ASSESSMENT OF IN VITRO CHONDROGENESIS AFTER TREATMENT WITH ALL-TRANS RETINOIC ACID

by

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(Under the direction of Mary Alice Smith)

ABSTRACT

Retinoids, derivatives of Vitamin A, are valuable in the treatment of certain forms of skin disorders and cancer; however, their therapeutic application is limited by their potential teratogenic potency. To date, mechanisms involving retinoic acid's teratogenicity are undetermined. Mesodermal cells grown *in vitro* differentiate into chondrocytes and produce cartilage-specific compounds such as proteoglycans. The purpose of this study was to determine the effects of *all-trans* retinoic acid (RA) on growth and differentiation of chondrocytes. Micromass cell culturing techniques were performed in which mesodermal limb bud cells were dispersed into a single cell suspension, placed into cell culture dishes, and incubated with *all-trans* RA for a 120hr period. After incubation, cells were fixed and stained for proteoglycan content with Alcian green and optical density (OD) determined at 600 nm. Subsequently, cultures were stained for cell proliferation using crystal violet (OD=570nm). The absorbances of stained cells were compared to vehicle controls (VC). Results indicated a dose dependent relationship of retinoic acid affecting proteoglycan content and cell proliferation at doses ranging from 10^{-9}-10^{-6}M. After treatment with 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M RA, proteoglycan content decreased to 49.8%, 39.3%, 28.6%, and 15.6% of VC, respectively. Cell proliferation decreased to 84.1%, 75.6%, 70.3%, and 69.3% of VC, respectively.

Temporally, RA decreased cell proliferation earlier (83% of VC @ 48) than proteoglycan content (20% of VC @ 72hrs). Using an ELISA, TNF α was measured in medium upon cell culture treatments with 10^{-9}-10^{-6}M *all-trans* RA. Results from the ELISA assay show a minimal detectable level of TNFα within 120hr chick embryonic cell cultures.

Research supported in part by Federal Hatch Funds to MAS.

INDEX WORDS: Micromass cultures, Limb development, Retinoic Acid, In vitro, Chondrogenesis, TNF

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CHAPTER 1

INTRODUCTION

All-trans retinoic acid (*all-trans* RA) is a known developmental toxicant and can be found in prescription drugs such as Accutane, an acne medication. Due to the teratogenicity of its components, Accutane is not prescribed for pregnant women, and women of childbearing age must provide their physicians with proof of birth control in order to receive the acne medication. Past studies have shown *all-trans* RA is a necessary component for normal mammalian development (Underhill and Weston, 1998). These studies have also shown *all-trans* RA adversely affects limb development in rodents and chick embryos. *All-trans* RA is also a well-known skeletal teratogen and a potent inhibitor of chondrogenesis (Smith et al., 1983). Because *all-trans* RA is necessary in normal development but teratogenic if provided in excess, understanding the normal and teratogenic mechanisms of *all-trans* RA could provide for therapeutic intervention.

Although, the actual mechanism of *all-trans* RA's teratogenicity is unknown, its effects on major components dealing with the developing limb are well documented. A*ll-trans* RA generally enhances cell differentiation on a variety of cell types (for review see Paulsen, 1994). It has been hypothesized that biochemically, *all-trans* RA influences the developing limb via its cellular retinoid binding proteins (CRABPs), and its nuclear receptors (RARs, RXRs), ultimately inhibiting the outgrowth of the apical ectodermal ridge (AER) (for review see Paulsen, 1994).

While past studies have investigated the effects of *all-trans* RA on binding proteins and cellular receptors, the present study examines the effects of *all-trans* RA on the growth factor tumor necrosis factor alpha (TNFα) and the developing limb of the chick by monitoring cell proliferation and proteoglycan content in an *in vitro* micromass culture system. The use of *in vitro* systems provides an isolated and homogeneous system that can be easily controlled when investigating mechanisms by which toxicants exert their effect (Smith and Kanti, 1997). Within this tissue culture system, mesodermal cells grown *in vitro* differentiate into chondrocytes and produce cartilage-specific compounds such as proteoglycans. Cell proliferation and abundance of proteoglycans are commonly measured variables in this micromass culture system.

TNFα is well recognized as an endogenous mediator of inflammation. However, TNFα has also been identified as a growth factor, differentiating agent or toxicant depending on the cell type exposed. For example, its stimulatory actions on the growth of cells have been demonstrated in normal human fibroblasts (Sugarman et al., 1985). TNFα may also act as a toxic, or differentiating agent depending on the target cell (Baglioni, 1992). Structurally, TNFα is a multifunctional peptide with well-established pathophysiological roles (Vassali, 1992), including the ability to precipitate DNA fragmentation (Larrick and Wright, 1990); a main characteristic of apoptotic cell death (Bortner et al., 1995). Within neuroblastoma SKNE cells, TNFα receptors are expressed when treated with *all-trans* RA, resulting in the differentiation of the SKNBE cells (Chambaut- Guerin et al., 1995). Because of TNFα's reported effects on growth, differentiation and apoptosis, and *all-trans* RA's ability to affect expression of TNFα

receptors, we hypothesized that TNFα is a mediator in differentiation of chondrocytes in our *in vitro* system.

The goal of this study was to determine the effects of *all-trans* RA on *in vitro* chondrogenesis by monitoring *all-trans* RA's effects on TNFα, cell proliferation and proteoglycan content.

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CHAPTER 2

REVIEW OF LITERATURE

The intent of this literature review is to summarize the current knowledge of the effects of *all-trans* RA on *in vitro* chondrogenesis. For over fifty years, the effects of excessive retinoids on development have been known. However, despite many years of research, the exact mechanism(s) of how this takes place is still unknown. Apoptosis, or programmed cell death, plays an important role in limb development. In separate systems, both *all-trans* RA and TNFα have been implicated as possible mediators in the cascade of events leading to apoptosis. To explore these relationships, the following literature information is divided into sections consisting of *all-trans* RA, TNFα, and micromass tissue cultures.

***All-trans* Retinoic Acid**. Retinoids, derivatives of vitamin A, are used to treat certain forms of skin disorders and cancer, such as psoraiasis, ichtyoses, skin cancers and their precursors (for review see Futoryan and Gilchrest, 1994). *All-trans* RA, a known developmental toxicant, is included in the formulation of Accutane, a drug prescribed by physicians to treat cystic acne. Due to the teratogenicity of its components, Accutane is not prescribed to pregnant women, and women of childbearing age must provide their physicians with proof of birth control in order to receive the acne medication. While *all-trans* RA is a known development toxicant, its teratogenic mechanisms are still unknown.

RA adversely affects limb development in rodents and chick embryos, however; past studies have shown *all-trans* RA is necessary for normal mammalian development (Underhill and Weston, 1998), and deficiencies of vitamin A are likely to

induce malformations in various mammalian species (Sharma and Kim, 1995). Endogenous RA is a signaling molecule required for normal development (Cash et al., 1997). Within normal limb development, mesenchymal cells derive positional information from regional differences in the concentration of *all-trans* RA (Paulsen et al, 1994). Thus, *all-trans* RA provides positional information to the developing limb, and disturbances in these normal signaling pathways can result in malformed limbs.

Helms et al., (1996) indicated *all-trans* RA plays a major role in early limb development concomitant with limb bud formation. Their conclusions were based on three observations: (1) Blocking the signaling pathway of *all-trans* RA resulted in down regulation of essential expressions of limb formation, such as sonic hedgehog expression (shh) and near complete loss of wing structures of chick embryos; (2) Wing bud tissue of chick embryos from stage 14 (Hamburger and Hamilton, 1951) coincides with a peak of *all-trans* RA synthesis; and (3) Exogenous *all-trans* RA induces factors in limb bud mesenchyme and ectoderm that substitute for stage 14 ectoderm or mesenchyme of the presumptive wing region (Helms et al., 1996). Thus, because *all-trans* RA is necessary in normal development but teratogenic in excess, understanding the normal mechanism of *all-trans* RA is needed to provide information for developing therapeutic intervention strategies.

All-trans RA plays a role in the zone of polarizing activity (ZPA). The ZPA is essential for limb development and specifies its pattern along the ante-posterior limb axis (Helms et al., 1996). During normal development, disrupting retinoid signaling pathways by applying antagonists LG629 and LG754 led to malformed hand plates and abnormal limb development (Helms et al., 1996).

In experimental animals, excessive intake of retinoids in the first trimester of pregnancy significantly increases the incidence of gross congenital defects (Cohlan, 1953). Depending on the dose and mode of delivery, exogenous retinoic acid has profound effects on limb skeletal pattern ranging from distal deletions to skeletal pattern duplications (for review see Paulsen, 1994). An *in vitro* study of mouse embryo limb buds treated with RA resulted in preaxial forelimb ectrodactyly and fusion of carpal bones (Shury et al., 1994). The effects of *all-trans* RA during this study were very bone specific, exhibiting different effects on various bone anlagen (Shury et al., 1994).

In rodents, specifically rats, *all-trans* RA induces limb defects by inhibiting cell proliferation in the distal mesenchyme of the rodent limb bud (Tsuiki and Kishi, 1999). Protein content was decreased in whole embryos at gestation days 12, 13, and 14 after treatment with RA (Tsuiki and Kishi, 1999). Test doses of *all-trans* RA were administered orally to the pregnant rats on (Day 12 of pregnancy) ranging from 50mg/kg to 100mg/kg. Cell death was monitored in the prechondrogenic areas of the humerus, ulna and radius. This study concluded that the decrease of protein content in the limb bud was related to the limb defects seen after treatment with *all-trans* RA.

Through further research, Paulsen et al. (1994) examined the specific effects of *all-trans* RA on chick limb bud mesenchymal cells in serum-free cultures. This study showed that the role of *all-trans* RA was related to the position and autonomous differences of mesenchymal cells. The primary interest was whether or not *all-trans* RA acted directly on limb mesoblasts or whether *all-trans* RA acted indirectly by altering the function of the ectoderm. The experiment was done in serum-free cultures, which allowed *all-trans* RA to promote the inhibition of chondrogenesis (Paulsen et al., 1994).

The findings suggested that distal and sub-distal cells of the limb bud generally showed greater stimulation and less inhibition of chondrogenesis and growth by *all-trans* RA than that of isolated cells from proximal limb regions (Paulsen et al., 1994).

Cellular Retinoic Acid Binding Proteins (CRABPs)

CRABPs are significant factors in cytoplasmic retinoid uptake (Howard et al., 1990). CRABPs form a gradient across the neuroblasts of the chick limb bud, which opposes the gradient formed by retinoic acid (Howard et al., 1990). While this study suggested CRABPs bind with retinoic acid, it is unknown if this process is obligatory for teratogenic activity (Howard et al., 1990).

Retinoid Receptors

Retinoid receptors compromise two distinct subfamilies of the nuclear receptor family, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Leid et al., 1992). Each receptor family is comprised of the members, α, β, and γ. RARα is present ubiquitously through the limb bud and its transcripts are found in the ectoderm and the mesoderm (Underhill and Weston, 1998). In micromass cultures, RARα was found to be down-regulated during chondrogenesis (Cash et al., 1997).

Although the actual teratogenic mechanism of *all-trans* RA is not yet known, past studies have indicated that the most of the observed teratogenic effects of *all-trans* RA arise during gene transcription. In particular, Cash et al. (1997) conducted a time-response study of *all-trans* RA, in which defects were found in rodent digits during embryonic treatment on day (E)9 and E10. During E11-E14, limb defects were found during mesenchymal condensation, due to changes in the apical ectodermal ridge (AER).

However after exposure to *all-trans* RA on gestation day E14, no observable effects were found on limb development.

The results of previous *in vitro all-trans* RA studies, such as the Cash et al. (1997) study, demonstrate the need to further investigate the actual mechanisms involving *all-trans* RA's potential teratogenicity. Knowledge of the effects of *all-trans* RA on specific growth factors during a time-response experiment using an *in vitro* chick limb bud culture would be novel. One growth factor of concern in this study is TNFα. To date, studies using rodent cell lines have suggested that exposure to retinoic acid resulted in the release of TNFα (Kawase et al., 1994).

TNFα Tumor necrosis factor α (TNFα) is a cytokine with the ability to act as a differentiating agent on various cells (Chambaut-Guerin et al., 1995). TNFα is a member of a group of cytokines that markedly influence mineral metabolism (Mundy, 1992). Members of the tumor necrosis factor family, specifically vascular endothelial growth inhibitor (VEGI), are endothelial cell-specific genes and inhibitors of endothelial cell proliferation and tumor growth (Yu et al., 2001). VEGI has been shown to mediate the activity of G1 arrest in G0/G1 cells and programmed death in proliferating cells (Yu, et al., 2001).

Whether acting alone or in conjunction with other proteins, these cytokines influence the rate at which bone cartilage is formed and destroyed (Mundy, 1992). TNFα has been detected in the nervous system and is believed to be a factor during mammalian development; however, its functions are poorly understood (Chambaut-Guerin et al., 1995).

Two cell-surface receptors, TNFR-1 and TNFR-2 have been identified for TFNα and are thought to initiate the biochemical actions of TNFα. Several comparisons of TNFα actions in TNFR-1 versus TNFR-2 knockout mice concluded that TNFR-1 was the major receptor responsible for increasing expression of genes associated with inflammation (Chambaut-Guerin et al., 1995).

TNFα is also a mediator of bone destruction in some disease states (Mundy, 1992). Specifically, TNFα has been implicated in tissue damage that occurs in the joints of patients with rheumatoid arthritis (Mundy, 1992). Rheumatoid arthritis is associated with local overproduction of TNFα and inhibition of TNFα ameliorates experimental arthritis (Campbell et al., 2001).

Within normal human fibroblasts, the growth stimulatory action of TNFα increases in a dose-dependent manner and plateaus when optimal TNFα dosage is reached (Sugarman et al., 1985).

While TNFα may be growth stimulatory to some cells, it may be cytotoxic to others (Mundy, 1992). TNFα has also been shown to increase collagenase and prostaglandin E_2 production by chondrocytes (Mundy, 1992). Finally, TNFα added to culture media of mouse germ cells stimulates proliferation (Kawase et al., 1994).

The present study evaluated *all-trans* RA within a chick embryo limb bud micromass system. The chick embryo limb bud micromass system has been used as both an alternative test for teratogenicity and a screening test for environmental contaminants (Brown, 1985; Kanti and Smith, 1997). The micromass system measures parameters of embryonic cellular development (Wiger et al., 1988). *In vivo* limb bud development involves a cartilaginous stage that is characterized by the biosynthesis of both collagen

and cartilage proteoglycans, while *in vitro* limb bud systems measure the effects of chemical treatments on synthesis and deposition of cartilage proteoglycans (Wiger et al., 1988).

Compared to rodent embryos, chick embryos have advantages when assessing developmental hazards in micromass cultures (Wiger et al., 1988). Advantages of chick embryos include: (1) reduction of the use of mammals in teratogenicity testing; and (2) chick embryos have fore and hind limb buds containing equivalent measurements of prechondrogenic mesenchyme during staging (Wiger et al., 1988). Testing from the present study utilized the fore limbs of the chick limb bud.

SUMMARY

Although much research has been conducted on the effects and mechanisms of *all-trans* RA on development, there remains a lack of understanding of the normal, complex mechanisms and interactions needed for normal limb development. Until these normal mechanisms are understood, logical strategies for preventing birth defects cannot be developed. Investigation into the mechanism of limb teratogenicity from *all-trans* RA exposure can provide information about normal and abnormal development. One approach is to monitor effects of *all-trans* RA on various growth factors. This study investigates that the effects of *all-trans* RA on TNFα, cell proliferation and proteoglycan content during limb development. Interest is given to growth factors and biological markers because their regulation by retinoids is poorly characterized (Seewaldt et al., 1999). Interest is also given to TNFα, because it has been found to have a unique and pivotal role in regulating the choice between pro-apoptotic and anti-apoptotic signaling

pathways, and in the control of cell proliferation and inflammation (Baud and Karin, 2001).

This research project determined if the teratogenicity of *all-trans* RA is correlated with a change in TNFα, alterations in cell proliferation and proteoglycan abundance. TNFα was monitored given previous studies that found it to have a growth stimulatory effect within normal human fibroblasts in a dose-dependent manner (Sugarman et al., 1985). Data collected from this study will be compared to previous *in vitro* studies and their findings. In one such *in vitro* study, Paulsen et al. (1994) concluded that *all-trans* RA exposures had adverse effects on limb patterning. *All-trans* RA exposures also caused a dose-dependent inhibition on chondrogenesis (Paulsen et al., 1994). Similarly, Wiger et al. (1988) found that *all-trans* RA inhibited proliferation, protein synthesis and cartilage proteoglycan synthesis in micromass cultures, thus, further confirming *all-trans* RA as a potent inhibitor of proteoglycan synthesis. The Wiger et al. (1988) study also resulted in *all-trans* RA inhibiting cartilage production 500 times more than cell proliferation. Finally, results from the present study will examine if that trend continues in our chick embryonic micromass culture system and examine the effects of *all trans* RA treatment on TNFα.

Hypothesis. *All-trans* RA affects *in vitro* chondrogenesis through a TNFα-mediated mediated pathway that subsequently affects proteoglycan content and cell proliferation.

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CHAPTER 3

EFFECTS OF *ALL-TRANS* RETINOIC ACID ON *IN VITRO* CHONDROGENSIS OF CHICK MESODERMAL CELLS [1]

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ABSTRACT

Retinoids, derivatives of Vitamin A, are valuable in the treatment of certain skin disorders and cancers; however, their therapeutic application is limited by their potential teratogenicity. To date, mechanisms involving retinoic acid's teratogenicity are largely undetermined. Mesodermal cells grown *in vitro* differentiate into chondrocytes and produce cartilage-specific compounds such as proteoglycans. The purpose of this study was to determine the effects of *all-trans* retinoic acid (RA) on growth and differentiation of chondrocytes. Micromass cell culturing techniques were performed in which mesodermal limb bud cells were dispersed into a single cell suspension, placed into cell culture dishes, and incubated with *all-trans* RA for a 120 hr period. After incubation, cells were fixed and stained for proteoglycan content with Alcian green and optical density (OD) determined at 600 nm. Subsequently, cultures were stained for cell proliferation using crystal violet (OD=570nm). The absorbances of stained cells were compared to vehicle controls (VC). Results indicated a dose-dependent inhibition of proteoglycan content and cell proliferation at concentrations ranging from 10^{-9}-10^{-6}M. After treatment with 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M RA, proteoglycan content decreased to 49.8%, 39.3%, 28.6%, and 15.6% of VC, respectively. Cell proliferation decreased to 84.1%, 75.6%, 70.3%, and 69.3% of VC, respectively. Temporally, RA decreased cell proliferation earlier (83% of VC at 48 hrs) than proteoglycan content (20% of VC at 72hrs). Using an ELISA, TNFα was determined in cell culture medium after treatments with 10^{-9}-10^{-6}M *all-trans* RA. Results from the

ELISA assay show a minimal detectable level of TNFα within 120 hr in chick embryonic cell cultures. When treated with *all-trans* RA, the greatest decrease in TNFα was seen at the highest concentration of *all-trans* RA (10^{-6}M) ($p<0.05$). While a dose-dependency was not found, a threshold response was found; *all-trans* RA 10^{-8}M had a stimulatory effect on TNFα ($p<0.05$). When added directly to culture media, TNFα had a stimulatory effect on protein content.

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INDEX WORDS: Micromass cultures, Limb development, Retinoic Acid, In vitro Chondrogenesis, TNFα

Introduction *All-trans* retinoic acid (*all-trans* RA) is a known developmental toxicant and is present in prescription drugs such as Accutane, a medication for cystic acne. Due to the teratogenicity of its components, Accutane is not prescribed for pregnant women. Women of childbearing age must provide their physicians with proof of birth control to receive the acne medication.

Past studies have shown *all-trans* RA is a necessary component for normal mammalian development (Underhill and Weston, 1998). These studies have also shown that *all-trans* RA adversely affects limb development in rodent and chick embryos. Reports from Tanaka et al. (1996) show inhibition of endogenous *all-trans* RA resulted in truncated cartilage elements and defects in organ culture systems. In contrast to their findings, Tanaka et al. (1996) also reported treatment with exogenous RA caused phocomelic limbs within the chick. Because *all-trans* RA is necessary in normal development but teratogenic if found in excess, understanding the normal and teratogenic mechanisms of *all-trans* RA could provide for therapeutic intervention.

While the actual mechanism of *all-trans* RA's teratogenicity is unknown, its effects on major components of the developing limb are well documented. *All-trans* RA generally enhances cell differentiation of a variety of cell types (Seewaldt et al., 1997). It has been hypothesized that biochemically, *all-trans* RA influences the developing limb via its cellular retinoid binding proteins (CRABPs) and its nuclear receptors (RARs, RXRs), ultimately inhibiting the outgrowth of the apical ectodermal ridge (AER) (Seewaldt et al., 1997). The AER consists of a narrow ridge of tall epithelial cells. Many scientists believe the AER is the most fundamental aspect of limb bud development.

Furthermore inhibition of the outgrowth of the AER may lead to distal limb deficiencies and apoptosis (inappropriate cell death) (Seewaldt et al., 1997).

TNFα is a cytokine with the ability to act as a differentiating agent on various cells (Chambaut-Guerin, 1995) and is a member of a group of cytokines that markedly influence mineral metabolism (Mundy, 1992). TNFα elicits a particularly broad spectrum of organismal and cellular responses including cell proliferation, cell differentiation, apoptosis, lymphocyte and leukocyte activation and migration, fever, acute phase response (Tracey and Cerami, 1993). Whether acting alone or in conjunction with other proteins, these cytokines influence the rate at which bone and cartilage are formed and destroyed (Mundy, 1992).

TNFα has been detected in many physiological systems and is believed to be a factor during mammalian development; however, its functions are poorly understood (Chambaut-Guerin, 1995). TNFα added to culture media of mouse germ cells stimulates proliferation (Kawase et al., 1994). While TNFα may be growth stimulatory to some cells, it may be cytotoxic to others (Mundy, 1992). TNFα has been shown to increase collagenase and prostaglandin E_2 production by chondrocytes (Mundy, 1992). Collagenase production is associated with tumor and arthritic processes, however in normal tissues is essentially restricted to bone formation (Jimenez et al., 2001).

The chick embryo limb bud micromass system has been used as both an alternative test for teratogenicity and a screening test for environmental contaminants (Brown, 1985; Kanti and Smith, 1997). The micromass system can be used to measure parameters of embryonic cellular development (Wiger et al., 1988). *In vivo* limb bud development involves a cartilaginous stage which is characterized by the biosynthesis of

both collagen and cartilage proteoglycans. *In vitro* systems can be more easily controlled allowing measurement of collagen and cartilage proteoglycans, and the effects of chemical treatments on synthesis and deposition of cartilage proteoglycans (Wiger et al., 1988).

Using the micromass culture system, this study investigates the effects of *all-trans* RA on proteoglycan content, TNFα, and cell proliferation. Further insight on *all-trans* RA's teratogenic activities will assist in designing better therapeutic drugs, and hopefully, preventing RA-induced birth defects in the future.

Methods

Tissue Culture Tissue cultures were prepared as previously described (Kanti and Smith, 1997). Briefly, eggs were obtained from a local hatchery and incubated for 5 days in a humidified, 37°C incubator. Chick embryos were removed from the eggs at stage 25 (Hamburger and Hamilton, 1951) and distal tips dissected from the chick wing bud. Mesenchymal cells were obtained by treating the wing buds with 1:3 trypsin (Sigma EDTA solution 1X)/tyrode's solution for 90 seconds and rinsed with a 1:3 serumax (Atlanta Biologicals, Norcross, GA)/Tyrode's solution. Cells were then dissociated by gently aspirating wing buds through a flamed bore glass pipette. Cells were filtered through two layers of Nytex (20μm mesh), and adjusted to a final concentration of 1.0×10^7 cells/ml. Cells were then plated onto a 48-well tissue culture plate (Corning, Corning NY) with 30μl aliquots (high density spot cultures), incubated for 3 hrs at 37°C in a humidified atmosphere of 5% CO_2, 95% air before adding 1.0 ml of appropriate test solutions and media. Medium consisted of Hams F12 with Hepes media (Sigma, St. Louis, MO), 0.005% gentamicin, 0.05% ascorbate (Sigma, St. Louis, MO),

and 6.8% Serumax (Atlanta Biologicals, Norcross, GA). One half of the medium was replaced daily with fresh medium for a maximum period of 120 hrs under conditions conducive for chondrogenesis. After the appropriate time period, cultures were removed from the incubator, medium was removed and cells prepared for future analysis.

Treatments For dose-response experiments, cell cultures were treated with *all-trans* RA in 0.85% ethanol (Kulyk and Hoffman, 1996). *All-trans* RA was dissolved in 100% ethanol and subsequently diluted to 0.85% in medium. Concentrations ranged from 10^{-9}- 10^{-6}M *all-trans* RA. Test solutions were applied to cultures on day 0 after cells attached to the culture dish. Finally, half of the medium with appropriate test dose was replaced daily (for a period of 120 hrs). 120 hr. period was chosen based on past studies performed by Wiger et al. (1987), and Smith and Kanti (1997). For time-course experiments, *all-trans* RA test doses ranging from 10^{-9}-10^{-6} M were applied to cell cultures for 24, 48, 72, 96 or 120hr periods. For histochemical analysis, at 24 hr intervals, cell cultures were removed from incubation, fixed in formalin, and stored at 4°C.

Histochemistry Alcian green staining of proteoglycans, as a measure of chondrogenesis, was performed according to the methods of Bjornsson (1993), as modified by Kanti and Smith (1997). Cell proliferation was evaluated in the same culture by crystal violet staining (Renault et al., 1989 and Anderson et al., 2001). After removing the formalin, the cultures were rinsed for 3 minutes with deionized water. A 0.5% alcian green (Sigma, St. Louis, MO) solution in 3% glacial acetic acid was added to the cell cultures for 15 minutes, followed by three rinses with tap water. Cultures were dried at room temperature and absorbance was determined at a wavelength of 600 nm in

the intact cell cultures using a spectrophotometer (Cambridge Scientific, Cambridge, MA) capable of reading 48-well plates. Subsequently, cultures were stained with 1% crystal violet (Sigma, St. Louis, MO) in 20% ethanol, rinsed with deionized water and the absorbance measured spectrophotometrically at 570 nm.

TNFα After culturing, cells were freeze thawed in Hams F12 media to lyse the cells. TNFα was determined using a commercially available ELISA kit (Cayman Chemicals, Ann Arbor, MI). Briefly, 100 μl aliquots of TNFα or test samples were added to each well. Following, 100 μl of acetylcholinesterase was added to standards, test sample, and controls. The ELISA plate was then covered and incubated overnight at 4°C. After incubation, the wells were emptied and rinsed six times with 200 μl per well of Ellman Reagent (Cayman Chemicals, Ann Arbor, MI). The plate was then incubated for 2 hrs for optimal detection and absorbance determined using a multi-well microplate reader spectrophotometer at 406-420 nm.

Statistics Analysis of variance (ANOVA) was used to determine significant differences among test groups. Differences between concentrations were determined using a Scheffe's and Tukey's test. All analyses were performed using Statistical Analysis Software (SAS version 6.08 CMS).

Results

Proteoglycan content and cell growth Proteoglycan content showed a dose dependent decrease after cell cultures were treated with *all-trans* RA at concentrations ranging from 10^{-9}-10^{-6}M, when compared to vehicle control and non-treated control. The vehicle control exhibited significant increases ($p<0.05$) in proteoglycan content of 38.3%. *All-trans* RA (10^{-9}M, 10^{-8}M, 10^{-7}M, and 10^{-6}M) decreased proteoglycan content to

49.8%, 39.3%, 28.6%, and 21.5% of vehicle control, respectively (Figure 3-1). The greatest inhibitory effect on proteoglycan content was found at the highest concentration tested (10^{-6}M) (Figure 3-1). When compared to non-treated controls, exposure to *all-trans* RA 10^{-6}M resulted in a decrease in proteoglycan content to 34.6% ($p<0.05$).

For cell proliferation, effects of *all-trans* RA were not as profound. As with proteoglycan content, vehicle control (0.85% ethanol) increased cell proliferation by 24.4% ($p<0.05$). After treatments with *all-trans* RA concentrations, cell proliferation decreased from approximately 15-30% in a dose-dependent manner. The greatest effect on cell proliferation was seen after treatment with the highest dose of *all-trans* RA tested (10^{-6}M) ($p<0.05$) (Figure 3-2).

Temporally, *all-trans* RA begin to affect proteoglycan content in the cell cultures between 48 and 72 hrs of treatment. After treatment with 10^{-6}M *all-trans* RA, proteoglycan content was decreased to 20% of vehicle control by 72 hrs ($p<0.05$) and proteoglycan content in these cultures did not increase between 72 and 120 hrs. All cultures treated with *all-trans* RA exhibited significant decreases in proteoglycan content when compared to both vehicle and non-treated controls at 120 hrs ($p<0.05$) (Figure 3-3).

Cell proliferation was also affected by treatment with *all-trans* RA. After treatment for 48 hrs with 10^{-6}M *all-trans* RA, cell proliferation was decreased to 83% of vehicle control. Figure 3-4 shows a dose-dependency of *all-trans* RA treatments on cell proliferation at every time interval, however, the greatest effect was seen at 120 hrs ($p<0.05$). Finally, time course data showed 10^{-9}-10^{-6}M *all-trans* RA decreased cell proliferation significantly when compared to vehicle and non-treated controls after treatment for120 hrs ($p<0.05$) (Figure 3-4). Overall analyses of temporal data show 10^{-9}-

10^{-6}M *all-trans* RA affects cell proliferation at an earlier stage than proteoglycan content during the 120hr culture period.

Vehicle control (0.85% ethanol) had a stimulatory effect on proteoglycan content and cell proliferation when compared to non-treated control cultures. However, data on the effect of varying concentrations of ethanol on proteoglycan content and cell proliferation show no statistically significant difference between concentrations ranging from 0.25% to 0.85% ethanol when compared to non-treated controls ($p>0.05$) (see Figures 3-5, 3-6). The concentration, 0.85% ethanol, used in treatments was determined based on the solubility of *all-trans* RA and research by Kulyk and Hoffman (1996). Dimethylsulfunyloxide (DMSO) was used a possible test vehicle at 0.4%; however, within this test system all trials resulted in massive cell death and abnormal tissue cultures (data not shown).

TNFα When added directly to culture medium daily for a 120 hr period at 0.5, 1.0 and 5.0 pg/ml, TNFα was significantly different ($p< 0.05$) when compared to control cultures within the micromass culture system (Figure 3-7). Results from the ELISA assay show a minimal detectable level of TNFα within 120 hr chick embryonic cell cultures (see Figure 3-8).

Within the ELISA assay, TNFα is detectable up to 250 pg/ml, however, the average TNFα found in control cultures was 5.25 pg/ml, while vehicle control cultures averaged 6.58 pg/ml. *All-trans* RA concentrations 10^{-9}-10^{-6}M averaged 5.39, 6.72, 5.60, 4.69 pg/ml respectively, demonstrating no clear dose-response pattern. Corresponding with the cell proliferation, proteoglycan content and time-course data, vehicle control (0.85% ethanol) increased the level of TNFα found on a pg/ml basis by 21% ($p<0.05$).

When analysis is based on the average pg TNFα per mg of total protein, the results did not change. 10^{-6}M *all-trans* RA decreased TNFα content to 69% of vehicle control and 86% of non-treated control ($p<0.05$) (Figure 3-8).

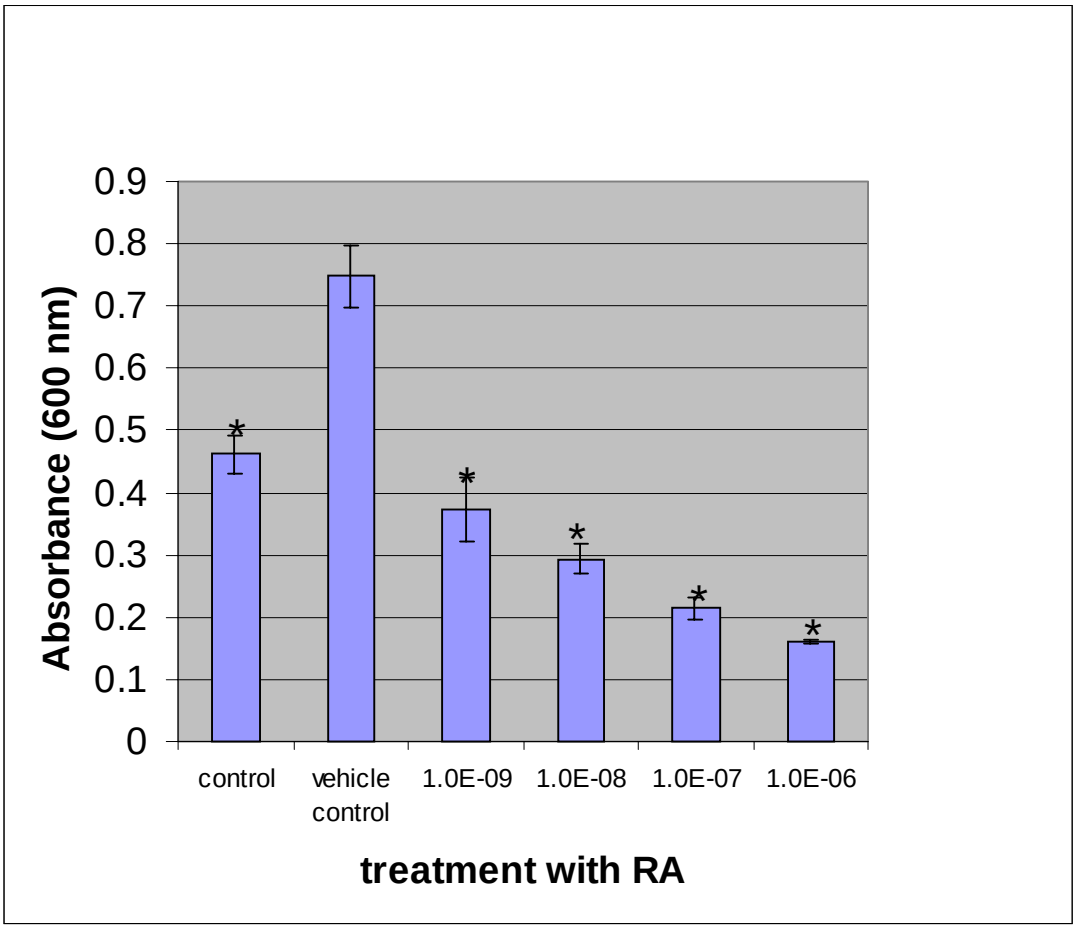

Figure 3-1. Effects of *all-trans* RA on proteoglycan content in tissue cultures of chondrocytes from chick embryonic limb buds. Significant differences ($p<0.05$) from vehicle control are denoted with an asterisk. Each point represents the mean of at least three experiments and within each experiment, each treatment was conducted in triplicate.

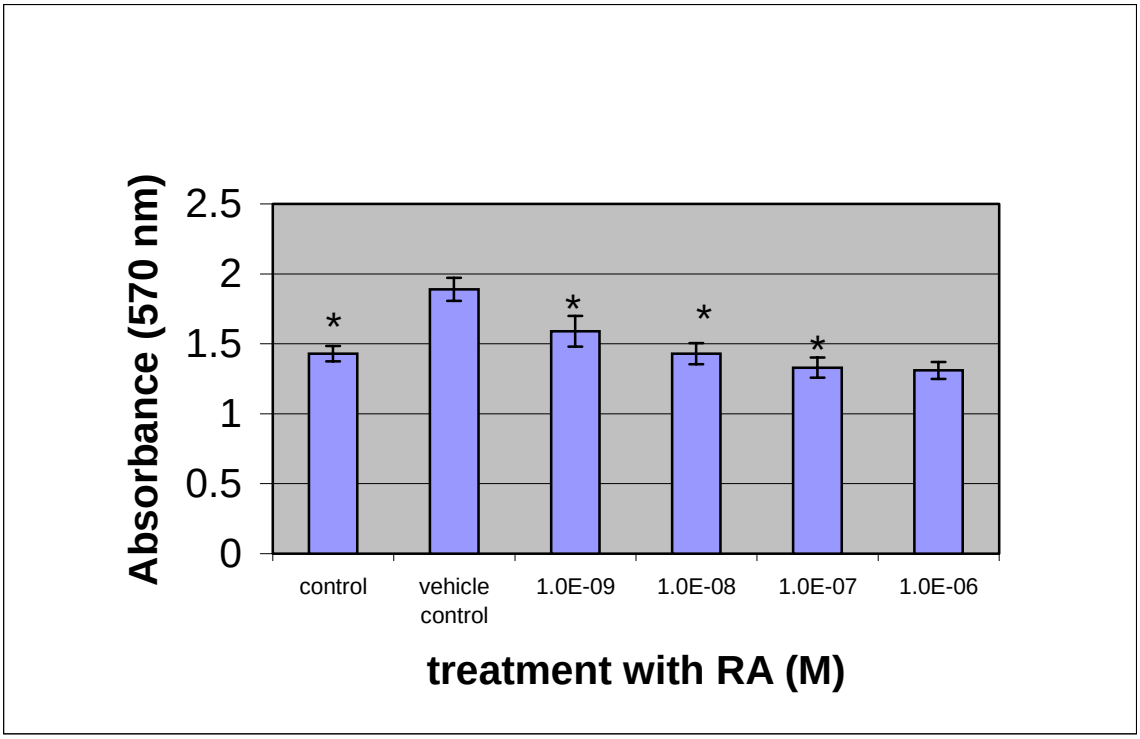

Figure 3-2. Cell proliferation in tissue cultures of chondrocytes from chick embryonic limb buds after treatment with *all-trans* RA. Significant differences ($p<0.05$) from vehicle control mean are denoted with an asterisk. Each point represents the mean of at least three experiments and within each experiment, each treatment was conducted in triplicate.

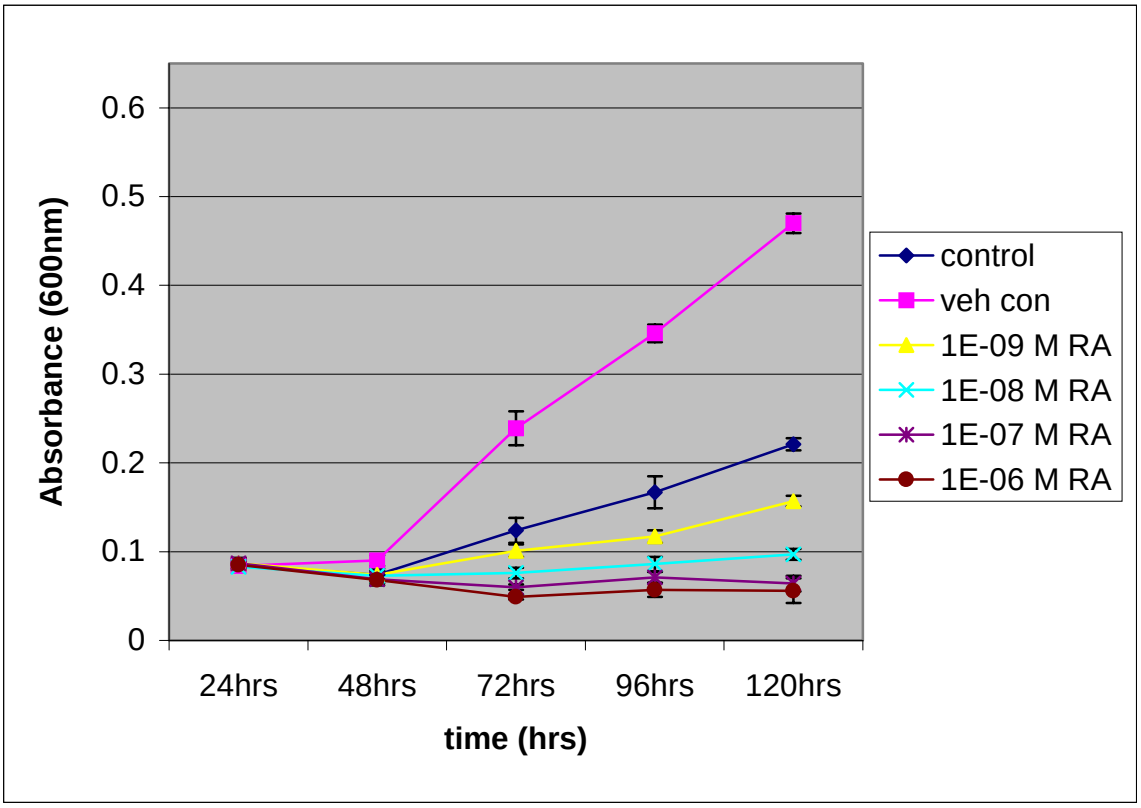

Figure 3-3. Time course of proteoglycan content after treatment of tissue cultures with varying concentrations of *all-trans* RA in media. Each experiment was repeated in triplicate.

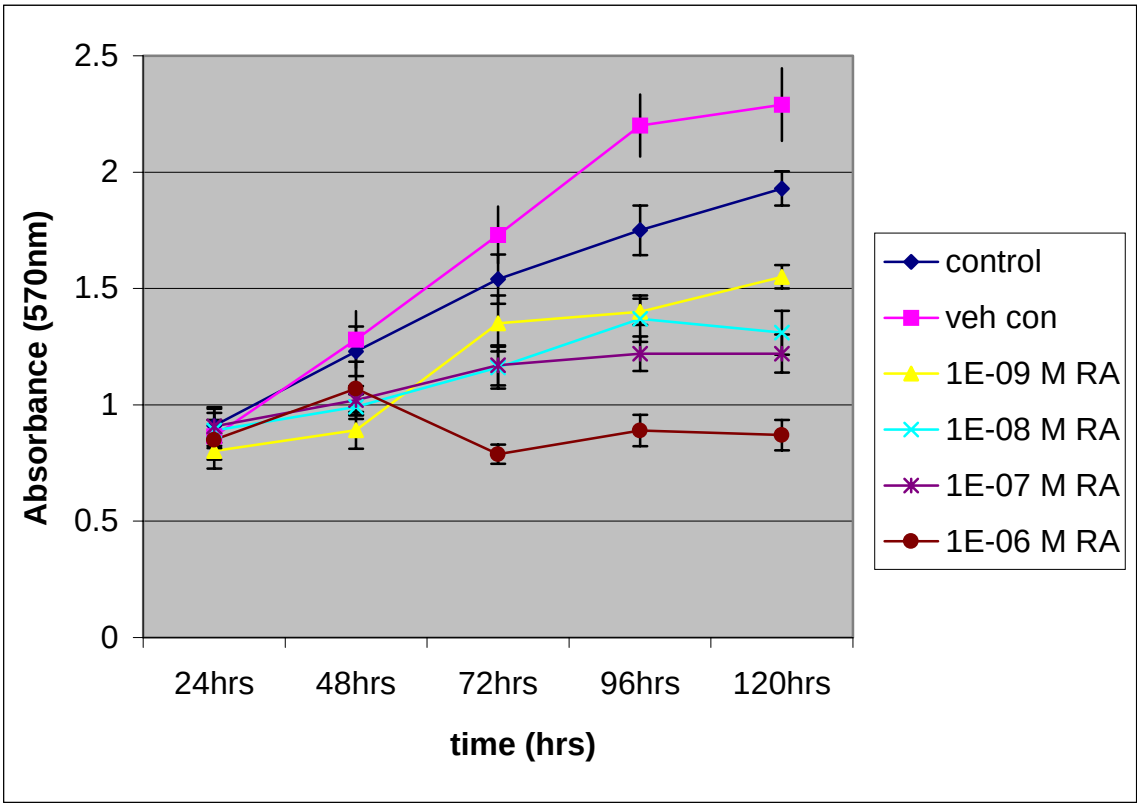

Figure 3-4. Time course of cell proliferation after treatment of tissue cultures with varying concentrations of *all-trans* RA in media. Each experiment was repeated in triplicate.

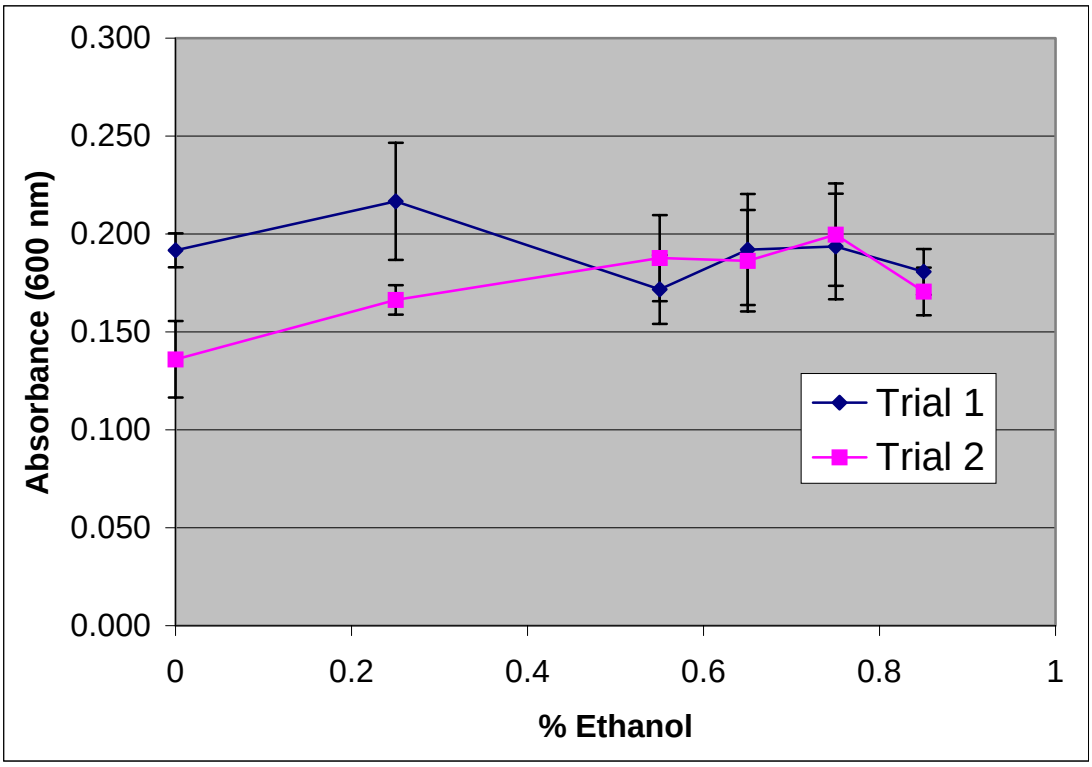

Figure 3-5. Effects of ethanol on proteoglycan content in tissue cultures of mesodermal cells from chick embryonic limb buds. n=2. Each concentration of ethanol was done in triplicate.

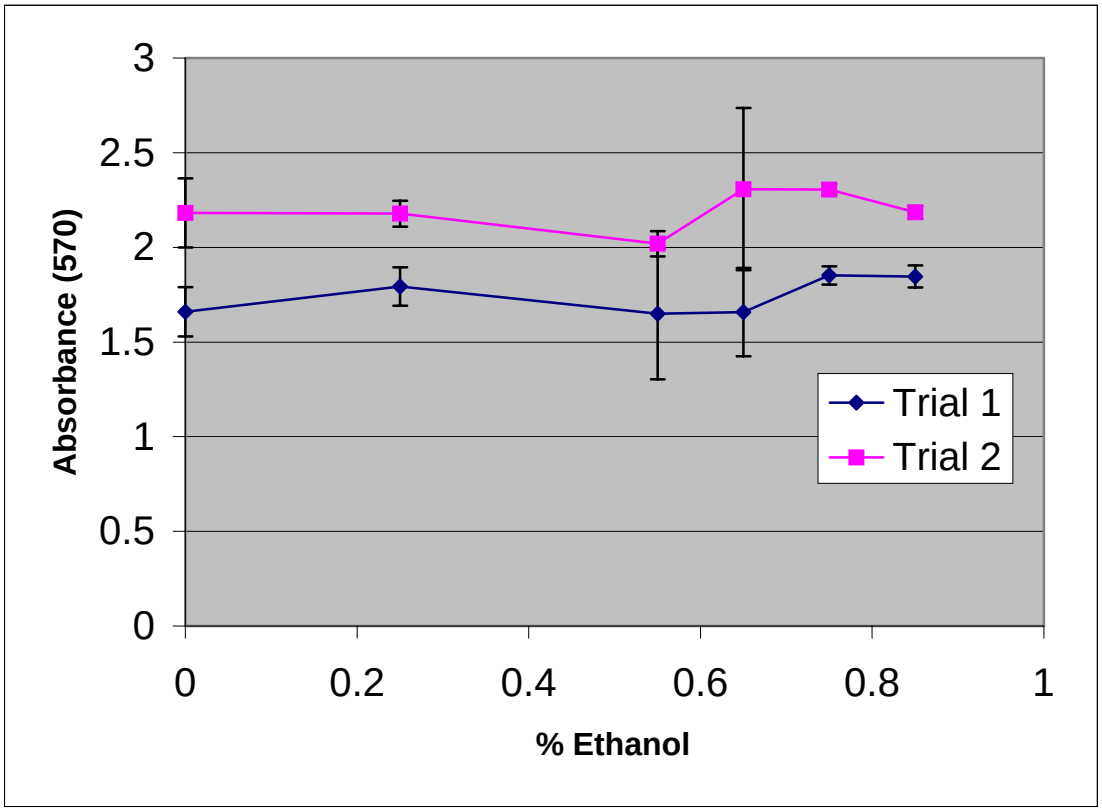

Figure 3-6. Effects of ethanol on cell proliferation in tissue cultures of mesodermal cells from chick embryonic limb buds. n=2. Each concentration of ethanol was done in triplicate.

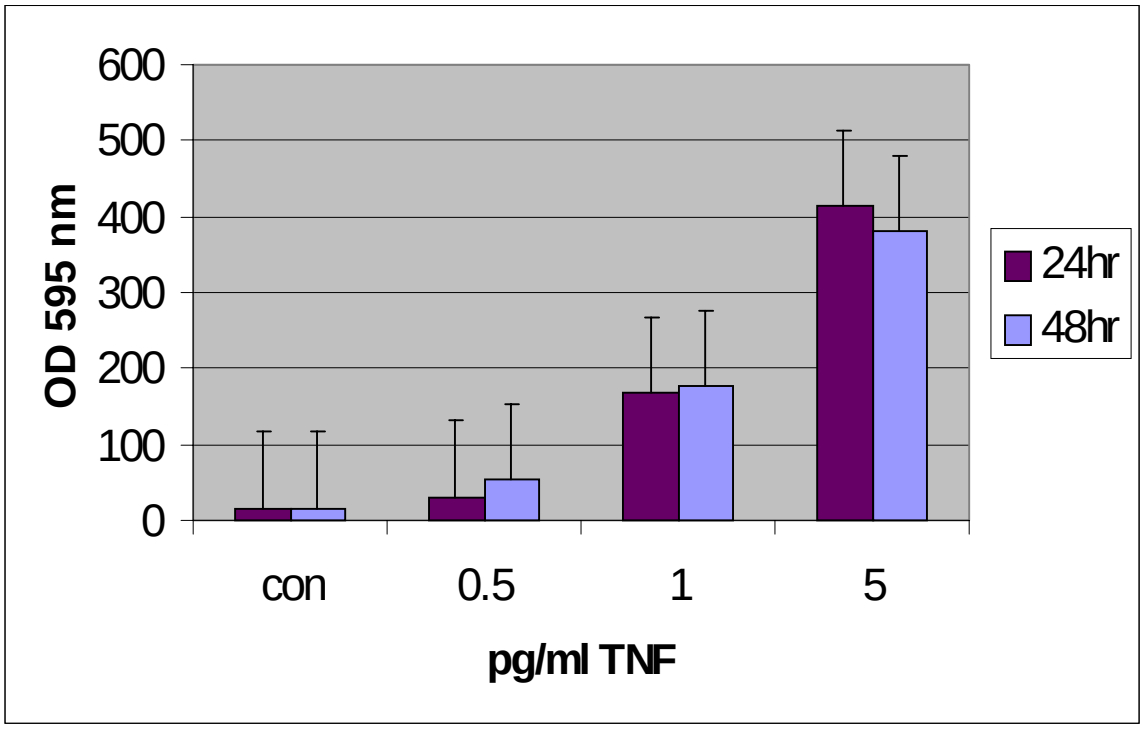

Figure 3-7. Protein determination of culture media extracts after treatment with TNFα.

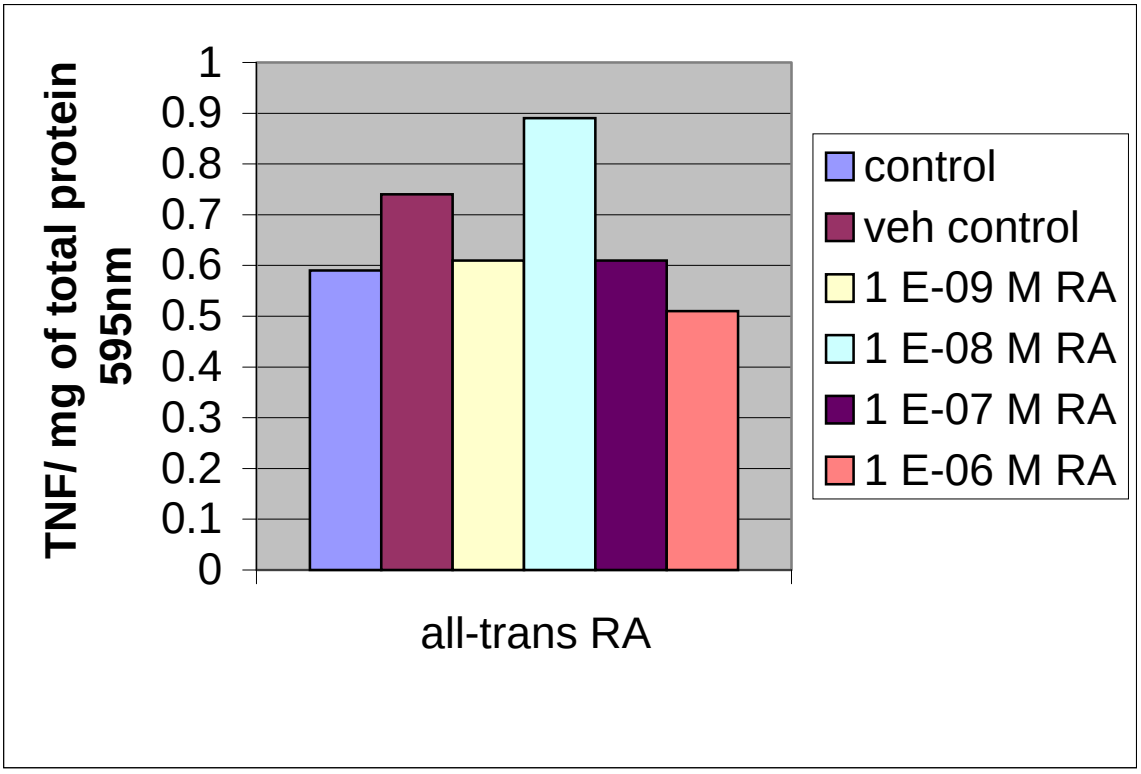

Figure 3-8. TNFα concentrations in cell cultures treated with *all-trans* RA. n=3. Average TNFα concentrations are recorded as pg of TNFα/ mg of total protein obtained from cell cultures. Each was repeated in duplicates.

Discussion Our study used alcian green and crystal violet to determine the effects of exogenous *all-trans* RA on proteoglycan content and cell proliferation of differentiating mesodermal cells obtained from embryonic chick limb buds. The techniques used during the present study were adapted from studies conducted by Renault et al. (1989) that established the proficiency and effectiveness of alcian blue and crystal violet to predict the teratogenicity of various chemicals to the skeletal system of rodent embryonic limbs. Based on studies performed by Smith and Kanti (1997) and Brown (1985) that determined the chick embryo limb bud tissue culture is comparable to the rodent tissue culture, alcian green and crystal violet were used during the present study as biomarkers of chondrogenesis in the chick limb bud cells.

Our data show a dose-dependent relationship of *all-trans* RA reduction of proteoglycan content and cell proliferation, thus illustrating the effects of exogenous *all-trans* RA on chondrogenesis. The data are in agreement with data from previous chick embryo studies (Ide and Aono, 1990), although the present study used broader concentrations of *all-trans* RA at 10^{-6}-10^{-9}M. Data from the present study are also similar to *in vitro* rodent studies performed by Tsuiki and Kishi (1999) and Wiger et al (1988), who found *all-trans* RA inhibited proliferation, protein synthesis and cartilage proteoglycan synthesis in micromass cultures. As stated previously, *all-trans* RA affected proteoglycan content more than cell proliferation, however, the differences in the inhibitory effect of *all-trans* RA on proteoglycan content compared to cell proliferation were not as great as the 500% inhibitory effect reported by Wiger et al. (1988).

Time-course data showed *all-trans* RA decreased cell proliferation earlier (by 48 hrs) than proteoglycan content (by 72 hrs), although the decrease was much greater for

the latter (Figures 3-3 and 3-4). The greatest inhibitory effects of *all-trans* RA were found at 120 hrs for both proteoglycan content and cell proliferation. Thus, chick limb bud cells were cultured to the 120 hr period in order to obtain the greatest effects of *all-trans* RA.

Vehicle control (0.85% ethanol) did have a stimulatory effect on proteoglycan content and cell proliferation, when compared to non-treated control cultures. However, proteoglycan content and cell proliferation were significantly lower in *all-trans* RA treated than in control cultures at all exposure levels except the lowest concentration (10^{-9}M) ($p<0.05$). This stimulatory effect of ethanol on cell growth was in agreement with data found of Kulyk and Hoffman (1996) who utilized ethanol concentrations from 1-4%. Data from the current study on effects of ethanol on proteoglycan content and cell proliferation did not show statistically significant difference between among concentrations of ethanol (0.85%).

Published evidence suggests that *all-trans* RA is a teratogen (Smith et al., 1983) although the teratogenic mechanism is not known. Thus, the present study examined the effects of *all-trans* RA on TNFα as a potential teratogenic mechanism. TNFα is present within the chick embryonic limb bud in our system. When added directly to culture medium, TNFα protein content of the cultures increased in a dose-dependent manner (Figure 3-7).

When treated with *all-trans RA*, overall pg/mg levels of TNFα were low. A dose-dependency was not found with *all-trans* RA affecting TNFα. *All-trans* RA dose 10^{-6}M had the greatest inhibitory effect on TNFα when compared to both treated and non-treated control ($p<0.05$). *All-trans* RA dose 10^{-8}M had the greatest stimulatory effect on

TNFα when compared to both treated and non-treated control ($p<0.05$). Based on the levels of TNFα recorded upon treatment with *all-trans* RA and the minimal detectable range of the ELISA assay, the results were not profound.

Given the actual mechanism in which *all-trans* RA is teratogenic has not been determined, future investigation should examine *all-trans* RA's effect on signaling pathways involved in bone formation and cartilage growth. Specifically, focus should be given to regulatory cascades initiated by *all-trans* RA and their effects on cell differentiation and chondrogenesis. Interest should also be given to the effects of *all-trans* RA on p38, a mitogen-activated protein kinase influential during bone formation.

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CHAPTER 4

CONCLUSION

Based on the results of this research, several conclusions can be drawn. In Chapter 3 it was shown that treatment with *all-trans* RA is correlated with a dose-dependent decrease in proteoglycan content and cell proliferation. These data are in agreement with previous chick embryo studies (Ide and Aono, 1990) although the present study used wider concentrations of *all-trans* RA at 10^{-6}-10^{-9}M. Cell proliferation was decreased earlier in the culture period than was proteoglycan content, however, proteoglycan content was decreased to a greater extent than was cell proliferation. Data found in the present study are also in agreement with *in vitro* rodent studies performed by Tsuiki and Kishi (1999) and Wiger et al. (1988).

It was also determined that 0.85% ethanol (vehicle control) exposures to the micromass system stimulated both proteoglycan content and cell proliferation when compared to non-treated controls. This finding was not unanticipated given the previous findings of Kulyk and Hoffman (1996). Significant changes were found with *all-trans* RA treatment when compared to either non-treated or vehicle control cultures.

This research project added supplementary information on TNFα activity within the chick limb bud system. TNFα was detectable within our test system. When treated with *all-trans* RA, the greatest decrease in TNFα was seen at the highest concentration of *all-trans* RA (10^{-6}M) ($p<0.05$). While a dose-dependency was not found, a threshold response was found; *all-trans* RA 10^{-8}M had a stimulatory effect on TNFα ($p<0.05$).

When added directly to culture media, TNFα had a stimulatory effect on protein content as was found during the Kawase et al. (1994) study.

Based on the results of this study, TNFα is detectable within the chick limb bud micromass system and can be used in future experiments as a biomarker when testing chemicals and their effects on limb development. The stimulatory activity of TNFα on protein content within this study in accordance with previous studies performed by Kawase et al., (1994). *All-trans* RA inhibits the expression of TNFα within the chick limb bud micromass system in a dose independent manner. *All-trans* RA continues to inhibit chondrogenesis within the micromass system, however, its exact route of teratogenesis is still unknown. Conclusions on *all-trans* RA inhibitory effects were reached based on its adverse effects on proteoglycan content and cell proliferation within the chick limb bud. The *all-trans* RA dependent TNFα pathway, we conclude, is one pathway in a cascade in which *all-trans* RA may be teratogenic. Further testing is needed concerning the effects of TNFα within the micromass system upon contact with *all-trans* RA. Future research on *all-trans* RA should investigate its effects on other cytokines and other mediators of apoptosis.

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Wiger, R., Stettum, A. and Brunborg, G. *Estimating chemical developmental hazard in a chicken embryo limb bud micromass system.* Pharmacology and Toxicology (1988) 62, 32-37.

APPENDIX

SOLUTIONS

Alcian green – 0.5%

0.5g of alcian green

100ml of 3% acetic acid

filter

pH 1.0

3% Acetic Acid

3ml of acetic acid

100ml of deionized water

Crystal Violet – 2%

2g of crystal violet

20ml of ethanol

80ml of deionized water

Alcian Green Stain Protocol

1- rinse with deionized water

2- rinse 3 minutes with 3% acetic acid

3- remove acetic acid

4- stain 15 minutes with alcian green

5- wash with deionized water 3 times

6- rinse for three minutes with 3% acetic acid

7- wash with deionized water

8- read, once dry, at 570nm

Crystal Violet Stain Protocol

1- rinse with deionized water

2- stain with crystal violet for 15 minutes

3- rinse with deionized water 3 times

4- read, once dry, at 570nm

Tyrode's

NaCl	8g
KCl	0.20g
MgCl, $6H_20$	0.21g
NaH_2PO4	0.055g
$NaHCO_3$	1g
Dextrose	1g
*$CaCl_2$	0.22g

1- weigh out all of the above ingredients and add all to a 2L flask except $CaCl_2$

2- add 900ml of sterilized deionized water to the 2L flask and mix on stir plate using an autoclaved stir bar

3- in a 100ml beaker, add 100ml of sterilized deionized water to the $CaCl_2$ and add it to the 2L flask after it is in solution

4- pH 7.0

5- filter 120 mls and place in sterile containers under refrigerated conditions

Media

Materials needed (all autoclaved) = 2L flask, stir bar, 1000ml volumetric, 1L of deionized water10 media bottles

Before beginning, weigh out 2.5g of sodium bicarbonate, remove one bottle of Hams F-12 Hepes Media from the refrigerator and calibrate the pH meter.

1- empty contents of Hams F-12 bottle into a flask

2- put stir bar into 2L flask

3- pour autoclave deionized water into 100ml volumetric, fill to 1000ml line

4- pour water from volumetric into 2L flask

5- do not pour the last 100ml (approximately) into the flask

6- turn on the stirrer and place the 2L flask on the stir plate

7- add 2.5g of sodium bicarbonate to 2L flask (the color will change from orange to pink in the flask)

8- pour last ~ 100ml of water from the volumetric into the Hams F-12 bottle, shake and pour into 2L flask (this gets the Hams residue from the bottle into flask)

9- let mixture stir for 3-5 minutes

10- follow the measure protocol for the pH meter and determine the pH of the mixture in 2L flask (want 6.85-6.88)

11- record this pH in your lab notebook

12- fill approximately 50ml of mixture under the hood using the vacuum Corning filter

13- pour into a 50ml centrifuge tube

14- follow the measure protocol again and take the pH of the post-filtered mixture (want 6.90-7.01)

15- record this pH in your lab notebook

16- filter the rest of the mixture and put Hams mixture into the media bottles, fill to 100ml line

17- label bottles with pH, date, initials, and Hams F-12

BSA Protein determination

1. Aliquot 50µl of BSA protein standard into 100µl of lysis buffer 9 (with protease inhibitors).
2. Aliquot 50µl of BSA protein standard into 150µl of lysis buffer 9 (with protease inhibitors).
3. Aliquot 50µl of BSA protein standard into 250µl of lysis buffer 9 (with protease inhibitors).
4. Aliquot 50µl of BSA protein standard into 450µl of lysis buffer 9 (with protease inhibitors).
5. Lyse samples on ice for 30 minutes with 50µl of lysis buffer and appropriate protease inhibitors.
6. Once samples have been lysed, aliquot 10µl drops of each standard and 10µl of samples onto a 48well plate for detection.
7. Add 1ml of Bio Rad dye to each well (both samples and standards).
8. After 10 minutes, read standards with the spectrophotometer at 600nm.