increased Max may be required in conjunction with increased c-Myc for transcriptional activation in differentiating neural cells. C-Myc regulates the cell cycle primarily in G_1 phase or G_1/S transition and has been known to repress

differentiation and cell adhesion while actively promoting cellular proliferation (49). C-Myc binds to the DNA in association with its dimerizing partner Max (50). Max-Myc heterodimers can effectively bind to specific DNA sequences to promote transcription, whereas c-Myc alone is unable to bind to DNA. Max lacks a transactivation domain and Max/Max homodimers can effectively block c-Myc function (51, 52). Furthermore, because of a longer half-life, Max interaction with the Mad family of proteins may logically influence c-Myc function.

We also noted an initial decrease in *mad* transcription after 24 h of differentiation. At this time we do not know the significance of transient decrease in *mad* mRNA during early part of spontaneous differentiation. However, increased c-*myc* gene expression in presence of Max is indicative of a Myc-Max heterodimer driven trans-activation process. Increased *mad* mRNA was observed during RA-mediated differentiation only on days 11, 14. The relative amount of *mad* mRNA was very low compared to c-*myc* and *max*. Coinciding with increased c-*myc* mRNA and protein, about a 3.5-fold increase in *mad* mRNA expression was observed by RPA during ES differentiation in the presence of RA on day 14 as compare with ethanol-treated controls. The findings suggest that RA-exposure during ES differentiation up-regulates Myc associated partners for optimal signaling. Evidence has been presented that accumulation of Mad/Max complexes during development of mouse embryo (53), myeloid leukemic cells (54) and keratinocytes (55) may facilitate differentiation. Accordingly, transcriptional properties of c-Myc may be influenced by binding with the Mad family of proteins, which in turn recruit sin3 and other co-repressors that compete for c-Myc binding sites and preclude c-Myc/Max-mediated gene regulation (26, 56). Consequently, increased expression of Mad proteins

has been associated with cellular differentiation and growth arrest. It can be envisioned that Mad and c-Myc inversely regulate differentiation and up-regulation of Mad in RA-treated cells may provide the signal for ES differentiation. It is likely that Mad acts as a switch between proliferation and differentiation, and retinoids may act as a master controller of the cellular fate in the decision-making process. The virtual absence of Mad at later stages of differentiation and is indicative of increased probability of Myc-Max or Max-Max dominated response.

The correlation of c-Myc expression to cellular proliferation and differentiation is fraught with variations, depending upon cell type and differentiating agent used and is incomplete without investigating the modulation of selected Myc associated partners. Our results show that RA can effectively modulate the expression of c-*myc* gene and protein during ES neural differentiation. We have also shown the ability of RA to effectively influence the c-Myc signaling process by differentially regulating the expression of genes encoding for c-Myc dimerizing partners, namely Max and Mad, suggesting that Myc family plays an important role during RA-mediated ES neural differentiation.

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CHAPTER 5

EXPRESSION OF SELECTED APOPTOSIS-RELATED GENES, MIF, IGIF, AND TNFα DURING RETINOIC ACID-INDUCED NEURAL DIFFERENTIATION IN MURINE EMBRYONIC STEM CELLS[1]

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Abbreviations:

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

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 (RA). The mRNA expression of caspase 3, caspase 6 and certain pro-inflammatory cytokines was also investigated simultaneously. ES cells exposed to 1 μM RA 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 RA treatment. Increased mRNA expression of caspase 3 and caspase 6 in RA-exposed ES cells suggested that caspases play an important role in RA-mediated apoptosis during ES differentiation. Increase in the expression of TNFα and macrophage migration inhibitory factor (MIF) was noted in RA-treated cells on day 14. Significant increase observed in interferon γ inducing factor (IGIF/IL-18) mRNA expression in RA-treated cells on day 14 and 17 did not translate to increased INFγ expression. No change in the expression of other pro-inflammatory cytokines was noted with RA-treatment. The function of TNFα, IGIF/IL-18 and MIF in RA-treated cells during ES differentiation and apoptosis is still

speculatory. 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/ RA/ differentiation/MIF/ IL-18

All-*trans*-retinoic acid (RA), 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 (Kochhar, 1975; Lammer *et al*., 1985; Alles and Sulik, 1989; De Luca *et al*., 1991). A temporal and dose-dependant effect of RA on embryos has been reported (Armstrong *et al*., 1994). Teratogenic response to excess dose of RA may be due to its ability to cause apoptosis (Dupe et al., 1988; 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, Glozac and Rogers, 2001). 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., 1998; Guzey 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 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 in 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 RA-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 (Evans and Kaufman, 1981; Doetchman 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. 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 were compared simultaneously by 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 RA-mediated apoptosis in ES cells during neural differentiation.

Material and Methods

ES cell culture and RA treatment

Murine embryonic stem cells were procured from American Type Culture Collection (ATCC, Manassas, VA) (ES-D3, ATCC # 1934-CRL) 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), 10-µM ß-mercaptoethanol and 1000 IU/ml leukemia inhibitory factor (LIF) (Sigma, St. Louis, MO). The medium was changed everyday. The ES cells were passaged every 2 days to maintain in an undifferentiated state. To induce differentiation, 2 x 10^5 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), 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^{-6} M in yellow light (to prevent photo isomerization) and was added to the media on days 8, 9 and 10 of differentiation. Our preliminary experiments have indicated the optimal dose and duration of treatment with RA 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 X 10^5 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 RA. To investigate RA-mediated differentiation, cells plated in similarly densities were concurrently treated with RA 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-^{32}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 X 10^5 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 RA-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 verses log neucleotide 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 ± standard error (s. e. m) of a representative experiment (n=2) are presented in the results. The difference between vehicle-and RA-treated samples was analyzed using Student's-t test assuming equal variances. Probability (p value) of ≤ 0.05 was considered significant.

Results

Results showed differential expression of apoptosis-related genes during RA-induced ES differentiation. Representative gels from the two RPA templates are shown (Figure 5. 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 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 (Figure 5. 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 (Figure 5. 2). Low levels of Bcl-2 were be detected at 0, 24 and 48 h. In RA-treated cells, significant increase in caspase-3 mRNA was detected at 11, 14 and 21 days as compared to the

vehicle-treated cultures (Figure 5. 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 RA-exposed cells. However, increased caspase 3 mRNA continued to be detected in RA-treated cells as compared to vehicle-treated cells even during this declining phase. Significant increase in caspase-6 was observed in RA–treated cells on days 11, 14, 17 and 21 compared to the vehicle-treated cells (Figure 5. 3b). A gradual decline in the expression of caspase 6 was seen in both RA-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 RA-treated cells compared to vehicle-treated cells as differentiation proceeded to the terminal phase.

Relative changes in Bcl-2 family were also detected by RPA following RA-treatment. When the ES cells were exposed to 1 μM RA on day 8, 9 and 10, Bax mRNA expression significantly increased in RA-treated cells on day 14 compared to the vehicle-treated cells (Figure 5. 4a). Increase in Bad mRNA levels was also noted in RA-exposed ES cells on days 11, 14, 17 and 21, compared to the vehicle-treated cultures (Figure 5. 4b). The expression of Bax and Bad declined from days 17 to 21, in both RA- and vehicle-treated cultures, although the level of mRNA expression of both proteins were relatively higher in the RA-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 RA-and vehicle-treated cells (data not shown). Ribonuclease protection analysis of TNFα from apoptosis signaling molecule template revealed relative absence of TNFα gene expression at 0, 24 and 48 h. After the ES cells were exposed to RA and/or treated with equal volume of

85% ethanol, TNFα gene expression was observed in both RA-treated and vehicle-treated cells on day 11 of differentiation (Figure 5. 5a). Expression of very low quantities of TNFα (normalized to L32, housekeeping gene) was noted only in RA-treated cells on day 14. Cytokine expression in ES cell with mCK-2b template was investigated after RA-exposure and compared to vehicle-treated ES cells. Very low to undetectable levels of (IL-1α, IL-1β, IL-1Ra, IL-6, IL-10, IL-12p35, IL-12-p40, and INFγ) were seen in both RA-and vehicle-treated cells (representative gel shown, Figure 5. 1b). Significant increase in the expression of Interferon gamma inducing factor (IGIF/IL-18) was seen on days 14 and 17 of differentiation in RA-treated cells compared to the vehicle-treated cells (Figure 5. 5b). Changes in mRNA expression of INFγ were not detected concurrent to increased IGIF/IL-18 in RA-treated cells. Significant increase in the expression of macrophage migration inhibitory factor (MIF) normalized to L32 was noted on day 14 in RA-treated cells as compared to the vehicle-treated cells (Figure 5. 6). No difference in MIF gene expression between RA-treated and vehicle-treated ES cells was detected on subsequent days of differentiation.

Discussion

The results of this study show that RA can effectively modulate caspase 3, caspase 6 and Bcl-2 related family gene expression during apoptosis occurring in ES neural differentiation. Although, increased TNFα mRNA, MIF and IGIF expression was detected in RA-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 it is the

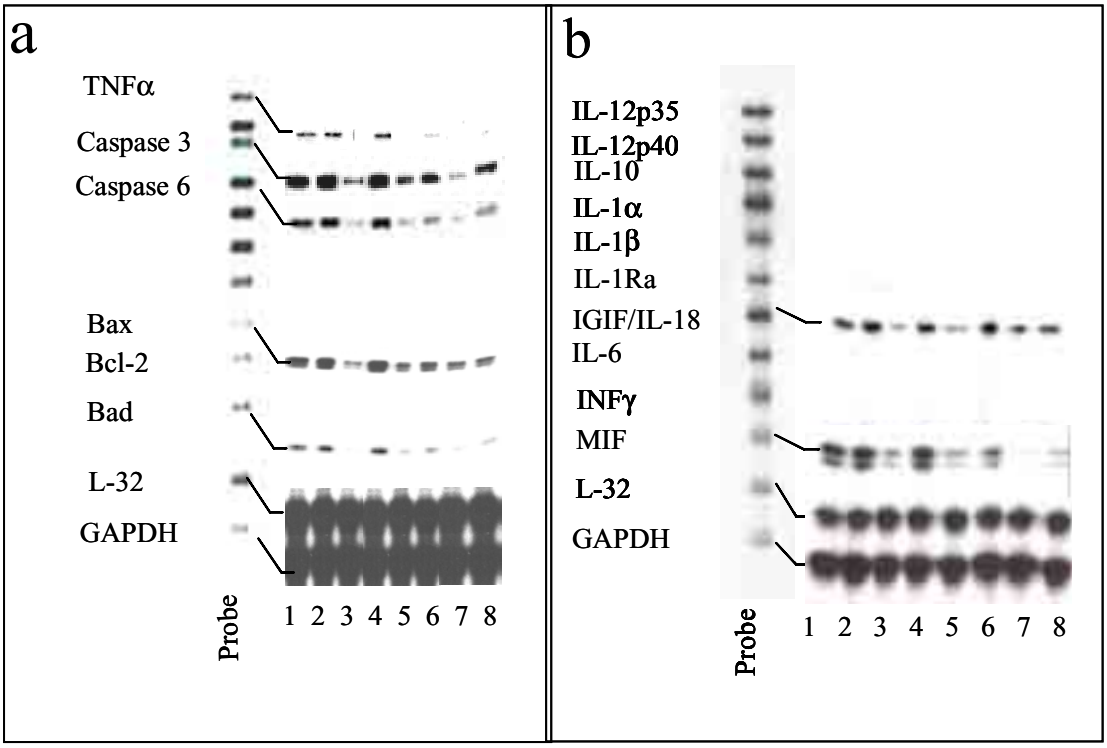

Figure 5. 1. Alteration in TNFα, caspases and Bcl-2 family expression during RA-mediated ES differentiation using Pharmingen Riboquant® templates. Panel (**a**): Representative gel of RNase protection assay using apoptosis signaling molecule probe. A non-hybridized probe was run as a size marker. The 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 γ inducing factor (IGIF/IL 18) and macrophage migration inhibitory factor (MIF) are indicated against L32 and GAPDH. Lane indications are same as panel (**a**).

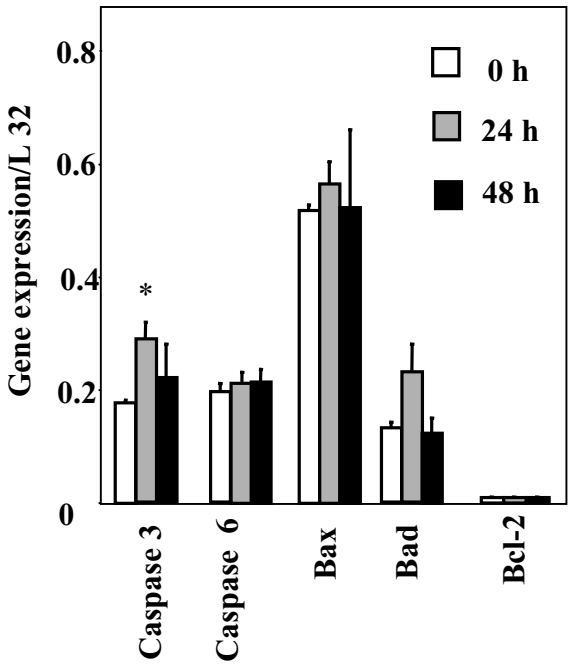

Figure 5. 2. Differential expression of caspase 3, caspase-6, Bax, Bad and Bcl-2 by RNase protection assay (RPA) during early spontaneous differentiation. The relative mRNA expression was normalized to house keeping gene L32 at 0=undifferentiated, 24 and 48 h after differentiation. Pooled RNA from three wells was used for the assay, and the results are expressed as mean ± standard error (n=2). * Indicates significantly different from concurrent vehicle-treated cultures at $P \leq 0.05$.

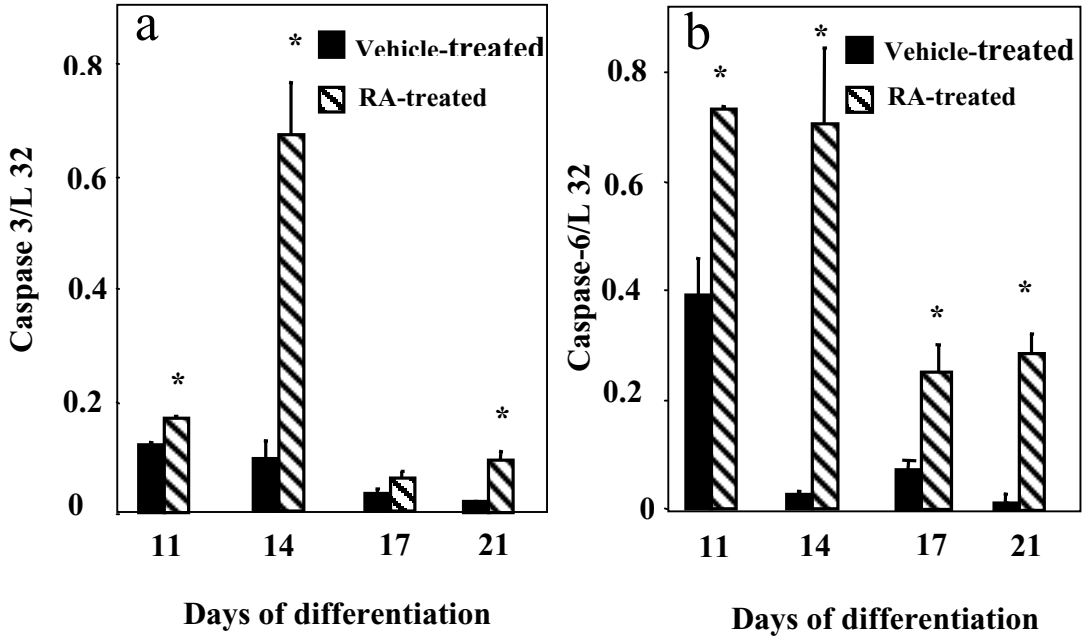

Figure 5. 3. Alterations in caspase mRNA expression during RA-mediated ES differentiation using apoptosis signaling molecule template. The cells were treated on day 8, 9 and 10 of differentiation with 1 μM all-*trans*-retinoic acid. (**a**) The relative mRNA expression of caspase 3 is indicated against 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 ± standard error (n=2). * Indicates significantly different from concurrent vehicle-treated cultures at $P \leq 0.05$.

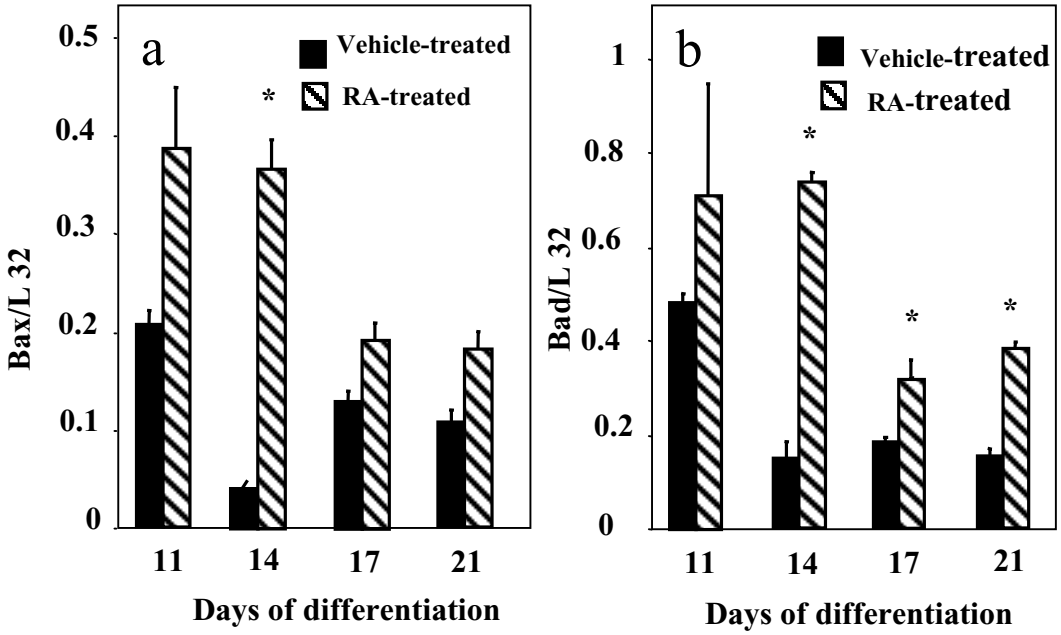

Figure 5. 4. Alterations in pro-apoptotic members of Bcl 2 family; Bax and Bad mRNA expression during RA-mediated ES differentiation using apoptosis signaling molecule template. (**a**) The relative mRNA expression of Bax is indicated against L 32 gene encoding for ribonuclear protein. (**b**) The relative mRNA expression of Bad is indicated against L 32, gene encoding for ribonuclear protein. The results are expressed as mean ± standard error (n=2). * Indicates significantly different from concurrent vehicle-treated cultures at $P \leq 0.05$.

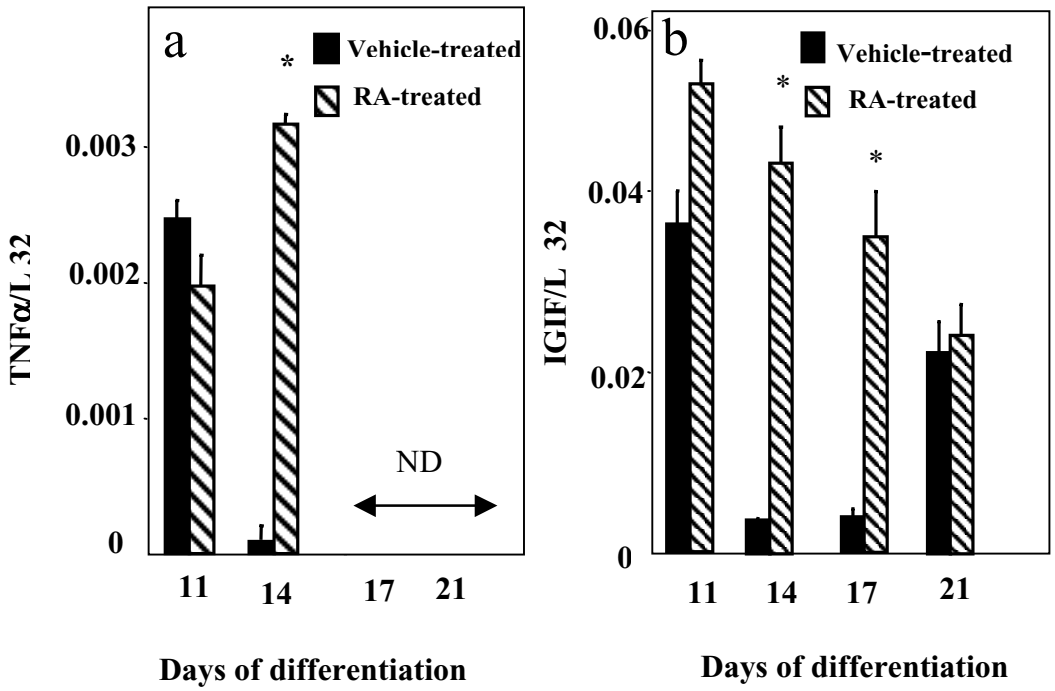

Figure 5. 5. Alterations in selected proinflammatory cytokine expression during RA-mediated ES differentiation using apoptosis signaling molecule template. (**a**) The relative mRNA expression of TNFα is indicated against L32, gene encoding for ribonuclear protein. (**b**) The relative mRNA expression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

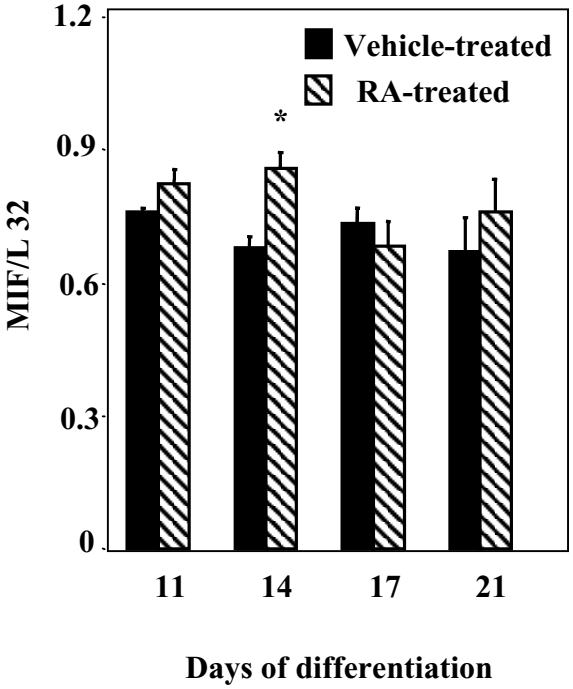

Figure 5. 6. Alterations in macrophage migration inhibitory factor (MIF) expression during RA-mediated ES differentiation using apoptosis signaling molecule template. The relative mRNA expression of MIF is indicated against L32, gene encoding for ribonuclear protein. The results are expressed as mean ± standard error (n=2). * Indicates significantly different from concurrent vehicle-control $P \leq 0.05$.

first report of temporal expression of these selected genes during RA-mediated murine ES-D3 cell differentiation.

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 RA-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 executioners 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 differentiation was accompanied by increased expression of effector caspases 3 and 6. It has been reported previously that RA 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 and Bad, and anti-apoptotic Ced 9/Bcl-2 can homodimerize and hetero dimerizes and the relative expression of pro-and anti-apoptotic fraction of the family is suggested to take part in the

cellular fate (Reed, 1997; Oltavi, 1999). Increased expression of Bad and Bax in RA-exposed cells on days 14, 17 and 21 suggests that Bax and Bad play an important pro-apoptotic role in RA-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 RA-and vehicle-treated cells suggests that Bcl-2 may not play an active role in RA-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 RA-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 RA-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 TNFα in RA–treated cell on day 14. Increased differentiation and reduced proliferation of ES cells with membrane bound and free TNFα has been noted earlier (Kohchi et al., 1996). Contrary to the above report, it has also been reported that TNFα decreased the formation of embryoid bodies in ES cells (Wuu et al., 1998). These reports suggest the temporal importance of TNFα signaling during differentiation.

Increased expression of IGIF/IL-18 was also seen in RA-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 TNFα, IGIF/IL-18 or MIF during ES differentiation and apoptosis, increased TNFα, IGIF/IL-18 and MIF mRNA in RA-treated ES cultures could signify a crucial role in neural differentiation of ES cells, as no neural differentiation is seen in the absence of RA. Retinoic acid has been long known to play an important role

in immuno-modulation and in this study, we add IGIF/1L-18 and MIF to the growing list of cytokines that can be directly or indirectly modulated by RA.

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 RA. The presence of Bcl-2 mRNA during early phase of spontaneous differentiation and undetectable levels of expression in both RA-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 TNFα during differentiation and apoptosis of ES cells will be attempted in our laboratory in future.

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CHAPTER 6

MODULATION OF P53 AFTER MATERNAL EXPOSURE TO ALL-*TRANS*-RETINOIC ACID IN SWISS WEBSTER MOUSE FETUSES[1]

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Summary

The response to exposure of all-*trans*-retinoic acid (RA) during development varies from physiologic to severe teratogenic outcomes and is dependent upon the dose and the stage of development in all species. Tumor suppressor, p53 regulates cellular proliferation, differentiation, and apoptosis. The aim of this study was to characterize the temporal and spatial pattern of p53 expression in fetuses following maternal treatment with RA during mouse organogenesis. Assessment of p53 in Swiss Webster mouse fetuses was undertaken after the administration of a single oral dose of 100-mg/kg body weight of RA on 10 days post coitum. RA-treatment resulted in decreased *p53* mRNA level in fetuses 24, 48, and 72 h after maternal treatment as detected by reverse transcriptase-polymerase chain reaction. This decrease was paralleled by a decrease in p53 protein expression at 24 and 48 h determined with Western blot. The spatial pattern of p53 expression by immunohistochemistry revealed decreased localization of p53 in the neuroepithelium of fetuses exposed to RA *in utero*. Elucidation of p53 signaling pathway of select downstream transactivation target p21 and another cell cycling inhibitor, p27, was investigated by Western blot to provide greater insights into cell cycle control during teratogenesis. Maternal RA-treatment resulted in decreased nuclear p21 and decreased expression of cytosolic as well as nuclear p27 at 72 h in the fetuses. Results demonstrated that RA-mediated teratogenesis is accompanied by a dramatic reduction in temporal and spatial pattern of *p53* gene and protein expression in addition to the disruption of the cell cycle by modulation of p21 and p27. Taken together, these findings indicate the involvement of p53 during development in RA-mediated teratogenesis.

Abbreviations: all-*trans*-retinoic acid (RA); cyclin dependent kinase inhibitor (CDKI); days post coitum (d. p. c); phosphate buffered saline-Tween (PBS-T); retinoic acid receptors (RAR); retinoid X receptor (RXR); reverse transcriptase-polymerase chain reaction (RT-PCR); sodium dodecyl sulphate (SDS).

Key words: p53/organogenesis/histochemistry/p21/RA/teratogenesis

Introduction

At physiological doses, naturally occurring retinoids (Vitamin A), their metabolites and synthetic derivatives mediate pleotropic events during cellular growth, differentiation, apoptosis, vision, and reproduction. However, it is well known that both excess and deficiency of retinoids can cause a spectrum of dose- and stage-specific malformations during development. All-*trans*-retinoic acid (RA) 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, 1995). Exposure of mouse embryos to excessive doses of retinoids during organogenesis was shown to cause a series of developmental malformations (Pillans et al., 1988; Morriss, 1973). The severity of the malformations has been correlated to the concentration of the retinoids in a dose-dependent manner and is also dependent on the stage of development across all species (Shenefelt, 1972; Rutledge et al, 1994; Armstrong et al, 1994). Inappropriate gene expression has been proposed as the mechanistic basis of retinoid teratogenicity. Morphological changes visible after retinoid modulation in embryos could be explained by the alteration of the spatial and temporal pattern of gene expression that control differentiation, proliferation, apoptosis and morphogenesis.

In addition to its role as a tumor-suppressor, p53 regulates cell cycle, differentiation and apoptosis (Almog and Rotter, 1997; Gottlieb and Oren, 1996). Initial experiments with *p53* null mice developing normally suggested the dispensability of *p53*. However, deregulation of *p53* function leads to spontaneous tumor formation at an early age of six months (Donehower et al, 1992). Studies have implicated the role of p53 in the development of the central nervous system and indicated defects like non-closure of neural tubes leading to exencephaly in *p53* null embryos (Armstrong et al, 1995; Sah et al, 1995). It has been reported that p53 is necessary in protecting embryos from chemical or radiation-induced damage. A teratological suppressor function for p53 in *p53*-deficient mice exposed to benzo[a]pyrene was also reported (Nicol et al, 1995), where increased resorption of *p53* -/- fetuses has been observed suggesting that p53 protects the developing embryo from DNA-damaging agents. Additionally, p53-dependent apoptosis has been reported to suppress radiation-induced teratogenesis (Norimura et al, 1996). Contrary to the above findings, the incidence of 2-chloro-2'-deoxyadenosine-induced eye defect reported was higher in *p53* +/+ mice compared with heterozygous (*p53* +/-) and null mutants (Wubah et al, 1996). The induction of p53 is generally characterized by increased transcription of p21, a cyclin-dependent kinase inhibitor (CDKI) (el-Deiry et al, 1993; Harper et al, 1993). It interacts with the cyclin-cyclin dependent complexes and regulates the cell cycle. However, it is becoming increasingly clear that p21 can be induced in a p53-independent manner (Gartel and Tyner, 1999). These results reinforce the important role of p53 in monitoring the integrity of the genome in the developing embryo by growth arrest, apoptosis, and DNA repair.

This study was undertaken to characterize the temporal pattern of *p53* mRNA and protein expression in response to maternal exposure to the classical teratogen, RA in developing Swiss Webster mouse fetuses. The expression of *p53* mRNA and protein was studied along with histochemical localization of p53 in response to maternal treatment with RA during the organogenesis phase. Changes in p53 were correlated to the downstream transactivation target of p53 function, p21, and another CDKI, p27. Results indicated that p53 is down-regulated in RA-induced teratogenesis and may provide further insight into the mechanistic basis of RA-induced developmental defects. Additionally, decreased levels of CDKI p21 and p27 in response to RA insult may further disrupt the cell cycle contributing to the developmental toxicity.

Results

RA Toxicity

A known teratogenic dose of RA was administered as a single oral treatment on 10 days post coitum (d. p. c). No difference in average maternal weight was noted between RA- and vehicle-treated dams at 24, 48, and 72 h. No other overt signs of retinoid toxicity were detected among RA-treated dams. In vehicle-treated group (n=3), 2 resorptions out of 32 implantation sites were observed, whereas in the RA-treated group, 5 resorptions out of 25 implantation sites were seen 24 h after *in utero* exposure (n=2). A decrease in average fetal weight was noted in fetuses from RA-treated dams compared with the fetuses from vehicle-treated group at 24 h ($P < 0.05$) (Table 6. 1). At 48 h, 2 resorptions were noted out of 37 implantation sites examined in the vehicle-treated group (n=3), and 1 resorption out of 34 implantation sites was observed in the RA-treated group (n=3). At 72 h, out of 34 implantation sites no resorptions were observed in the vehicle-treated

dams (n=3) and 1 resorption was noted out of 35 implantation sites in the RA-treated dams (n=3). The average weight of the fetuses was decreased in RA-treated group compared with vehicle-treated group at 72 h ($P < 0.05$).

p53 Gene Expression by Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Three fetuses each from two independent dams (n=2) were used for gene-expression analysis for RA-treated group at 24 h. For analysis of gene expression at all other time points, three fetuses from three dams (n=3) were used for vehicle as well as the RA-treated group. RA treatment resulted in a significant decrease ($P < 0.05$) in *p53* mRNA in fetuses 24, 48, and 72 h after maternal treatment (Figure 6. 1).

p53 Protein Expression by Western Blot

Three fetuses from three dams were used in the vehicle-treated group (n=3), whereas three fetuses each from two dams (n=2) was used in the RA-treated group for protein analysis by Western blot at 24 h. Maternal RA treatment resulted in decreased expression of p53 in four out of six fetuses at 24 h. Two fetuses from one of the RA-treated dams showed increased expression of cytosolic p53. At all other time points, three fetuses from three dams (n=3) were used for vehicle as well as RA-treated group. RA-treatment resulted in decreased cytosolic p53 in the fetuses at 48 h compared with the fetuses from the vehicle-treated dams. No difference in p53 protein expression was noted at 72 h between RA-and vehicle-treated groups as some fetuses from the RA-treated group showed equivalent levels of p53 expression (Figure 6. 2). No change was detected

in the nuclear expression of p53 at 24, 48, and 72 h in the fetuses after maternal treatment with RA compared with the fetuses whose mothers were treated with the vehicle only (data not shown).

Histochemical Localization of p53

During early developmental stages of nervous tissue in mouse, on 11 d.p.c., the fetuses from vehicle-treated dams showed extensive p53 localization in all the neuroepethelium (Figure 6. 3a). Ten fetuses from the vehicle-treated group and 8 fetuses from the RA-treated group were examined by immunohistochemistry. The floor of the 4^{th} ventricle, trigeminal ganglion, fascio-acoustic ganglion complex as well as inferior ganglion of vagus and dorsal root ganglion showed increased localization of p53. Strong staining was also observed in the mandibular process, lining of the buccal cavity, olfactory pit, and the lateral nasal process. The inner lining of endolymphatic diverticulum and otic vesicle also revealed p53 localization. Twelve fetuses/group were examined 48 h after maternal vehicle and RA treatment. In fetuses from vehicle-treated dams, an overall decrease in the intensity of p53 localization was seen on 12 d. p. c. compared to 11 d. p. c. The pattern of p53 localization was more tissue specific with the increase in the gestational age of the fetuses (Figure 6. 3b). Decreased intensity of staining was observed in the neuroepithelial bases of ventricles, the region of trigeminal ganglion and spinal cord. The dorsal root ganglia in the caudal region showed increased p53 localization. P53 was also localized in the genital tubercle. Decreased p53 localization was observed in the hind limb bud. On 13 d. p. c., 9 fetuses/group were examined, and a further decline in the staining for p53 was noted compared with 11 d. p. c. -12 d. p. c. in

fetuses from dams treated with the vehicle (Figure 6. 3c). Localization of p53 was also seen in the ventral part of the spinal cord, areas around diancephalic nucleus, the maxillary area, and areas around the nasal turbinates. P53 was also localized in the enamel of the developing tooth. The characteristic budding pattern of the lung was clearly visible with tracheal lining cells staining strongly for p53, and slightly diffuse staining was also noted in the surrounding mesoderm.

Maternal RA-treatment resulted in gross abnormalities in the fetuses after 24 h (Figure 6. 3d). The fetuses were smaller and developmental defects in the formation of the ventricles and ventricular floors were noted. A drastic decline in p53 localization in the neuroepithelium was noted in fetuses from RA-treated dams compared with fetuses from vehicle-treated group. The absence of p53 in the areas lining the ventricular floors, ganglion areas including the floor of the 4th ventricle, the trigeminal ganglion, the fascio-acoustic ganglion complex as well as the inferior ganglion of vagus and dorsal root ganglion was noted. Abnormalities in the mandibular processes were also observed in the RA-treated group in addition to decreased p53 positive staining compared with the fetuses from the vehicle-treated group. After 48 h, RA-treatment resulted in further decline in p53 in the fetal neuroepethilium and trigeminal ganglion (Figure 6. 3e). Decreased p53 localization was observed in the spinal cord, and in the dorsal root ganglia in the RA-treated group. Decreased p53 staining was also observed in the developing genital tubercle compared to the vehicle-treated group. In contrast to the fetuses from vehicle-treated dams, some fetuses from RA-treated dams showed increased p53 localization in the developing hind limb bud. After 72 h of *in utero* RA-exposure, variation in the localization of p53 was noted in some fetuses. Although a majority of the

fetuses showed decreased p53 localization as compared to the concurrent controls, some fetuses from RA-treated dams showed similar pattern of p53 localization compared with the fetuses from vehicle-treated group. Positive staining for p53 in the primodial lungs and heart were similar to the findings observed in fetuses of dams treated with vehicle only. However, decreased p53 localization was seen in the brain, developing nasal turbinates and the mandible (Figure 6. 3f). Localization of P53 was however observed in formation of vertebral discs and the developing hind limb bud in the RA-treated group.

In fetuses of vehicle-treated dams, dramatic localization of p53 was noted in the developing optic sac on 11 d. p. c. Increased p53-positive staining was observed in the developing lens. Differentiating epithelium of the retina showed positive p53 staining in the outer layer and diffuse staining in the intermediate and inner layers (Figure 6. 4a). Absence of p53 localization in the developing lens was observed in fetuses from RA-treated dams. In contrast to the organized pattern of p53 localization in the retina, patchy localization of p53 in the retina was noted in fetuses from RA-treated dams (Figure 6. 4b). Localization of p53 was also observed in the developing atrial and ventricular chambers of the heart and hepatic primodium in the fetuses from vehicle-treated dams 48-72 h after *in utero* exposure (Figure 6. 4c). In fetuses from RA-treated dams, decreased localization of p53 was observed in the atrial and ventricular walls (Figure 6. 4d). Decrease in p53 localization was also detected in the developing liver in fetuses from RA-treated dams 48-72 h after *in utero* exposure.

p21 and p27 Expression in Cytosolic and Nuclear Extracts

No change in cytosolic p21 was observed in fetuses 24, 48, and 72 h after maternal treatment with RA compared with the fetuses from vehicle-treated dams (Figure 6. 5).

Similarly, no change in the expression of p21 was observed in the nuclear fraction of the protein at 24 and 48 h after maternal RA treatment. Maternal RA-treatment resulted in decreased expression of nuclear p21 in the fetuses after 72 h (Figure 6. 6). No change in fetal cytosolic p27 was noted 24 and 48 h after maternal treatment with RA. However, a decreased expression of cytosolic p27 was noted in the fetuses after 72 h of maternal treatment with RA (Figure 6. 7). Because the effect of RA on CDKIs appeared to be a delayed event, only 48 h and 72 h protein extracts were examined for expression of p27 in the nuclear fraction of protein by Western blot. The decreased cytosolic expression of p27 was reflected in the decreased level of p27 in the nuclear fraction of protein extracts of the fetuses from RA-treated dams compared with fetuses from vehicle-treated group at 72 h ($P < 0.05$) (Figure 6. 8).

Discussion

Our results indicate that maternal administration of a single oral dose of 100 mg/kg body weight of RA during the organogenesis phase (10 d. p. c) of Swiss Webster mouse development leads to decreased expression of *p53* mRNA and protein. At 72 h, p53 protein expression in fetuses exposed to RA *in utero* varied from normal to decreased compared with the concurrent fetuses from dams treated with vehicle only. Examination of the fetuses by histochemical localization of p53 corroborated our findings from Western blots.

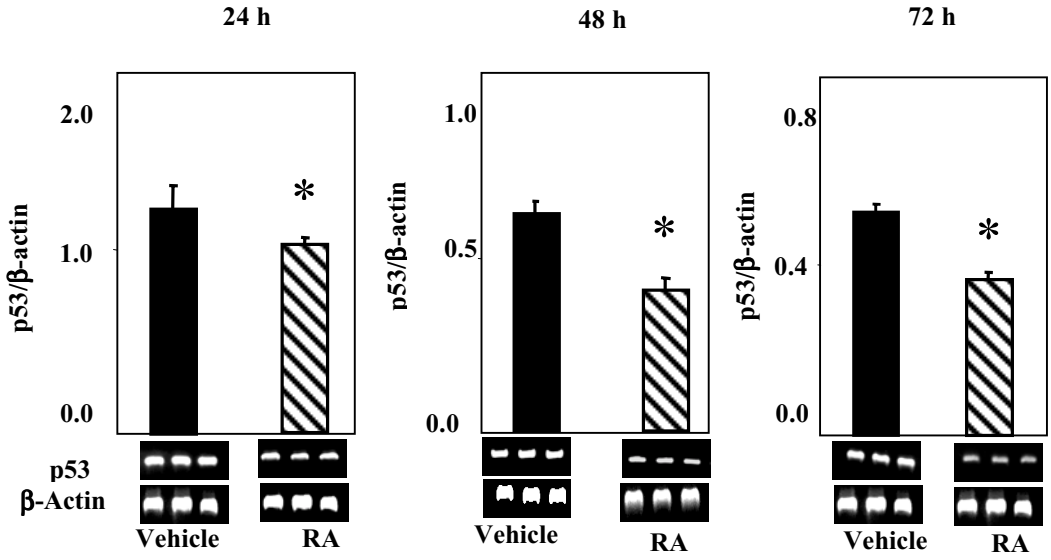

Figure 6. 1 Expression of *p53* mRNA in Swiss Webster mouse fetuses by RT-PCR. The *p53* mRNA expression was normalized against ß-actin. The photographs of representative gel are shown below. Results are expressed as mean ± standard error (n=3 dams). * Indicates significantly different from vehicle-treated group at $P < 0.05$.

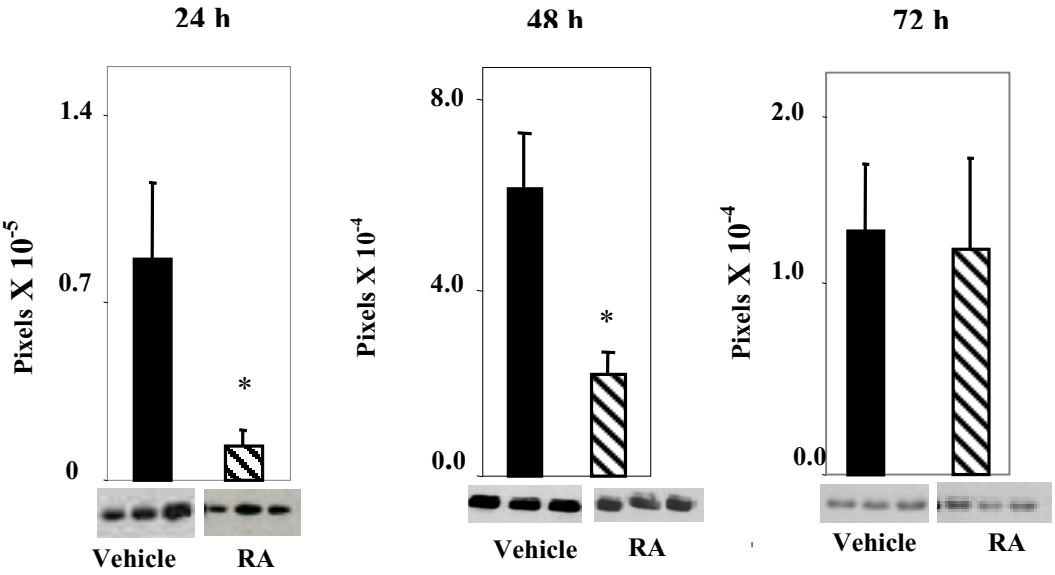

Figure 6. 2. Expression of cytosolic p53 protein in fetuses after *in utero* exposure to RA or vehicle on 10 d. p. c. Cytosolic expression of p53 protein was analyzed by Western blot 24, 48, and 72 h after maternal treatment. Representative gels are shown below respective bars. Results are expressed as mean ± standard error (n=3 dams). * Indicates significantly different from vehicle-treated group at $P < 0.05$.

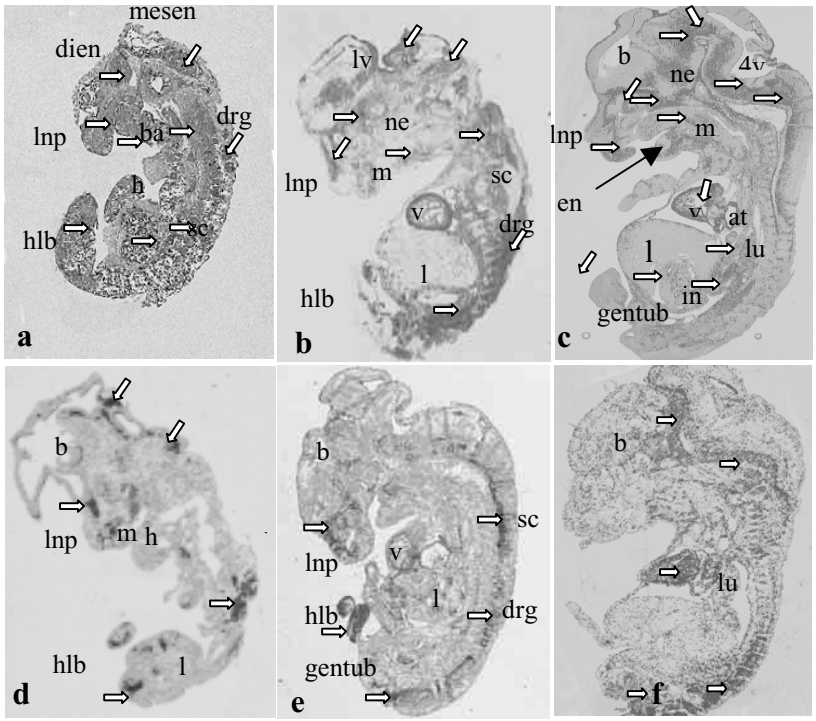

Figure 6. 3 Immunohistochemical localization of p53 in section of Swiss Webster fetuses. Panels (**a-c**) represent vehicle-exposed fetuses 24, 48, and 72 h after maternal treatment. Panels (**d-f**) show concurrent fetuses from RA-treated dams. Arrows indicate prominent expression of the protein. Decreased localization of p53 was seen in the neuroepithelia, spinal cords and the hearts in the RA-treated group. All fetuses are magnified 20 X. Abbreviations used in the above figure are atrial walls (a) brain (b), branchial arches (ba), diencephalons (dien), dorsal root ganglia (drg), eye (e), heart (h), intestine (i), genital tubercle (gentub), hind limb bud (hlb), lateral nasal process (lnp), lateral ventriclular floor (lv), liver (l), lungs (lu), mandible (m), mesencephalon (mesen), mesenchyme (l), neuroepithelium (ne), ventricular wall of the heart (v), spinal cord (sc).

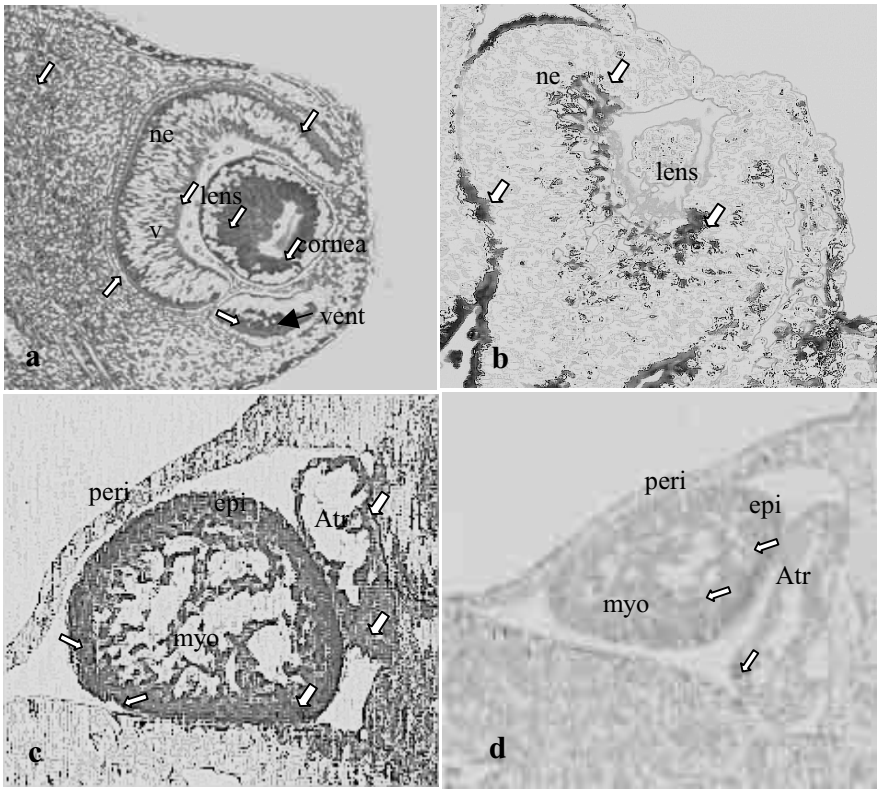

Figure 6. 4. Histochemical localization of p53 in the developing eye after RA or vehicle-exposure *in utero*. Panel (**a**), fetal eye from vehicle-treated fetus showing increased p53 in the lens, retinal neuroepithelium and surrounding mesoderm. Panel (**b**), fetal eye from RA-treated dam on day 11 d. p. c showing absence of p53 in the lens and patchy localization in the neuroepithelium. Panel (**c**), developing fetal heart on 12 d. p. c., 48 h after maternal treatment with vehicle only, showing presence of p53 in the ventricular and atrial walls. Panel (**d**), RA-exposed fetus on the concurrent day showing diffuse p53 localization in the heart. Arrows indicate the presence of stain with anti-p53. Abbreviations in the above figure are atrial wall (atr), epicardium (epi), retinal neuroepithelium (ne), ventricular myocardium (myo), ventricular zone (vent), vitreous cavity (vitr).

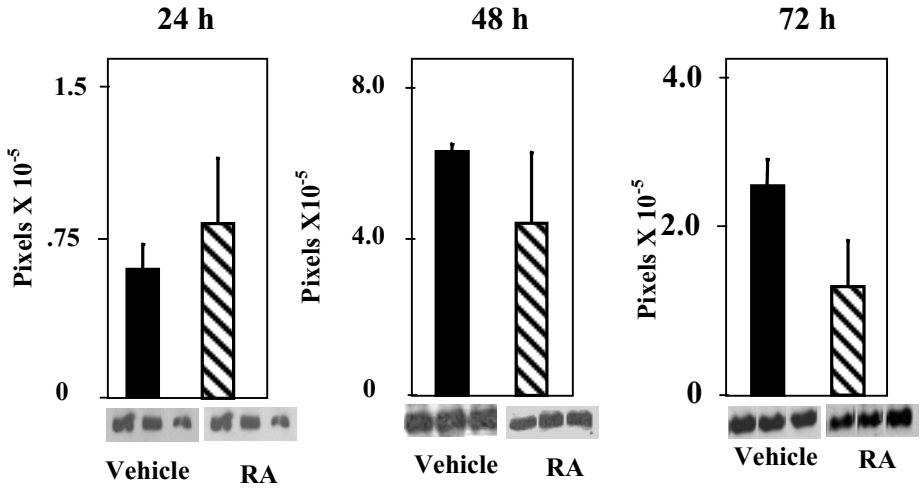

Figure 6. 5. Expression of cytosolic p21 protein in fetuses after *in utero* exposure to RA or vehicle on 10 d. p. c. Cytosolic expression of p21 protein was analyzed by Western blot 24, 48, and 72 h after maternal treatment. Representative gels are shown below respective bars. Results are expressed as mean ± standard error (n=3 dams). * Indicates significantly different from vehicle-treated group at $P < 0.05$.

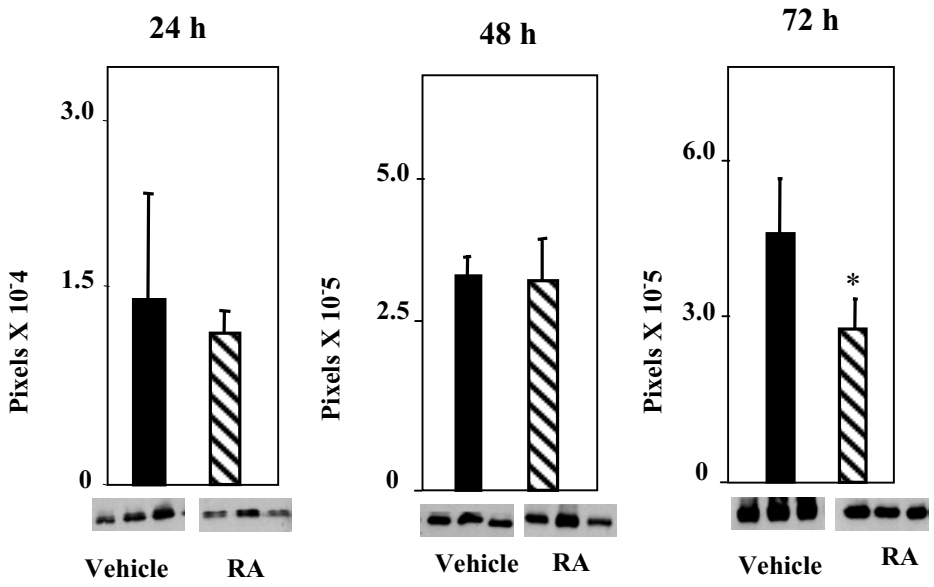

Figure 6. 6. Expression of nuclear p21 protein in fetuses after *in utero* exposure to RA or vehicle on 10 d. p. c. Nuclear expression of p21 protein was analyzed by Western blot 24, 48, and 72 h after maternal treatment. Representative gels are shown below respective bars. Results are expressed as mean ± standard error (n=3 dams). * Indicates significantly different from vehicle-treated group at $P < 0.05$.

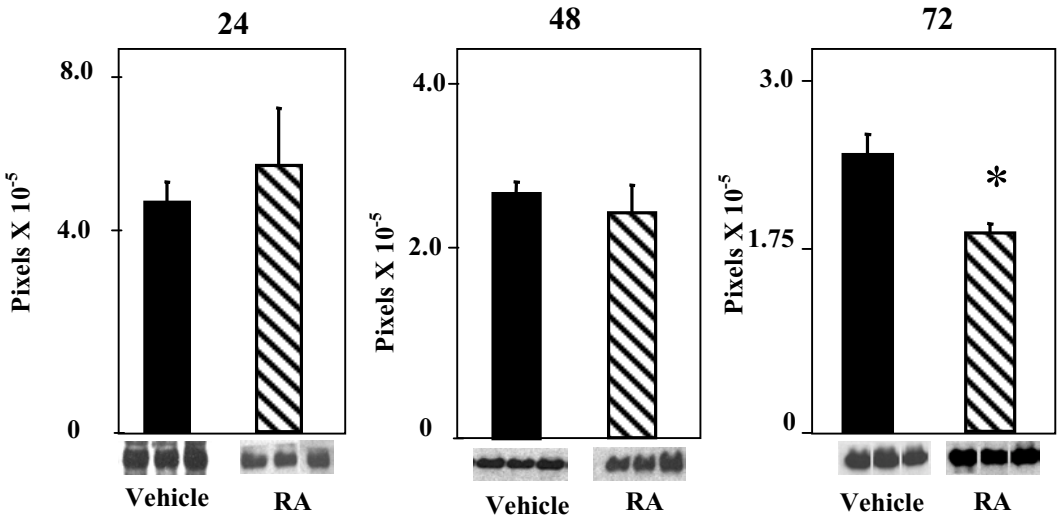

Fig. 6. 7**.** Expression of cytosolic p27 in fetuses after *in utero* exposure to RA or vehicle on 10 d. p.c. Cytosolic expression of p27 protein was analyzed by Western blot 24, 48, and 72 h after maternal treatment. Representative gels are shown below respective bars. Results are expressed as mean ± standard error (n=3 dams). * Indicates significantly different from vehicle-treated group at $P < 0.05$.

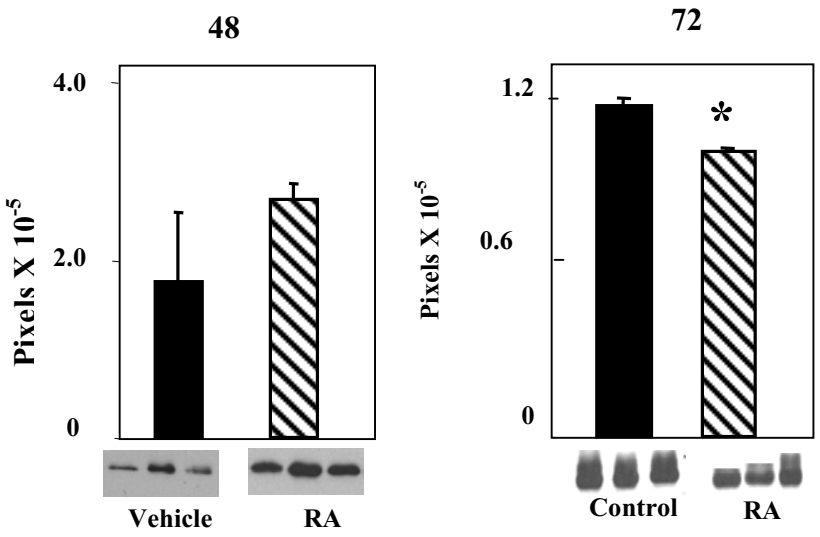

Figure 6. 8. Expression of nuclear p27 protein in fetuses after *in utero* exposure to RA or vehicle on 10 d. p. c. Nuclear expression of p27 protein was analyzed by Western blot 48 and 72 h after maternal treatment. Representative gels are shown below respective bars. Results are expressed as mean ± standard error (n=3 dams). * Indicates significantly different from vehicle-treated group at $P < 0.05$.

Table 6.1. Effect on fetal weights 24, 48, and 72 h after oral administration of RA and vehicle in 10 d. p. c. Swiss Webster dams.

Time in hours	n	Treatment	Weight (g) mean ± (s. e. m)
24	30	Vehicle	0.0544 ± 0.0002
	20	RA	0.0332 ± 0.0004∗
48	30	Vehicle	0.1031 ± 0.0006
	31	RA	0.0999 ± 0.0010
72	34	Vehicle	0.1550 ± 0.0012
	34	RA	0.1365 ± 0.0005∗

Weight data was analyzed by ANOVA. ∗ Indicates significantly different from vehicle control ($P < 0.05$).

Several retinoids function as endogenous ligands that in physiological doses regulate growth, differentiation, morphogenesis, and apoptosis during normal development. The pleotropic effects mediated by RA are caused by its interaction with specific nuclear receptors termed retinoic acid receptors (RAR) and retinoid X receptors (RXR) (Leid et al, 1992). The retinoid-receptor complex influences gene regulation by modulating gene transcription. A known oral teratogenic dose of RA was chosen and administered at the crucial stage of organogenesis to study the temporal effect of RA on p53. At 100 mg/kg b. w., RA is well known to cause neural tube defects, in addition to cleft palate and limb defects (Kochhar et al., 1998). Reduction in average weight of the

fetuses was noted in this study in RA-treated group after 24 and 72 h of maternal insult with RA compared with the group whose mothers were treated with vehicle only.

The role of p53 in teratological research has been a recent focus. It has been reported that treatment of pregnant mice with 2-chloro-2'-deoxyadenosine resulted in several craniofacial abnormalities, microphthalmia, retinal dysplasias, and tooth abnormalities in a p53-coupled mechanism (Wubah, 1996). In radiation-induced teratogenesis, p53 has been assigned a dual role of teratogen-inducer or suppressor. Irradition of mouse fetuses by high dose gamma rays on 9.5-10.5 day of gestation was not teratogenic for *p53* (+/+) mice but teratogenic for *p53* (-/-) mice (Kato et al, 2001).

Expression of p53 in mouse fetuses examined by *in situ* hybridization has been linked to specific tissues undergoing differentiation, and a decline has been noted in cells undergoing terminal differentiation (Schmid et al, 1991). A reduction in the *p53* mRNA in embryo tissues from day 11 was reported (Rogel et al, 1985). In situ hybridization studies (Mora et al, 1980) reported *p53* expression in primary cell cultures of 12-14 days mouse embryo.

Our results were consistent with the previous reports of down-regulation of p53 during organogenesis phase of mouse-embryo development as p53 expression levels decreased progressively from 11 to 13 d. p. c. We have noted that as differentiation and maturation of the fetal brain proceeds the p53 staining in the neuroepithelium gradually decreases. P53 expression becomes tissue specific during the later part of gestation. In the current study, decreased localization of p53 expression in the neuroepethilium of fetuses exposed to RA *in utero* was detected. The role of p53 in the development of the central nervous system has been studied in *p53* -/- knockout mice that show a spectrum of

neural tube defects, exencephaly and anencephaly, along with craniofacial abnormalities (Armstrong et al, 1995). In contrast to the fetuses from dams treated with vehicle only, fetuses from RA-treated dams showed thinning of the neuroepithelium, neural tube defects, together with decreased p53 localization in the neuroepethilium implicating the role of p53 in the RA-mediated toxicity. Localization of p53 in the rat brain was associated with apoptosis during development (Poulaki et al, 1999). In contrast, another group has reported an absence of localization of p53 in the cells undergoing apoptosis in the developing brain (van Lookeren Campagne and Gill, 1998). Retinoid receptors are abundantly found in the developing mouse brain (Yamagata et al., 1994); it is highly likely that exogenous exposure of retinoids can alter the gene expression of p53 in a spatial and temporal manner to cause a spectrum of CNS abnormalities.

In this study, decreased p53 protein was a direct result of decreased *p53* mRNA as detected by RT-PCR. Increase in p53 protein in some fetuses could be caused by contamination with extraembryonic membranes as placental membranes express high levels of p53. At 72 h, a decrease in *p53* mRNA detected by RT-PCR was not paralleled by uniform p53 protein expression seen by Western blot and histochemical localization in fetuses exposed to RA *in utero*. A lack of postive correlation between gene expression and translated protein has been reported for p53 (Kochbin et al, 1992). Some of the fetuses from RA-treated dams showed comparable p53 expression to the fetuses from the vehicle-treated dams. The variation among littermates from a single litter was smaller than variation among different litters. Swiss Webster mice are an out-bred strain and the there could be inherent variability in p53 response to RA. Fetal susceptibility to retinoid teratogenesis is often determined by the variations in maternal retinoid homeostasis and

trans-placental transfer. Similarly, it was consistently seen that fetuses exposed to exogenous RA *in utero* were not equally affected, and the diversity of teratogenic response ranges from normal to severely deformed.

The role of retinoids in the induction of palatal and other craniofacial abnormalities is well known. A dramatic increase in tissue RA was reported after the administration of a teratogenic dose of RA on day 10.5 and was associated with lower jaw and palate abnormalities, in addition to vertebrae, tail, and limb malformation (Horton and Madden, 1995). RA-treatment resulted in decreased localization of p53 in the mandibular process, lining of the buccal cavity, olfactory pit and the lateral nasal process indicating that decrease in p53 may determine the RA-mediated teratogenic outcome in these tissues.

The developing lens and the retina in fetuses from vehicle-treated dams exhibited strong p53 localization as compared with the fetuses whose mothers were treated with RA. P53 promoter-driven bacterial chloramphenicol acetyl transferase expression system has previously revealed p53 activity in the cornea as well as in the retina of the eye (Tendler et al, 1999). Induction of p53-dependent apoptosis by 2-chloro-2'-deoxyadenosine was strongly linked to congenital eye defects (Wubah, 1996). After the administration of a low dose RA on 7 d. p. c., microphthalmos, anophthalmos, faulty closure of the embryonic fissure, developmental abnormalities of the vitreous, aphakia, and faulty separation of the lens vesicles were (Ozeki and Shirai, 1998). The incidences of optic dysplasias and aplasias have been reported in response to RA (Pauken, 1999) although the mechanism by which RA induces optic dysplasia is not known. We found absence of p53 localization in the lens of the fetuses in response to maternal RA

treatment. It is easy to conceive that changes in gene expression related to apoptosis or differentiation that can be mediated by p53 may have profound effects on the development of the eye.

In human embryos, p53 was localized in the proliferative areas of the developing heart (Lichnovsky et al, 1998). Decreased p53 localization in the atrial and ventricular walls of the developing heart of the RA-treated fetuses seen in this study could be correlated to abnormal cardiac morphology noted in some of the RA-treated fetuses, as p53 can perform the dual function of both apoptosis and differentiation.

P21/waf-1/Cip 1, a direct target for p53, encodes for protein that inhibits cyclin-dependent kinase (cdk) (El-Deiry et al, 1993; Harper et al, 1993). Irradiated cells accumulate cdk/cyclin E that are inactivated by p21 association (Dulic et al, 1994). The inhibition of G_1 phase-specific kinase results in a hypophosphorylated retinoblastoma gene product, pRb, which blocks E2-F specific transcription of genes required for S phase entry (Xiong et al, 1993). Thus, p53-mediated cell cycle block is seen mainly in the G_1 phase. P21 also binds to and blocks proliferating-cell nuclear antigen, thus interfering in DNA replication (Waga et al, 1994).

No change in cytosolic p21 expression was noted in fetuses 24, 48, and 72 h after *in utero* exposure to RA. Decreased p21 was noted in the nuclear fraction at 72 h after maternal RA-treatment. Because p53 expression in fetuses is cell specific, the transactivation of p21 by p53 is dependent on the level of p53 in a cell specific manner and may not be reflected by the lack of changes in p21 in the total cytosolic or nuclear pool. Disruption of p27 gene during development was shown to cause abnormal pituitary and disorganization of retinal layers (Nakayama et al, 1996). Moreover, localization of

p53 with p27 in the ventricular and subventricular zone of rat brain rather than the transactivation target p21 has been reported (van Lookeren and Gill, 1996). Modulation of cell cycle inhibitors, p21 and p27, by RA during development could disrupt the progression of the cell cycle resulting in a teratogenic outcome.

Embryonic growth and development entails differentiation, growth, apoptosis and morphogenesis in a highly coordinated and controlled environment. Numerous complex proliferative signals mediated by various transcription factors, cyclins and cyclin-dependent kinases, are suppressed for cell differentiation. Consequently, any alteration in the interactions between genes controlling these critical events during the cell cycle leads to abnormal homeostasis and growth. The present work concludes that RA in teratogenic doses can modulate the expression of p53 in a spatial and temporal manner in Swiss Webster mouse fetuses. The data suggest that the mechanism of RA-mediated teratogenesis is complex and may be caused by disruption of p53 function by changes in the expression and localization together with derailment of cell cycle control by decreased expression of CDKIs, p21 and p27.

Materials and Methods

Animal Housing and Retinoic Acid Treatment

Time-pregnant Swiss Webster mice were purchased from Harlan (Harlan Inc. Indianapolis, IN). The day of detection of the vaginal plug was considered 0 day post coitum (d. p. c). The dams were acclimatized in the University of Georgia Animal Resources facility for 2 days at 23ºC and 50% relative humidity with a 12 h light/dark cycle. The dams were allowed pelleted food and fresh water *ad libitum*. The animals

were also weighed everyday to note weight gain during pregnancy. The animal-use protocols complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. To investigate the effect of RA on p53 and other related genes during organogenesis, 10 d. p. c. dams (3/group) were given a single oral dose of 100 mg/kg body weight (b. w.) of RA (Sigma, St. Louis, MO) dissolved in 5 % acetone and Tween-20 (Sigma) at the rate of 1 ml/100 g b. w. The control dams were treated only with 5% acetone and Tween-20 (vehicle). RA was formulated and dispensed under yellow light to prevent photo-isomerization. The females were killed by carbon dioxide asphyxiation at 24, 48, and 72 h after RA or vehicle treatment. The fetuses from RA-and vehicle-treated dams were collected surgically by abdominal incision, freed from extra-embryonic membranes, weighed and quick frozen in liquid nitrogen and stored at -80ºC for future analysis. Some fetuses were preserved in 4% paraformaldehyde solution for histochemical analysis of p53.

***P53* gene expression by RT-PCR**

The RNA was isolated from the fetuses and converted to cDNA by RT-PCR using a similar protocol described earlier (Sharma et al, 2000). For semi-quantitative measurement, the number of cycles within an exponential phase was determined by initial trials to ensure gene amplification in the linear exponential phase. Samples were amplified for 25 cycles for β-actin and 30 cycles for *p53* in a thermal cycler (Eppendorf Scientific, Westbury, NY). The annealing temperatures were optimized using a gradient. An annealing temperature of 48°C was used for β-actin and 55°C for *p53*. The primers

for p53 and *β-actin* (house-keeping), chosen by the Primer3 program (Whitehead Institute, Cambridge, MA), were 5'-GTACCTTATGAGCCACCCGA-3' (sense) and 5'-CTTCTGTACGGCGGTCTCTC-3' (antisense) for *p53*, and 5'-ATGGATGACGATATC GCT-3' (sense) and 5'-ATGAGGTAGTCTGTCAGGT-3' (antisense) for *β-actin*, respectively. Negative controls with the same primers but no cDNA in the PCR reactions were used. After amplification, 10 µl of PCR products were mixed with DNA dye and run on 2.0 % agarose gel stained with ethidium bromide for electrophoretic separation. The gels were photographed in a backlighted UV transilluminator and quantified on UN-SCAN-IT™ automated digitizing system, (Silk Scientific, Inc. Orem Utah). Total pixel counts for p53 were normalized to β- actin.

Determination of p53, p21 and p27 Protein in Cellular and Nuclear Extract by Western Blot

The proteins from tissue were isolated using a similar protocol described earlier (Deryckere and Gannon, 1994). Briefly, whole embryos frozen at –80°C were ground to a fine powder with a mortar and pestle in liquid nitrogen and homogenized in lysis buffer containing protease inhibitors. The supernatant was incubated on ice for 5 m, centrifuged and stored at –80°C; and the pellets were resuspended in lysis buffer with high salt and glycerol to obtain nuclear extracts. Protein concentration was determined by Bradford reagent (BioRad Laboratory, Hercules, CA). An aliquot of 15 µg of the supernatant protein from each sample was heated with reducing buffer and separated electrophoretically on 12% SDS–polyacyramide gels for p21 and 27. For p53, 10% gels were used. The proteins were transferred onto 0.45 µm pore size nitrocellulose

membranes and blocked overnight with 5% milk Tween-Tris buffer saline. Membranes were exposed to p53 (clone 421, generated by immunizing with p53 protein and fusing with NS-1mouse melanoma cells), p21 and p27 primary monoclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) in blocking buffer and later incubated with horseradish peroxide-conjugated polyclonal anti-mouse IgG (Sigma). Proteins were visualized autoradiographically on X ray films by enhanced chemiluminescent substrate (ECL® Amersham Pharmacia, Piscataway, NJ). The radiographs were scanned and digitized using UN-SCAN-IT™ automated digitizing system as described for PCR gels above.

P53 localization by Histochemistry

Immunohistochemistry was performed to localize of p53 in fetuses. Briefly, the fetuses preserved in 4% paraformaldehyde were embedded in paraffin blocks and 4 μm sections were mounted on slides and heated for 10 min at 70°C. The sections were deparffinated in Citrasolv, (Fisher Scientific, Fair Lawn, NJ) and rehydrated by serially decreasing the ethanol concentration. P53 antigen was retrieved by steaming in 0.1 M citrate buffer pH 6.0 and 0.01% trypsin application in a humid chamber at 37°C. Endogenous peroxidase was quenched by 5% hydrogen peroxide. The sections were blocked with goat serum (Sigma) for 30 min at 37°C and incubated with p53 antibody at 1:10 dilution in phosphate buffered saline-Tween (PBS-T) overnight at 4°C in humid chamber. The sections were incubated with biotinylated secondary goat anti mouse IgG, (Sigma) for 2 h followed by avidin-biotin peroxidase (Vector Laboratories, Burlingame, CA), and diaminobenzidine (Sigma) peroxidase substrate was added develop the stain. The

sections were counterstained with double strength Gill's hematoxyilin, serially dehydrated with increasing concentrations of ethanol, mounted, and cover slipped with Permount (Fisher).

Statistical Analysis

For analysis of fetal weight between groups, one-way analysis of variance (ANOVA) was used. Probability (*P* value) of ≤ 0.05 was considered significant. Three fetuses each from two independent dams (n=2) were used for analysis for RA-treated group at 24 h. For analysis of gene expression at all other time points, three fetuses from three independent dams (n=3) were used for vehicle as well as RA-treated group. The differences between vehicle and treated groups were analyzed using Student's *T* test assuming equal variances. The error bars represent standard error of mean (s.e.m.). Probability (*P* value) of ≤ 0.05 was considered significant.

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CHAPTER 7

EXPRESSION OF C-MYC AND OTHER APOPTOSIS-RELATED GENES IN SWISS WEBSTER MOUSE FETUSES AFTER MATERNAL EXPOSURE TO ALL-*TRANS*-RETINOIC ACID[1]

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Abstract

C-Myc regulates proliferation, differentiation, and apoptosis. To investigate the temporal pattern of c-Myc expression during all-*trans*-retinoic acid (RA)-mediated teratogenesis, Swiss Webster mice were treated with a single oral dose of 100 mg/kg RA or vehicle on 10 days post coitum. The mice were sacrificed 24, 48, and 72 h after treatment and the fetuses were collected surgically by abdominal incision. Reverse transcriptase-polymerase chain reaction and ribonuclease protection assay revealed decreased c-*myc* expression at 48 h followed by an increase at 72 h in fetuses from RA-treated dams. Increased c-Myc protein was detected at 72 h in RA-treated group. The gene expression of *Max*, *Mad,* caspases, and pro-apoptotic members of Bcl-2 family decreased at 48 h after maternal RA-treatment. Increased *Max* and *Mad* were detected 72 h after *in utero* exposure to RA. Apoptosis by terminal uridinetriphosphate nick end-labeling analysis revealed increased apoptosis 24 and 48 h after maternal RA-treatment. Decreased apoptosis was observed in the fetuses 72 h after *in utero* RA-exposure, which correlated with decreased expression of pro-apoptotic genes, noted at 48 h. Further investigations are needed to understand the role of Myc family during RA-mediated teratogenesis.

Keywords: Apoptosis; c-Myc; Caspase; Max; Organogenesis; RA

Abbreviations: bw, body weight; dpc, days post coitum; RA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RPA, ribonuclease protection assay; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulfate; TUNEL, terminal uridinetriphosphate nick end-labeling

Introduction

Naturally occurring retinoids (Vitamin A), their metabolites and synthetic derivatives, at physiological doses mediate pleotropic effects that regulate cellular growth, differentiation, apoptosis, vision, and reproduction. It is well known that both deficiency and exogenous exposure to retinoids in large doses cause a spectrum of malformations in a dose and stage specific manner during development across all species.[1] All-*trans*-retinoic acid (RA), an important metabolite of vitamin A, plays an important physiological role in embryonic development and is teratogenic in large doses in all species.[2,3,4,5] Excessive doses of retinoids during the organogenesis stage of murine development cause a series of developmental malformations.[6,7] Inappropriate gene expression has been proposed as the basis of retinoid teratogenesis. Morphological changes observed after retinoid modulation in embryos could be explained by alteration in spatial and temporal pattern of genes that control differentiation, proliferation, apoptosis and morphogenesis.

Oncogenic transcription factor, c-Myc, regulates cellular proliferation, differentiation, cellular adhesion, metabolism and apoptosis.[8] Activation of c-Myc by different pathways has been noted in oncogenic transformation of cells. Normally, c-Myc is extensively regulated, however, deregulation is the cardinal feature in malignant transformations of cells. It has been suggested that c-Myc plays an important role during embryonic development and growth.[9] Homozygous deletion of *c-myc* leads to embryonic lethality at 10.5 days post coitum (dpc).[10] c-Myc expression in mice is highest in the proliferating tissues of mesoderm origin.[11] Conversely, ectodermal and endodermal tissues express little or no c-Myc.[12, 13] The mechanism by which c-Myc influences differentiation and

apoptosis has not been fully understood. It has been speculated that c-Myc in conjunction with Max family of proteins, promotes the activation of genes that control proliferation[14,15] and the process is inhibited by Mad.[16] Max/Max and Max/Mad heterodimers are thought to repress c-Myc targets and promote differentiation.[17,18]

Cyestine aspartate-specific proteases (c-asp-ases) are synthesized as biologically inactive precursors and play an important role in the initiator and effector phase of apoptotic cell death.[18] Physiological regulation of the caspases occurs through 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.[19,20] Bcl-2 can heterodimerize with the pro-apoptotic members of the family and sequester them, thereby preventing their apoptotic function in response to death signal. [21]

The aim of this study was to investigate the temporal modulation of Myc family of genes during RA-induced teratogenesis. The expression of several apoptosis-related genes including caspase 3 and caspase 6, Bad, Bax, and anti-apoptotic Bcl 2 was investigated simultaneously to elucidate the mechanism of RA-mediated teratogenicity during mouse organogenesis.

Results

No change in c-*myc* mRNA was detected by reverse transcriptase-polymerase chain reaction (RT-PCR), 24 h after maternal per oral (po) RA-treatment. After 48 h of maternal treatment with vehicle or RA, a decrease in the level of expression of c-*myc*

mRNA was noted in fetuses from RA-treated dams compared with the vehicle-treated group. However, 72 h after treatment, an increase in the level of c-*myc* mRNA was detected in the fetuses from RA-treated dams compared with the vehicle-treated group (Figure 7. 1).

Detection of c-Myc protein in fetuses by western blot analysis resulted in no observable change 24 and 48 h after maternal po treatment. The c-Myc protein expression did not parallel the gene expression at 72 h. Decreased expression of c-Myc protein was detected at 72 h in fetuses from RA-treated dams compared with the vehicle-treated group (Figure 7. 2).

In order to better understand the changes in gene expression of c-*myc* at 48 and 72 h after maternal treatment with RA and vehicle, ribonuclease protection assay (RPA) was undertaken to simultaneously analyze the gene expression of c-Myc dimerizing partners, Max and Mad, along with several other proapoptotic and anti-apoptotic members of Bcl-2 family and effector caspases. RPA corroborated the results of c-*myc* gene expression detected by RT-PCR. Decreased expression of c-*myc* mRNA was detected in fetuses 48 h after maternal RA-treatment compared to fetuses from vehicle-treated dams. However, 72 h after treatment, an increased expression of c-*myc* mRNA was detected in the fetuses from RA-treated dams compared with the fetuses from vehicle-treated dams (Figure 7. 3).

Simultaneous analysis of gene expression of the c-Myc dimerizing partners in fetuses from vehicle-and RA-treated dams revealed an initial decline in *Max* expression at 48 h, which was followed by increased expression of *Max* at 72 h. The mRNA expression of *Mad* was similar to c-*myc* and *Max* expression, with decreased level of

Mad mRNA observed at 48 h followed by increased levels at 72 h in fetuses from RA-treated dams (Figure 7. 3).

Expression of both *caspase 3* and *caspase 6* declined at 48 h in fetuses from RA-treated dams. After 72 h of maternal treatment, increased variability in *caspase* mRNA expression in the fetuses from RA-treated group was detected (Figure 7. 4).

Decreased expression of the pro-apoptotic *Bax* in the fetuses from RA-treated dams at 48 h was noted. No change in *Bax* mRNA expression was detected in the fetuses 72 h after maternal RA-treatment. The expression of *Bad* was not altered after RA-treatment. Very low expression of anti-apoptotic *Bcl 2* was detected in comparison to *Bax* and *Bad*. Maternal treatment with RA on 10 dpc did not result in any observable change in the level Bcl-2 mRNA expression in fetuses (Figure 7. 5).

Analysis of apoptosis by terminal uridinetriphosphate nick end-labeling (TUNEL) revealed TUNEL positive cells in the neuroepithelium of the ventricular floors, spinal cord, branchial arches, liver, lumen of the developing gut, heart, liver, hind limb bud and the tail area of the developing fetus (Figure 7. 6 a, b and c). In fetuses from RA-treated dams, increased positive staining for apoptotic cells by TUNEL was seen in the neuroepithelium, branchial arches and the spinal cord area at 24 and 48 h compared to fetuses from vehicle-treated dams (Figure 7. 6 d and e). At 72 h, the intensity of staining for apoptotic cells in the neuroepithelium and the spinal cord declined in the fetuses from RA-treated dams (Figure 7. 6f).

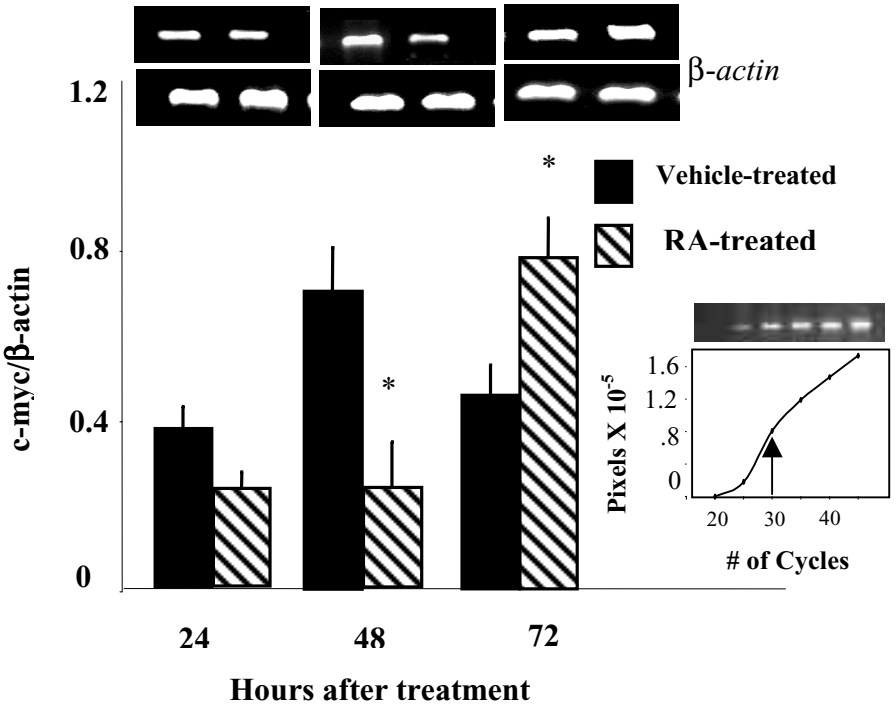

Figure 7. 1. Expression of c-*myc* mRNA in Swiss Webster fetuses after maternal RA-treatment. The c-*myc* mRNA expression were quantified by RT-PCR and normalized to ß-actin. The mRNA of three representative embryos at respective times is indicated. Inset shows amplification of PCR products in the exponential phase of increase in product size. The results are expressed as mean ± sem (n=3). * Indicates significantly different from vehicle-treated group at $P \leq 0.05$.

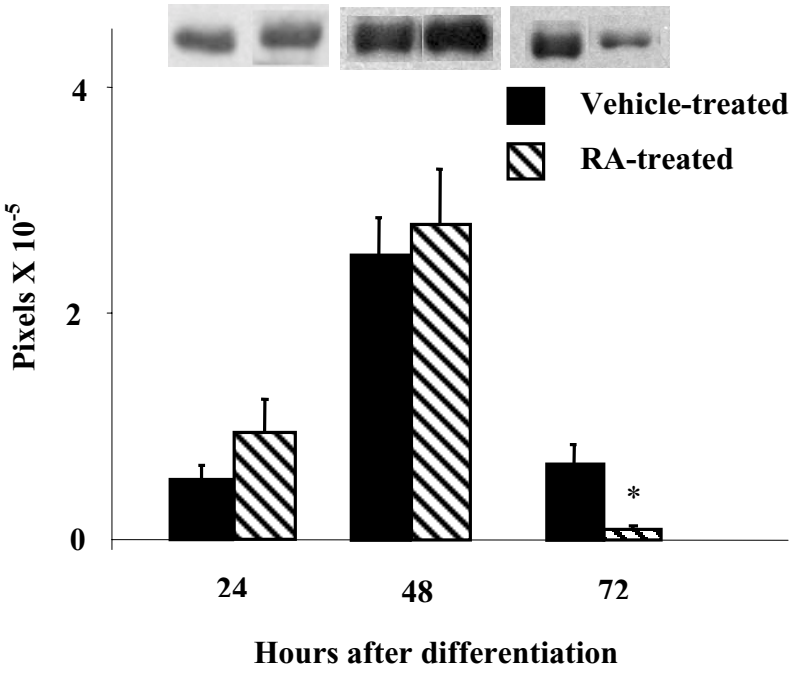

Figure 7. 2. Determination of cytosolic expression of c-Myc protein in fetuses by western blot. Representative gels are shown above respective bars. Results are expressed as mean ± standard error (n=3 dams) except 24 h RA-treated group (n=2 dams). * Indicates significantly different from vehicle-treated group at $P < 0.05$.

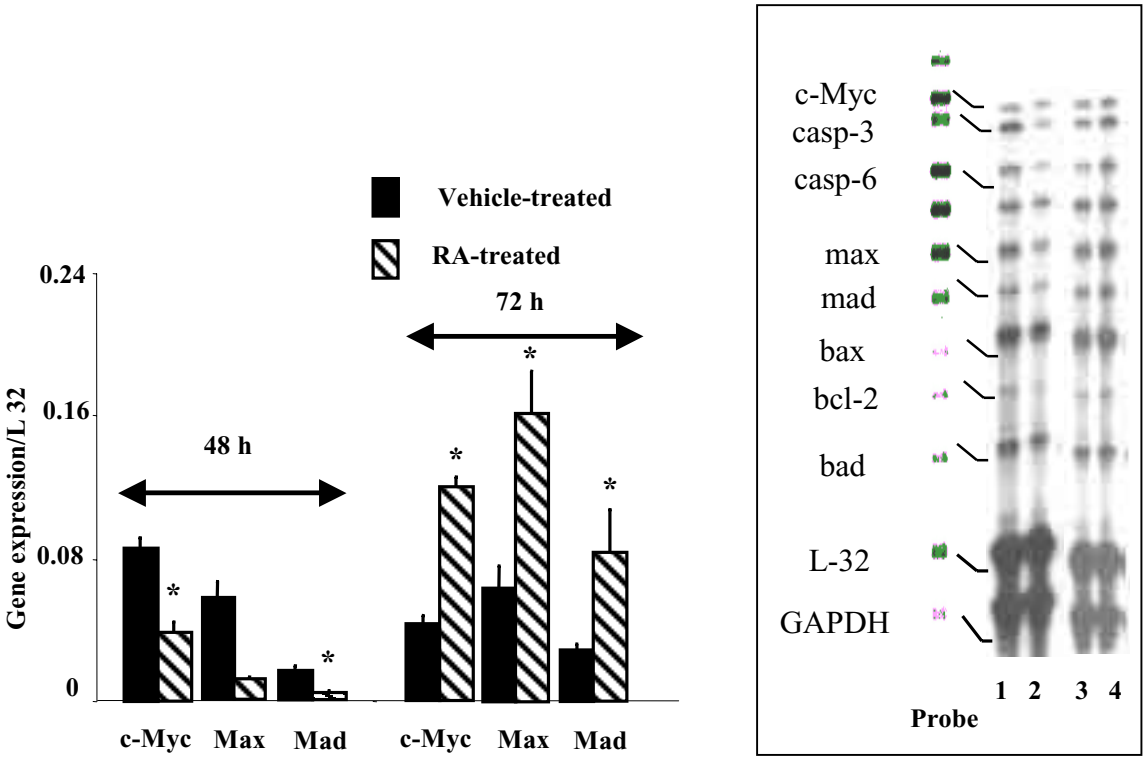

Figure 7. 3. Expression of c-*myc*, *Max* and *Mad* by RPA. The relative mRNA expression was normalized to housekeeping gene L32. The results are expressed as mean ± sem (n=3) except 48 h RA-treated group (n=2). * Indicates significantly different from concurrent vehicle-treated group at $P \leq 0.05$. Representative gel of RNase protection assay is shown on the left 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) 48 h vehicle-treated, (2) 48 h RA-treated, (3) 72 h vehicle-treated, (4) 72 h RA-treated.

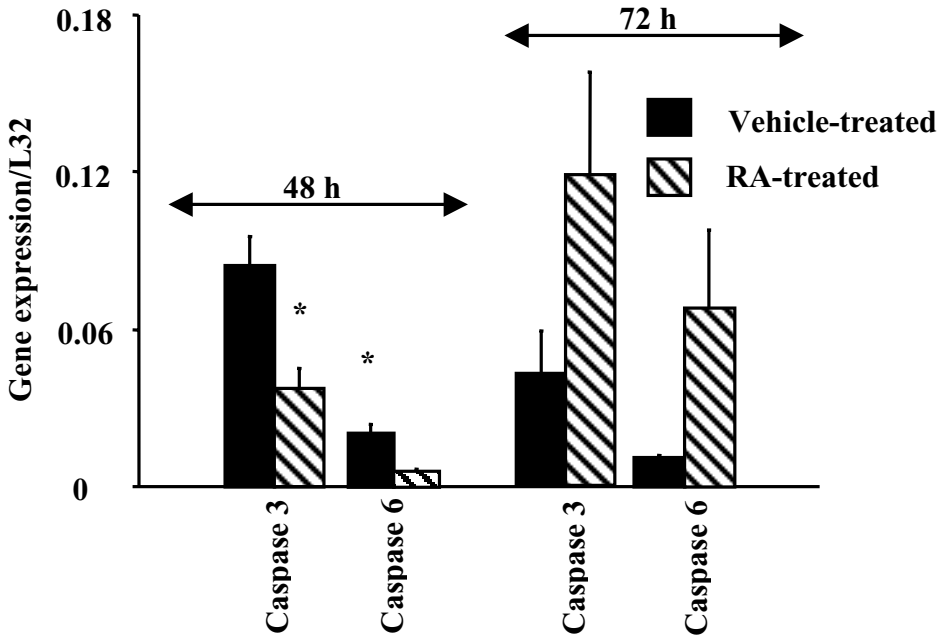

Figure 7. 4. Expression of caspase 3 and caspase 6 by RPA. The relative mRNA expression was normalized to housekeeping gene L32. The results are expressed as mean ± sem (n=3) except 48 h RA-treated group (n=2). * Indicates significantly different from concurrent vehicle-treated group at $P \leq 0.05$.

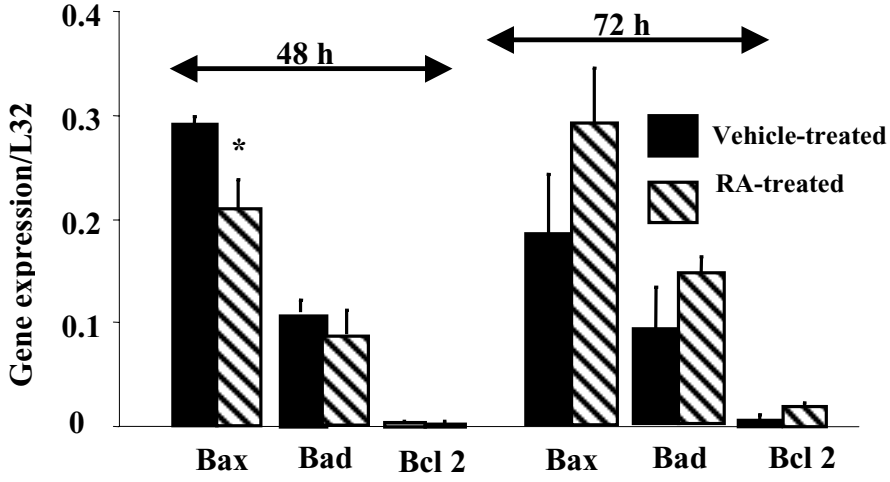

Figure 7. 5. Expression of Bax, Bad and Bcl 2 by RPA. The relative mRNA expression was normalized to housekeeping gene L32. The results are expressed as mean ± sem (n=3) except 48 h RA-treated group (n=2). * Indicates significantly different from concurrent vehicle-treated group at $P \leq 0.05$.

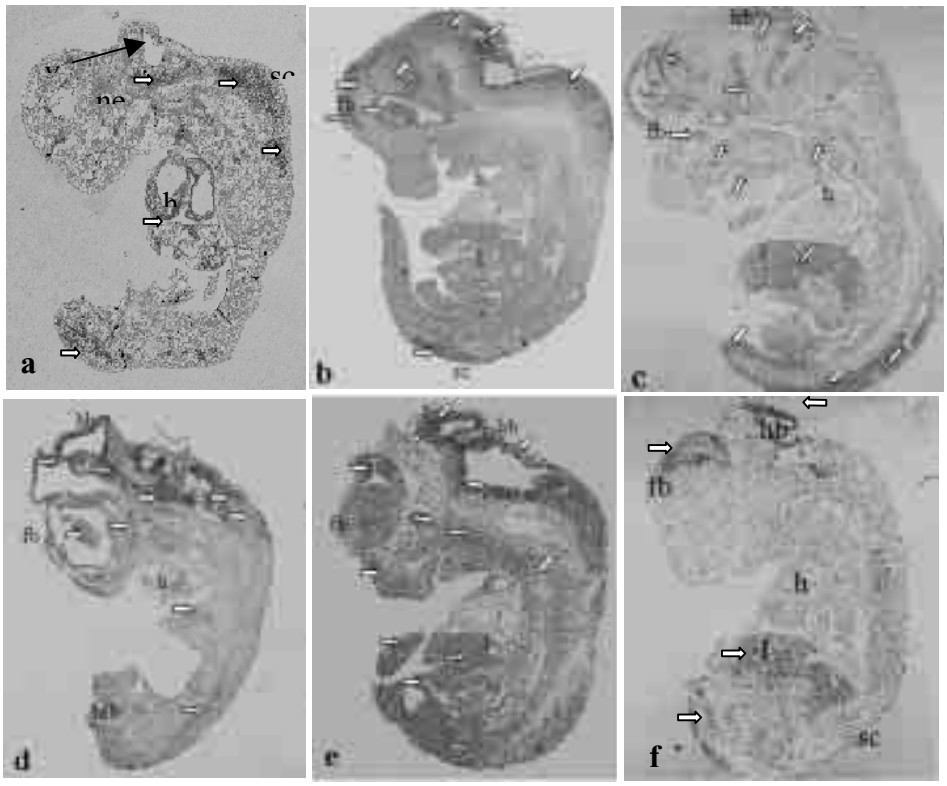

Figure 7. 6. Section of Swiss Webster mice fetuses from dams treated with RA or vehicle showing apoptosis by TUNEL procedure at 11, 12 and 13 (dpc). Panel (**a-c**) represents control fetuses and RA-treated fetuses are shown in panel (**d-f**). Fetuses exposed to RA *in utero* show increased TUNEL positive cells in the neuroepithelium and the spinal cord on 11-12 d. p. c. Arrows indicate prominent localization of TUNEL positive cells. The tissues indicated are neuroepithelium (ne), ventricles (v), hindbrain (hb), heart (h), spinal cord (sc), liver (l) and tail (t). Magnification for all section is 20X.

Discussion

To our knowledge this is the first report on a systemic evaluation of several genes involved in apoptosis as influenced by RA. Results demonstrate that during the organogenesis phase, a known teratogenic dose of RA modulated the expression of several genes of Myc family, namely c-Myc, Max and Mad in mouse. The expression of apoptosis-related genes was repressed 48 h after RA-treatment suggesting that RA modulated common transcription factors that resulted in a generalized repression of several apoptosis-related genes.

Embryonic growth and development entails differentiation, growth, apoptosis and morphogenesis in a highly coordinated and controlled environment. RA mediates cellular differentiation apoptosis and morphogenesis by its interaction with retinoid alpha receptor (RAR) and retinoid X receptor (RXR).[22] The retinoid-receptor complex influences gene regulation by modulating gene transcription. Consequently, any alteration in the spatial and temporal expression of genes controlling these critical events during the cell cycle leads to abnormal homeostasis and growth. It has been previously noted that during the critical phase of organogenesis higher doses ($\geq$ 100 mg/kg) of RA are needed to elicit developmental toxicity.[23] A known teratological dose of 100 mg/kg body weight (bw) RA was used that consistently produced neural tube and craniofacial defects during the organogenesis phase of development.[24]

C-Myc regulates cell cycle primarily in G_1 phase or G_1/S transition and represses differentiation and cell adhesion while actively promoting cellular proliferation.[25] We have found no change in c-*myc* gene and protein expression at 24 h in response to a known teratogenic does of RA during the organogenesis phase in Swiss Webster mouse.

C-*myc* comprises of the group of early immediate response genes that are activated by mitogenic stimulation, without any requirement for protein translation, [26,27] and the subtle changes in c-*myc* gene and protein expression in response to RA may have been missed at 24 h. While proliferative cells express abundant c-Myc, a decreased c-*myc* gene and protein expression has been linked to growth arrest and initiation of differentiation [28, 29] suggesting that exogenous exposure of excessive doses of RA during development could adversely affect proliferation, apoptosis, growth arrest or premature differentiation in cell specific manner. At 48 and 72 h, the expression of c-*myc* gene and translated product were not correlated. Several groups have shown that decrease in c-*myc* gene does not result in decreased transcription activity, implicating post-transcriptional regulatory mechanisms that play an important part in c-*myc* expression.[30, 31]

Myc family is a group of transcription factors that act together to influence apoptosis, differentiation and growth arrest. Investigation of c-*myc* gene and protein expression alone can only be partially correlated to the molecular mechanism of RA-mediated teratogenesis. Contrasting its expression with the other interacting partners can better elucidate the implications of c-Myc response to RA-mediated teratogenesis. The c-Myc signaling effectively requires dimerizing partners Max and Mad, as c-Myc by itself is unable to bind to DNA. C-Myc binds to the DNA in association with its dimerizing partner Max.[32] Max-Myc heterodimers can effectively bind to specific DNA sequences to promote transcription. Max lacks a transactivation domain and Max/Max homodimers can effectively block c-Myc function.[33, 34] It is generally agreed that Max expression does not change during cell cycle or differentiation.[35] Due to longer half-life, Max interaction with Mad family of proteins may logically influence c-Myc function.[36]

Overexpression of Max may thus lead to reduced growth and delayed differentiation.[37] In fetuses from dams treated with RA, modulation of *Max* and mRNA was similar to c-*myc* expression. Decreased expression of c-*myc*, *Max* and *Mad* mRNA at 48 h was followed by an increased expression at 72 h after RA treatment. Our results suggest that RA can modulate the expression of Myc family during mouse organogenesis through transcription factors that can globally activate or repress the transcription of Myc family.

Increased apoptosis in fetuses by TUNEL analysis 24 to 48 h after maternal RA-treatment was observed. Apoptotic cells were localized in the neuroepithelial lining around the ventricle and the spinal cord, heart, liver mesenchyme and hind limb bud. Apoptosis or programmed cell death is a highly conserved evolutionary process and plays a vital role in development by removing unwanted cells.[38,39] Apoptosis has been observed during vertebrate development as early as the blastocyst stage.[40,41] We observed that in fetuses from RA-treated dams, increased apoptosis is followed by a transient decline in the expression of c-*myc*, *Max, Mad* and other apoptosis associated genes for caspase 6, caspase 3 and Bax at 48 h. Cysteine proteases (caspases), play an important role during initiation and effectors phase of apoptotic cells death.[42] Caspases are secreted as proenzymes that can be activated autocatalytically[43] and by protein-protein interactions[44] thereby unleashing the "caspase cascade" that can amplify the signals leading to apoptosis. Decreased expression of pro-apoptotic genes in the fetuses exposed to RA *in utero* at 48 h is followed by decreased apoptosis in the neuroepithelium and the spinal cord at 72 h in fetuses from RA-treated dams.

Alteration or abolition of caspase activity by the use of physiological or pharmacological agents has been known to rescue the cells from apoptotic cell death.[45]

Among the physiological agonists and antagonists is the Bcl 2 family of proteins, that act as modulators of caspase activity. The pro-apoptotic members, Bax and Bad, and anti-apoptotic Ced 9/Bcl 2 can homodimerize and heterodimerize with each other. The relative expression of pro-and anti-apoptotic fraction of the family is suggested to take part in the cellular fate leading to apoptosis or survival.[46] When Bax is in excess, the prevalent homodimer complex enhances the susceptibility of the cell to undergo apoptotic cell death.[47] Decreased expression of *Bax* in fetuses from dams treated with RA was seen at 48 h in this study. Maternal RA-treatment did not change the expression of *Bad* in the fetuses 48 and 72 h after treatment, and no change in *Bcl 2* was observed. These findings suggest that decreased Bax could exist in a heterodimeric complex with Bcl 2 and suppress the apoptotic process transiently, thus limiting further damage to the fetus from chemical insult.

After 72 h of *in utero* RA-exposure, increased expression of *c-myc, Max* and *Mad* was seen, suggestive of regenerative processes taking place in the fetus. Increased c-*myc* expression during regeneration has been noted in both rat liver[48] and in *Xenopus* forelimb.[49] Contrary to the c-*myc* gene expression, decreased c-Myc protein was detected in fetuses from RA-treated dams suggestive of complex regulation of c-Myc during murine embryonic development. A transient decrease in the expression of pro-apoptotic genes observed 48 h after maternal treatment with RA was followed by decreased localization of TUNEL positive cells at 72 h. These findings suggest that subtle changes in gene expression of Myc family and other apoptosis-related genes with RA treatment, during organogenesis, could result in teratogenic phenotype.

The molecular mechanism of RA-mediated teratogenesis is complex. RA can modulate the expression of genes during development in tightly-coupled spatial and temporal compartments. We have shown that cellular response in Swiss Webster fetuses as a result of RA-treatment resulted in increased early apoptosis compared with the fetuses from dams treated with vehicle only. We speculate that transient decline in apoptosis related genes caspase 3, caspase 6 and Bax could decrease the susceptibility of the cells to undergo apoptotic death in response to maternal RA-treatment and prevent further damage. Temporal modulation of Myc family and caspase will be further studied between 0-24 h duration to better elucidate the effect of RA on Myc and other apoptosis-related genes.

Materials and Methods

Animal housing and retinoic acid treatment

Time pregnant Swiss Webster mice were purchased from Harlan (Indianapolis, IN). The day of detection of vaginal plug was considered 0 days post coitum (dpc). The dams were acclimatized in the University of Georgia Animal Resources facility for 2 days at 23ºC and 50% relative humidity with a 12 h light/dark cycle. The females were allowed pelleted food and fresh water *ad libitum.* The animals were weighed everyday to observe weight gain during pregnancy. The animal use protocols complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee. Females were divided randomly in groups of three and given a single oral dose of 100 mg/kg bw of RA (Sigma, St. Louis, MO) dissolved in 5 % acetone and Tween-20 (Sigma) at the rate of 1 ml/100 g bw on 10 d. p. c. The control dams were treated with vehicle only. RA was handled under yellow

light to prevent photo-isomerization. The dams were killed at 24, 48, and 72 h after treatment by carbon dioxide asphyxiation and the fetuses were collected surgically by abdominal incision, freed from extra-embryonic membranes, weighed and quick frozen in liquid nitrogen and stored at -80ºC for future analysis. Some fetuses were fixed in freshly prepared 4% paraformaldehyde for TUNEL assay.

C-*myc* gene expression by RT-PCR.

The RNA was isolated from the fetuses, converted to cDNA by RT-PCR using a similar protocol described earlier.[50] For semi-quantitative measurement, the number of cycles within an exponential phase of product accumulation was selected. Initial trials were run to ensure that the genes are amplified in the linear exponential phase (inset: Figure 1). Samples were amplified for 25 cycles for β-actin and 30 cycles for *c-myc* in a thermal cycler (Eppendorf Scientific, Westbury, NY). The annealing temperatures were optimized using a gradient. Annealing temperature of 48°C was used for β-actin and 56°C for c-*myc*. The sense and antisense primers for β-actin (house-keeping) and c-Myc was chosen by Primer3 program (Whitehead Institute, Cambridge, MA), 5'-ATGGATGACGATATCGCT-3' (sense), and 5'-ATGAGGTAGTCTGTCAGGT-3' (anti-sense), 5'-ATCTGCGACGAGGAAGAGAA-3' (sense), and 5'-ATCGCAGATGAAGCTCTGGT 3' (anti-sense) for β-*actin* and c-*myc,* respectively. After amplification, PCR products were electrophoretically separated on 2.0 % agarose gel stained with ethidium bromide. The gels were photographed in a backlighted UV transilluminator, scanned and quantified on UN-SCAN-IT™ automated digitizing

system, (Silk Scientific Inc., Orem Utah). Total pixel count for c-Myc was normalized to β-actin.

Determination of c-Myc protein by Western blot

The proteins from tissue were isolated using a similar protocol described earlier.[51] Briefly, whole embryos frozen at –80°C were ground to a fine powder with a mortar and pestle in presence of liquid nitrogen and homogenized in lysis buffer containing protease inhibitors. The supernatant was incubated on ice for 5 m, centrifuged and stored at –80°C. Protein concentration was determined using Bradford reagent (BioRad Laboratory, Hercules, CA). An aliquot of 15 µg of the supernatant protein from each sample was heated with reducing buffer and separated electrophoretically on a 12% SDS–polyacyramide gel. The proteins were transferred onto 0.45 µm pore size nitrocellulose membranes for 90 m, and blocked overnight with 5% milk Tween-Tris buffer saline. Membranes were then exposed to c-Myc primary monoclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h in blocking buffer and later incubated for 1 h with horseradish peroxide-conjugated polyclonal anti-mouse IgG (Sigma). c-Myc protein was visualized autoradiographically on X ray films by enhanced chemiluminescent substrate (ECL® Amersham Pharmacia, Piscataway, NJ). The radiographs were scanned and digitized using UN-SCAN-IT™ automated digitizing system as described for PCR gels above.

RNA isolation and RPA

RNase protection assay was performed with Pharmingen's riboquant, multi-probe RNase assay containing apoptosis signaling molecules (TNFα/c-Myc/caspase-3/caspase-6/Max/Mad/Bax/Bad/Bcl 2) (Pharmingen, San Diego CA) using a similar protocol described earlier.[52] [α-^{32}P]-UTP (ICN Biomedicals, Costa Meas, CA) labeled anti-sense RNA probe was synthesized from DNA templates driven by T7 polymerase and hybridized overnight to 40 μg of target RNA according to manufacturers protocol. The free probe and single-stranded RNA were treated with RNase A and RNase TI that was followed by proteinase K digestion, thus destroying all single-stranded RNA. Appropriate positive control in the form of mouse ribosomal RNA and a yeast negative t-RNA control provided by the manufacturer were simultaneously hybridized. The cRNA/mRNA complexes were purified, electrophoresed, dried, and visualized by autoradiography on XAR films (Kodak, Rochester, NY) after 16 h of exposure. The gene expression was quantified by using densitometer imaging UN-SCAN-IT software (Silk Scientific INC., Orem UT). The gene expression was normalized against ribosomal gene L32 and glyceraldehyde-3 phosphate dehydrogenase (GAPDH). For appropriate normalization the gels were exposed for 6 h for housekeeping genes. For RPA analysis at 48 h, three fetuses from the vehicle-treated group (n=3) and two fetuses from the RA-treated group (n=2) were used. Three embryos each from vehicle-and RA-treated dams were used for RPA analysis at 72 h (n=3). The RPA was repeated twice for consistency.

Detection of Apoptosis TUNEL

Paraformaldehyde-fixed embryos were embedded in paraffin; 5 μm sections were prepared and used for analysis of apoptosis using terminal UTP nucleotide transferase end-labeling (TUNEL) of apoptotic cells with peroxidase based ApoTag® Plus kit (Intergen, Purchase, NY) using diaminobenzide (DAB), according to manufacturer's protocol. The tissues were counterstained will double strength Gill's hematoxylin for 5 s.

Statistical analysis and Replications

Three fetuses each from two independent dams (n=2) were used for analysis for RA-treated group at 24 h. At all other time points, three fetuses each from three independent dams (n=3) were used for vehicle as well as RA-treated group. The difference between vehicle and treated groups were analyzed using Student's-t test assuming equal variances. The error bars represent standard error of mean (sem). Probability (*P* value) of ≤ 0.05 was considered significant.

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CHAPTER 8

SUMMARY AND CONCLUSIONS

Differentiation, growth arrest and apoptosis are highly controlled and regulated processes during development and embryogenesis. All-*trans*-retinoic acid (RA), an important metabolite of vitamin A, plays an important physiological role in embryonic development and is teratogenic in large doses in all species.

Temporal modulation of p53 by RA was investigated in murine embryonic stem cells during differentiation and apoptosis. Undifferentiated embryonic stem cells express a high level of p53 mRNA and protein followed by a decrease in p53 levels as differentiation proceeds. The addition of RA during 8-10 days of differentiation increased the levels of p53 mRNA and protein, accompanied by accelerated neural differentiation and apoptosis. Marked increase in apoptosis was observed at 10 h to 20 h after RA treatment when compared with untreated controls. Retinoic acid-induced morphological differentiation resulted in predominantly neural-type cells. Maximum increase in p53 mRNA in RA-treated cells occurred on day 17, whereas maximum protein synthesis occurred on days 14-17 that coincided with increased neural differentiation and proliferation. Increased p53 levels did not induce p21 transactivation, and a decrease in p21 was observed on day 17 after RA exposure. The level of p53 declined by day 21 of differentiation. The results demonstrated that RA-mediated apoptosis preceded the changes in p53 expression, suggesting that p53 induction does not initiate RA-induced apoptosis during development. RA accelerated neural differentiation

and increased the expression of p53 in proliferating neural cells, corroborated by decreased p21 levels, indicating the importance of cell type and stage specificity of p53 function.

c-Myc regulates cellular proliferation, differentiation, and apoptosis. Temporal expression of c-Myc during RA-mediated murine embryonic stem cell neural differentiation was investigated. Correlation to the modulation of dimerizing partners Max and Mad that may influence c-Myc signaling and transcription regulation was investigated for the first time in these cells. In RA-treated cells, increase in c-*myc* mRNA was detected by RT-PCR on day 11 and 14 of differentiation as compared to the vehicle-treated controls. The results were further corroborated by RPA. Western blots revealed an increase in c-Myc protein only on day 14 in RA-treated cells. Increases in *Max* and *Mad* genes transcription at times of elevated c-Myc in RA-treated embryonic stem cells suggest that a transient increase in c-Myc protein expression may influence differential dimerization of Myc partners needed for signaling in the neural differentiation of embryonic stem cells.

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 ES by RA. The mRNA expression of caspase 3, caspase 6 and certain pro-inflammatory cytokines was also investigated simultaneously. ES cells exposed to 1 μM RA 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 RA treatment. Increased mRNA expression of

caspase 3 and caspase 6 in RA-treated ES cells suggested that caspases play an important role in RA-mediated apoptosis during ES differentiation. Increase in the expression of TNFα and macrophage migration inhibitory factor (MIF) was noted in RA-treated cells on day 14. Significant increase observed in interferon γ inducing factor (IGIF/IL-18) mRNA expression in RA-treated cells on day 14 and 17 did not translate to increased INFγ expression. No change in the expression of other pro-inflammatory cytokines was noted with RA-treatment. The function of TNFα, IGIF/IL-18 and MIF in RA-treated cells during ES differentiation and apoptosis is still speculatory. Results suggest that RA mediated apoptosis during neural differentiation of ES cells involves up-regulation of caspase 3, caspase 6, Bad and Bax.

RA-treatment resulted in decreased *p53* mRNA level in fetuses 24, 48, and 72 h after maternal treatment as detected by reverse-transcriptase polymerase chain reaction. This decrease was paralleled by decrease in p53 protein expression at 24 and 48 h determined with western blot. The spatial pattern of p53 expression by immunohistochemistry revealed decreased localization of p53 in the neuroepithelium of fetuses exposed to RA *in utero*. Elucidation of p53 signaling pathway of select downstream transactivation target, p21 and another cell cycling inhibitor p27, was investigated by western blot to provide greater insights into cell cycle control during teratogenesis. Maternal RA-treatment resulted in decreased nuclear p21 and decreased expression of cytosolic as well as nuclear p27 at 72 h in the fetuses. Results demonstrated that RA-mediated teratogenesis is accompanied by dramatic reduction in temporal and spatial patterns of *p53* gene and protein expression, in addition to the

disruption of the cell cycle by modulation of p21 and p27. Taken together, these findings indicate the involvement of p53 during development in RA-mediated teratogenesis.

The temporal pattern of c-Myc expression was investigated in Swiss Webster mice fetuses after a single oral dose of 100 mg/kg body weight of RA on 10 d. p. c. Analysis of mRNA by RT-PCR and RPA revealed decreased c-*myc* expression at 48 h followed by an increase at 72 h in fetuses from RA-treated dams. Increased c-Myc protein was detected at 72 h in RA-treated group. The gene expression of *Max*, *Mad,* caspases, and pro-apoptotic members of Bcl-2 family decreased at 48 h after maternal RA-treatment. Increased *Max* and *Mad* were detected 72 h after *in utero* exposure to RA. Apoptosis by TUNEL revealed increased apoptosis 24 and 48 h after maternal RA-treatment. Decreased apoptosis was observed in the fetuses 72 h after *in utero* RA-exposure, which correlated with decreased expression of pro-apoptotic genes, noted at 48 h. Further investigations are needed to understand the role of Myc family during RA-mediated teratogenesis.

The major finding of the present study is that RA can modulate several proto-oncogenes and tumor-suppressors during ES differentiation and murine organogenesis. The precise mechanism and cellular events due to altered gene expression need to be investigated further. Application of RA modulation of proto-oncogenes and tumor suppressors during development may help in elucidating “retro-differentiation” in carcinogenesis.

APPENDIX

Mouse embryonic stem cell culture (ES-D3)

American Type Culture Collection CRL 1934

1. Sterile filtered ES culture medium (pH 7.2)

   Dulbeccco's modified Eagles medium with 4 mM L-glutamine

   1.5 g/L sodium bicarbonate

   4.5 g/L glucose

   1.0 mM sodium pyruvate

   100 µM 2-mercaptoethanol

   1000 units/ml mouse recombinant leukemia inhibitory factor (LIF)

   15% Knock-Out serum replacement (Gibco)

2. Maintenance of undifferentiated state (humidified atmosphere of 5% CO_2 in air at 37°C)

   Preparation of fibroblast feeder layers ATCC CRL (ST0-1503)

   Grow mouse fibroblasts to completely confluent monolayer absence of LIF and 2-mercaptoethanol

   Supplement DMEM with 5% fetal bovine serum instead of Knock-Out serum replacement

   Treat confluent fibroblast monolayers with 10 µg/ml of mitomycin c for 2 h in the incubator

   Trypsinize fibroblasts and reseed at 8 X 10^4 cells/sq. cm

Fibroblast feeder layers can be kept for 2-3 days after mitotic inhibition by mitomycin c

3. ES cell seeding (humidified atmosphere of 5% CO_2 in air at 37°C)

   Rapidly thaw ES cells in 37°C in a water bath and seed on mitotically inhibited fibroblast layers

   Supplement with ES culture medium containing LIF and 2-mercaptoethanol

   Medium is changed everyday

4. ES cells can be passaged after every 2 days to maintain undifferentiated state for prolonged period

5. Induction of differentiation

   ES cells may be plated on 6-well plates at a density of 2 X 10^5/ ml in the absence of fibroblast feeder layers

   Alternatively plates may be coated with sterile 0.1% Type I gelatin (Sigma)

   Differentiating media (DMEM and Knock-Out serum replacement) is devoid of LIF and 2-mercaptoethanol

   Knock-Out serum replacement prevents the attachment of fibroblast feeder cells

   ES cells aggregate into embryoid bodies in the absence of feeder layers and LIF. The embryoid bodies further differentiate into cells of various lineage

   Medium is changed everyday

Histochemistry Protocol

1. Heat paraffin embedded section at 70°C for 10 min

2. De-paraffination

    Citrasolv (Fisher) for 7 min

    Citrasolv for 7 min

3. Serial rehydration

    100% ethanol for 2 min

    100% ethanol for 1 min

    95% ethanol for 2 min

    95% ethanol for 1 min

    70% ethanol for 1 min

    Tap water for 1 min

4. Antigen retrieval

    4.2 g of citric acid in 20 ml water

    14.7 g of sodium citrate in 500 ml of water

    Add 18 ml of citric acid solution to 82 ml of sodium citrate

    Bring the volume up to 1 L (pH 6.0)

    Pre-warm citrate buffer in a coplin jar and steam slides for 25 min in the buffer in a steamer

    Cool slides to room temperature

5. Wipe off excess buffer and circle with a hydrophobic histology pen

6. Trypsin treatment

    Add 0.01% trypsin to 0.1 M Tris (pH 7.5), with 1% calcium chloride for 30 min in humid chamber at 37°C

7. Rinse in 0.2 M Tris with 0.1 M glycine buffer (pH 7.5) for 5 min to inactivate trypsin
8. Rinse in 0.1 M Tris glycine buffer (pH 7.5) for 5 min
9. Endogenous peroxide quenching

   Immerse slides in 5% hydrogen peroxide for 10 min

   Rinse in tap water for 10 min
10. Block with 2% (goat) serum of species in which the biotinylated secondary antibody was made in phosphate buffered saline-0.1% Tween (PBS-T) (pH 7.5) for 30 min at 37°C in humid chamber
11. Blot of excess buffer and incubate with primary antibody at 1:10 ratio overnight at 4°C in humid chamber
12. Rinse in PBS-T for 5 min
13. Rinse in PBS-T for 5 min
14. Incubate with secondary antibody diluted 1:250 in PBS-T at 37°C for 2 h in humid chamber
15. Rinse in PBS-T for 5 min
16. Rinse in PBS-T for 5 min
17. Incubate with Avidin–Biotin peroxidase solution for 1 h at 37°C in a humid chamber. To 5 ml of PBS-T add 2 drops of Vector ™ Sol A and mix. Add 2 drops of Sol B and mix. The solution is made 30 min in advance and warmed to room temperature.
18. Rinse slides in PBS-T for 5 min
19. Rinse slides in PBS-T for 5 min

20. To 5 ml of water add of diaminobezidine tablets A and B (Sigma) and mix

21. Incubate for 5-15 min in dark

22. Rinse slides in de-ionized water

23. Counterstain with double strength Gill's hematoxylin

24. Rinse slides in running tap water

25. Dehydrate serially

    70% ethanol for 1 min

    70% ethanol for 2 min

    95% ethanol for 1 min

    95% ethanol for 2 min

    100% ethanol for 1 min

    100% ethanol for 2 min

    Citrasolv for 7 min

    Citrasolv for 7 min

25. Mount and coverslip with Permount (Fisher)