EFFECTS OF A SELECTED HETEROAROTINOID ON INDUCTION OF DIFFERENTIATION OF F9 MOUSE TERATOCARCINOMA CELLS

By

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LIST OF ABBREVIATIONS

A	adenine
ARAT	acyl-CoA:retinol acyltransferase
bp	base pair(s)
C	cytosine
9-*c*-RA	9-*cis*-retinoic acid
13-*c*-RA	13-*cis*-retinoic acid
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CoA	coenzyme A
CRABP	cellular retinoic acid-binding protein
CRBP	cellular retinol-binding proteins
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenedinitrilo-tetraacetic acid disodium salt
FBS	fetal bovine serum
G	guanine
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
HRA	heteroarotinoid
Kb	kilobase(s)
KDa	kilodalton

LB	Luria Broth
LRAT	lecithin:retinol acyltransferase
LWS-3	*(E)-p*-[2-(4,4-dimethylchroman-6-yl)propenyl]benzoic acid
M	molar
mg	milligram
mRNA	messenger RNA
mm	millimeter
nM	nanomolar
ng	nanogram
nm	nanometer
^{o}C	degree centrigrade
^{32}P	radioisotope of phosphorus
PBS	phosphate buffered saline
PDGF	platlet-derived growth factor
RA	retinoic acid
RAR	retinoic acid receptor
RARE	RAR response elements
RBP	retinol binding protein
RNA	ribonucleic acid
RT	room temperature
RXR	retinoid "X" receptor
RXRE	RXR respnsive elements
T	thymine
TAE	Tris-acetate electrophoresis
TE	Tris-EDTA
t-RA	all-*trans*-retinoic acid
TTNBP	*(E)*-4[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-hephthalenyl)-1-propenyl]benzoic acid

µl	microliter
U.V.	ultraviolet
V	volt(s)

CHAPTER I

INTRODUCTION

Cancer is a disease involving loss of cellular growth control and disruption of cell differentiation. Recent approaches to therapy for various types of cancer have focused on drugs that induce the differentiation of maturation-resistant cells causing the disease (Stevens et al., 1990). Some compounds such as retinoic acid, which inhibit the Epstein-Barr virus activation induced by tumor promoters (Tokuda et al., 1986; Nishino et al., 1988), have been shown to act as inhibitors of tumor promotion in vitro (Verma et al., 1980). Furthermore, it is known that retinoids can suppress the process of carcinogenesis in vivo in experimental animals and prevent human solid tumors (Sporn and Roberts, 1983; Hong et al., 1990; Lee et al. 1993). Clinical trials of differentiation-inducing agents in patients with leukemia, myelodysplastic syndromes, and solid tumors have included the use of *t*-RA and it's metabolites. These agents induce complete or partial remissions of some kinds of tumors (Breitman and He, 1990).

However, all of these retinoids exhibited considerable toxicity and the desired therapeutic potential has not been achieved (Roberts et al., 1994). Consequently, if new compounds could be found with good efficacy, but much less toxicity compared with most natural retinoids, a significant advance might be realized in the control of cell differentiation as related to cancer treatment. On the basis of such considerations, a group of new compounds named "heteroarotinoids" has been synthesized in recent years. Heteroarotinoids, which are modified on the basis of arotinoids, contain aromatic ring(s) and at least one heteroatom within the skeletal framework (Spruce et al., 1987). Recently,

heteroarotinoids have shown important anticancer activity and much lower or equal toxicity when compared to *t*-RA, 13-*c*-RA, and *(E)*-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-hephthalenyl)-1-propenyl]benzoic acid, also known as TTNPB (Spruce and Berlin, 1994). *(E)-p*-[2-(4,4-dimethylchroman-6-yl)propenyl]benzoic acid, also known as LWS-3, is a new compound of this heteroarotinoid family. Recent data have shown that it is approximately equal in toxicity to *t*-RA, but 630-940 fold less toxic than TTNBP (Spruce and Berlin, 1994). More interestingly, this compound has a binding affinity for retinoic acid receptors (Benbrook, 1995).

The objective of the present study was to evaluate the effect of the specific heteroarotinoid, LWS-3, on the induction of F9 cell differentiation. The F9 mouse teratocarcinoma cells were used as a model system to study cell differentiation and to monitor expression of the SPARC gene, a marker of differentiation of F9 cells (Gudas et al., 1994).

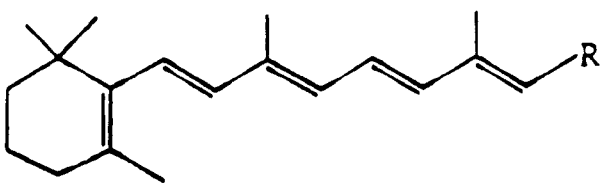
CHARTER II

LITERATURE REVIEW

Historical Perspective

The term, "retinoids" is a general term that refers to both naturally occurring compounds with vitamin A activity, including all-*trans*-retinol (vitamin A), retinal, and retinoic acid (Figure 1), and synthetic analogs, with or without the biological activity, (Sporn et al., 1984). The IUPAC-IUB Joint Commission on Biochemical Nomenclature in 1982 recommended that, "The term vitamin A should be used as the generic descriptor for retinoids exhibiting qualitatively the biological activity of retinol. This term should be used in derived terms such as vitamin A activity, vitamin A deficiency, vitamin A antagonist" (IUPAC-IUB Joint Commission, 1982).

The existence of an essential nutritional factor, later termed vitamin A, was reported and its physiological effects described long before the vitamin itself was isolated or its chemical structure determined. McCollum and Davis reported that a fat-soluble substance not only can promote rat growth but can also prevent xerophthalmia (McCollum and Davis, 1913). Drummond named this essential fat-soluble substance vitamin A in 1920 (Drummond, 1920). Subsequently, the determination of the chemical structure of β-carotene by Kerrer's group clarified the relationship between vitamin A in animals and provitamin carotene in plants (Karrer et al., 1930). Almost at the same time, Karrer et al. first determined the structure of retinol using a highly purified vitamin A extract obtained from shark liver oil (von Euler and Karrer, 1931). A decade later, Arens and Van Dorp succeeded in synthesis of (all-*E*)-retinoic acid (Arens and Van Dorp, 1946). More effort in



R	$= CH_2OH$	**Retinol**
	$= CHO$	**Retinal**
	$= CO_2H$	**Retinoic Acid**

Figure 1. The Naturally-occurring Retinoids.

the field of metabolism of natural retinoids accompanied by the rapid development of analytical methods, especially high-performance liquid chromatography (HPLC).

A more detailed review of the "classical" discoveries in the field of vitamin A research, including the original description of vitamin A, the recognition of carotenoid as a provitamin, the elucidation of the chemical structure and then the total synthesis of naturally occurring retinoids, unique pathology of both hypovitaminosis A and hypervitaminosis A in experimental animals and men, the fundamental role of vitamin A in vision, and the adequate nutrition in human and animals can be consulted in several references (Karrer and Jucker, 1950; McCollum, 1957; Isler, 1971; Wald, 1968; and Wolf, 1980).

In the late 1970s, the discovery of additional biological activities, such as growth promotion, reproduction, bone remodeling, and the induction of epithelial cell differentiation, led to rapid progress in the field of retinoids. Retinoids are prime candidates for cancer chemoprevention since they regulate cell proliferation and differentiation and cancer is associated with abnormal growth and loss of differentiation (Hicks, 1983). Within the last decade, the discovery of retinoid receptors as members of the steroid receptor superfamily of transcription factors has spurred molecular approaches in the study of retinoids. A variety of retinoid receptors have been discovered recently. These receptors are ligand-dependent transcriptional factors which can activate many genes, and repress the expression of other genes (Means and Gudas, 1995).

Retinoid-Binding Proteins and The Retinoid Receptors

In the late 1970s, Ong and Chytil first discovered a cellular retinoic acid-binding protein (CRABP). They demonstrated that the binding protein was a small cytoplasmic protein (M_r 15.5 kDa) that binds *t*-RA selectively and with high affinity (Kd = 4 nM) (Ong and Chytil, 1978). There are at least two species of this protein, CRABP I and CRABP II. Both proteins have been well studied and both genes have been isolated. These two closely related proteins show distinct patterns of expression in the adult as well as during

development (Giguere et al., 1990; Maden et al., 1988). Cellular retinoic acid-binding proteins are thought to prevent RA from interacting with the retinoic acid receptors. Overexpression of CRABP I can reduce the ability of RA to stimulate transcription, and also will decrease the rate of RA metabolism (Means and Gudas, 1995). The function of CRABP II is less clear. The induction of the CRABP II gene by RA suggested that CRABP II may function to sequester or degrade retinoids when high RA levels saturate CRABP I (Astrom et al., 1994). However, there is no direct evidence that CRABP can regulate transcription by *t*-RA. Some cells can respond to *t*-RA without the binding protein, and in these cells, *t*-RA can have effects that do not require CRABP (Mangelsdorf et al., 1990).

There is another type of protein that belongs to the retinoid-binding protein family, the cellular retinol-binding proteins (CRBPs). Like retinoic acid-binding protein, CRBPs are small and localized in the cytoplasm. CRBPs also have two species, CRBP I and CRBP II. Cellular retinol-binding proteins are thought to be involved in the metabolism of retinol into retinyl esters, or into retinadehyde and then retinoic acid (Levin, 1993; Napoli et al., 1991). Thus, the CRBPs may facilitate the synthesis of active retinoids in the embryo.

In 1987, Petkovitch et al. and Giguere et al. reported that they isolated a human orphan receptor cDNA which encoded the first known RA-activated transcription factor. This human RA receptor (hRAR-α) has a DNA-binding domain and ligand-binding domain, and can activate the transcription of target genes with *t*-RA at the effective concentrations in vivo (Petkovitch et al, 1987; Giguere et al., 1987). Evans later found that hRAR-α belongs to the family of nuclear receptors because the two domains of this receptor are structurally and functionally conserved with other members of the nuclear receptor superfamily (Evans, 1988). Now, three different subtypes of RARs (RAR-α, RAR-β, and RAR-γ) are known to exist in mammals, birds, and amphibians (Giguere et al., 1987; Benbrook et al., 1988; and Krust et al., 1989). The ligand-binding domains of

the RARs are highly conserved (>75% amino acid identity) suggesting that they all came from a common ancestral RAR gene (Mangelsdorf et al., 1994).

The second class of retinoid receptors, RXRs (retinoid "X" receptor) were discovered in the early 1990s (Mangelsdorf et al., 1990). cDNAs were isolated that encoded three RXR proteins, termed RXR-α, RXR-β, and RXR-γ. Like RARs, the RXRs are closely related in both their DNA-binding and ligand-binding domains, and act as sequence specific transcription factors for a variety of genes (Mangelsdorf et al., 1992; Leid et al., 1992).

The homologies of each class of retinoid receptor indicate that they likely bind common ligands and regulate common target sequences. The RAR protein can bind to *t*-RA and/or 9-*c*-RA (9-*cis*-RA). However, RXRs have a lower affinity for *t*-RA than do the RARs. RXRs exhibit a higher affinity for 9-*c*-RA, a geometric isomer of *t*-RA (Heyman et al., 1992). All the members of the retinoid receptor family regulate gene expression by binding to very similar DNA sequence elements called RAREs (RAR response elements) and RXREs (RXR response elements) (Umesono et al., 1988). These elements consist of tandem repeats of a six base pair consensus sequence (AGGTCA) separated by 1-5 base pairs of variable sequences. The strength of binding to each of the receptors is affected by the slight variations in these hexamer sequences and in the distance by which they are separated (Means and Gudas, 1995).

RARs and RXRs bind target genes and regulate transcription by forming homo- or heterodimers. RXRs not only can form a heterodimer with RAR but can also serve as a heterodimeric pattern with other members of the steroid hormone superfamily, such as the thyroid hormone receptor (TR), vitamin D receptor (VDR), and peroxisome proliferation activated receptor (PPAR) (Yu et al., 1991; Kliewer et al., 1992). The ability of RXR to bind other receptors heterodimers establishes a central role for RXR in hormonal signaling pathway. Mangesdorf et al. demonstrated two retinoid signaling pathways. RXR homodimer is stabilized to favor the activation of RXR target genes at high 9-*c*-RA

concentrations. When there is a low 9-*c*-RA concentration or a high RAR concentration or both, the RAR-RXR heterodimer is stabilized. Because the RXR-RAR heterodimer has a higher affinity for DNA than the RXR-RXR homodimer does, the heterodimer may outcompete the homodimer for binding to RXREs (Mangelsdorf et al., 1994).

In addition to receptor dimerization, the activity of the retinoid receptors may be modulated by other nuclear transcription factors, and in particular by AP-1. AP-1 is a complex of the proto-oncoproteins Jun and Fos, whose activity is stimulated by tumor promoters and growth factors. Recent studies suggest that RAR may form a nonproductive complex with Jun, thereby inhibiting AP-1 transaction. These results provide a possible mechanism for how RA can limit cell growth (Schule et al., 1991; Nicholson et al., 1990).

Every subtype of retinoid receptors has its distinct pattern of expression. In general, RAR-α, RXR-α, and RXR-β are all ubiquitously expressed and thus may mediate retinoid signaling, while RAR-β, RAR-γ, and RXR-γ genes exhibit more limited patterns of expression. The different patterns of expression suggest that individual subtypes of RAR and RXR families plays an important role in diverse aspects of development, from implantation of the embryo to organogenesis, limb development, and central nervous system differentiation as well as in adult physiology and metabolism (Mangelsdorf et al., 1994).

Metabolism of Retinol and Retinoic Acid

Metabolism of Retinol

All retinoids in the body originate in the diet either as plant carotenoid pigments, such as β–carotene, or as the long-chain retinyl esters found in animal tissue. Glover indicated two pathways of carotenoid conversion to retinol in mammals in 1960: 1. central oxidative cleavage of carotenoid to retinal, then reduction to retinol. Two soluble enzymes are involved in this process: β-carotene 15, 15-dioxygenase and retinaldehyde reductase, and 2. excentric oxidative cleavage through a series of β-apocarotenals to retinal,

followed by reduction to retinol (Glover, 1960). In intestinal mucosa, the first pathway is known to be the predominant one to retinol (Lakshman et al., 1989). The enzymes involved in excentric cleavage have not yet been reported.

Dietary retinyl esters are hydrolyzed in the intestinal lumen and intestinal mucosa. Two enzymatic activities have been clarified. One of them, with hydrolase activity that preferentially hydrolyzed short-chain retinyl esters, such as retinyl caproate. The other one with hydrolytic activity is most likely to hydrolyze long-chain retinyl esters, such as retinyl palmitate (Ong et al., 1987).

The retinol from the reduction of retinaldehyde or absorbed by the intestinal mucosa is then converted to retinyl ester to nascent chylomicrons for uptake into the body (Quick and Ong, 1990). Quick and Ong demonstrated that for the human intestinal Caco-2 cell line, esterification of retinol requires that retinol bind to CRBP II first. Thus, CRBP II plays a central role in directing and channeling dietary retinol into the general circulation (Quick and Ong, 1990).

After being transported into the lymphatic system, nascent chylomicrons undergo lipolysis catalyzed by lipoprotein lipase to convert them to chylomicron remnants (Goodman and Blaner, 1984). After entering the plasma, chylomicrons acquire the C and E apoliproteins from plasma high-density lipoproteins (HDL). The apoC II can activate the enzyme lipoprotein lipase, beginning with the hydrolysis of chylomicron core triglycerides resulting in the shrinkage of the chylomicron core and transfer of redundant surface components into HDL (Green and Glickman, 1981). Fatty acids produced by lipolysis can either circulate as free fatty acids bound to serum albumin or are taken up by adjacent tissues for oxidation or reesterification (Goodman, 1984). These processes form chylomicron remnants (Redgrave, 1983). Most chylomicron remnants are removed from circulation by the liver (Mahely and Hussian, 1991).

The liver plays a major role in the body in the uptake and metabolism of newly absorbed retinol (retinyl esters). In the liver, two different types of the cells are involved in

retinoid storage and metabolism: the parenchymal cells and steller cells (Blaner et al., 1985; Batres and Olson, 1987). The parenchymal cells are directly involved in uptake of chylomicron remnants and in the synthesis and secretion of RBP. The steller cells are major storage cells for retinyl esters in the liver (Hendricks et al., 1988). Hendricks et al. indicated that the retinyl esters of chylomicron remnants are first hydrolyzed in the parenchymal cells and then transferred to the steller cells for storage (Hendricks et al., 1988). Recently, Gad and Harrison reported that a retinyl ester hydrolase localized in the parenchymal cell plays an important role in hydrolysis of newly absorbed dietary retinyl ester. The hydrolysis is very quick after the chylomicron remnant is taken up by the hepatic parenchymal cell (Gad and Harrison, 1991). In addition to hydrolysis of retinyl esters, the parenchymal cells are the major cellular site for the synthesis and secretion of RBP in the liver (Yamada et al., 1987). Some dietary retinol may be directly secreted into circulation, bound to newly synthesized RBP. The remainder of the dietary retinol will be transported to the steller cells for storage (Yamada et al., 1987).

During hepatic storage and metabolism, retinyl esters are hydrolyzed and esterified to long-chain fatty acid esters by lecithin:retinol acyltransferase (LRAT) and acyl-CoA:retinol acyltransferase (ARAT) (Randolph et al., 1991). Randoph et al. demonstrated that when retinol and fatty acid CoA were present at the normal concentrations, LRAT played a predominate role in catalyzing the formation of retinyl esters. However, if the concentrations of retinol or fatty acid CoA are much higher, ARAT might play a larger role in retinol esterification (Randolph et al., 1991).

The questions of how the retinol is transferred between parenchymal cells, stellate cells, and plasma is still unsolved. Several hypotheses were proposed (Blaner and Olson, 1994). (1) The RBP is solely responsible for the intracellular transfer and release of retinoids in the cells. (2) An unidentified protein carrier plays a crucial role in intercellular transfer. (3) The retinol is transferred directly between perenchymal and stellate cells by means of membrane contacts.

Since the nuclear receptors for RA have been discovered, scientist have paid increasing attention to the processes that generates and maintains cellular RA levels. Blood plasma *t*-RA can be derived from dietary sources and from endogenous metabolism in tissues. Most cells and tissues seem to have the ability to oxidize retinol to RA. However, the biochemical process by which RA is enzymatically formed within tissues from the oxidation of retinol is still unknown. The current hypothesis is that retinol is first oxidized to retinaldehyde, then oxidized to RA (Blaner and Olson, 1994). Although the alcohol dehydrogenase (ADH) of liver can catalyze the oxidation of retinol to retinaldehyde (Zachman and Olson, 1961) and the aldehyde oxidase can convert retinaldehyde to RA (Frolik, 1984), the exact role of these two enzymes in the conversion of retinol to RA is unclear.

13-*c*-RA and 9-*c*-RA are metabolites of *t*-RA produced in vivo. These isomers are active in mediating RA function. Cullum and Zile indicated that 13-*c*-RA was a naturally occurring metabolite of *t*-RA. They injected a dose of *t*-RA intrajugularly into vitamin A-depleted rats and found 13-*c*-RA in the plasma and small intestine within 2 minutes after injection (Cullum and Zile, 1985). However, little is known about the enzymatic formation and catabolism of the 13-*c*- and 9-*c*-isomers.

Induction of Differentiation of F9 Teratocarcinoma Cells by Retinoids

Teratocarcinomas are malignant tumors composed of a large variety of sometimes highly organized and well-differentiated cell types derived from all three primary germ layers (Stevens, 1967). Characteristically, these tumors contain a rapidly dividing, undifferentiated cell population known as embryonal carcinoma cells which are the stem cells for the differentiated cells and which are responsible for the formation of the tumor.

Embryonal carcinoma cells have been isolated from primary cultures of cells derived from teratocarcinomas, and from these cells, several clonal cell lines have been developed. The relationships between embryonal carcinoma cells, early embryonic cells,

and teratocarcinomas have been established (Martin, 1981). These relationships indicate that the embryonal carcinoma cell system can serve as a model for the study of early embryonic development and the regulation of gene expression which is involved in this process.

F9 is a clonal embryonal carcinoma cell line which is mostly used as a model to study the control of differentiation by retinoids, since F9 stem cells show very low spontaneous differentiation into two stages of mammalian cell development (Strickland and Saway, 1980). Strickland and Mahdavi (1978) first showed that treatment with *t*-RA can induce differentiation of F9 cells very efficiently. This differentiation can be induced at concentrations of *t*-RA as low as 10^{-9} M. The process of F9 cell differentiation induced by *t*-RA can be divided into two stages. In the presence of *t*-RA, F9 cells differentiate first into endoderm cells with marked changes in morphology, stimulation of plasminogen activator secretion, synthesis of new types of collagen, and disappearance of the cell surface antigen SSEA-1 (Strickland et al., 1980; Evain and Anderson, 1981). After the first differentiation stage, the F9 endoderm cells have the potential to express the parietal endoderm phenotype in low density cultures or in the presence of dibutyryl cAMP, whereas in suspension or high density cultures they express the visceral endoderm phenotype (Hogan et al., 1981). However, in the absence of *t*-RA, elevation of cAMP levels has no effect on the differentiation of F9 cells (Strickland et al., 1980). Wang and Gudas (1983) have demonstrated that *t*-RA treatment of F9 cells causes an increase in the level of collagen IV and laminin B mRNA expression. Treatment with both *t*-RA and dibutyryl cAMP further elevates the mRNA level, whereas single dibutyryl cAMP has no effect. Retinoids have been found to be very effective inducers of differentiation of almost every embryonal carcinoma cell line tested (Jetten, 1986). It has been demonstrated that both retinoic acid and retinol can induce differentiation (Jetten and Deluca, 1983; Eglitis and Sherman, 1983).

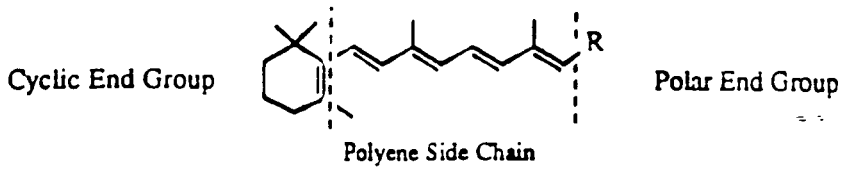
The levels of a variety of different transcription factor genes are altered by *t*-RA treatment of F9 cells. The SPARC gene is one of these transcription factor genes and it has been widely used as a marker to monitor the differentiation of F9 cells induced by *t*-RA (Gudas et al., 1994). Mason et al. (1986b) reported that the expression of the SPARC gene was increased approximately 20-fold in response to the *t*-RA and dibutyryl cAMP. In F9 stem cell differentiating into parietal endoderm in response to *t*-RA and dibutyryl cAMP there was a small decrease during the first 24 hours, followed by a 20-fold increase in SPARC mRNA levels over the next 5.5 days. They also indicated that the differentiation of F9 cells into parietal endoderm was accompanied by an increase in SPARC gene transcription (Mason et al., 1986b). The SPARC gene is positively regulated by *t*-RA via sequences in its promoter, although the exact sequences have not been identified (McVey et al., 1988).

The SPARC gene was cloned and the protein encoded by this gene, SPARC protein, was characterized in 1986. It is a M_r 43,000 secreted, acidic, cysteine-rich, calcium-binding glycoprotein homologous to 43K bovine endothelial 'culture shock' protein (Mason et al., 1986a). It is encoded by a single gene localized in the central region of mouse chromosome II (Mason et al., 1986b). Recently, this protein has been identified as a member of a group of proteins that exert anti-spreading effects on various cultured cells (Everitt and Sage, 1992). SPARC can induce the expression of type I plasminogen activator inhibitor (PAI-1) in cultured bovine aortic endothelial cells (Hasselaar et al., 1991), and can inhibit the binding of PDGF-BB and PDGF-AB complexes to the PDGF receptor on human dermal fibroblasts (Raines et al., 1992). Other transcription factor genes of F9 cells modulated by retinoids include the Hox 1.6 gene. Hox1.1 gene, jun Family gene, etc. (Means and Gudas, 1995).

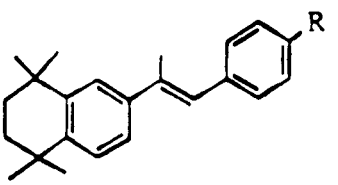
Synthetic Retinoids

The fundamental role of retinoids in controlling the state of cellular differentiation and preventing excessive proliferation raises the possibility that these compounds may be useful in inhibiting carcinogenesis, for the treatment of cancer, and for dermatological diseases such as psoriasis. Though retinoids might be valuable as noncytocidal chemotherapeutic agents, their high toxicity prevents their use conventionally or for a prolonged period. In addition, the retinoids thus far known are lipophilic, are very slowly eliminated from the body, cause serious prolonged toxicity, and in particular, teratogenic effects. Great interest has been generated in the last decade in the production of synthetic retinoids with better therapeutic effects and lower toxicities. The basic structure of retinoids consist of three portions: a cyclic end group, a polyene side chain, and a polor end group (Figure 2). Synthetic retinoids have been developed based on the alteration in one or more parts of the basic retinoid skeleton (Lippman and Meyskens Jr., 1988). Extensive chemical modification of retinoic acid led to the synthesis of arotinoids. The most extensively studied arotinoid is (*E*)-4-[2-(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-hephthalenyl)-1-propenyl]benzoic acid, also known as TTNPB. These compounds are very potent in several standard screening tests and in the treatment of keratinizing and hyperproliferative disorders including psoriasis (Tsambaos et al. 1990).

Heteroarotinoids (Figure 2) are a class of newly synthesized retinoids which normally consist of at least one aryl ring and a heteroarotom within the retinoid skeletal framework (Spruce et al., 1987). Several separate assays, including the tracheal organ culture (TOC) assay, the ornithine decarboxylase (ODC) assay and the ability to induce the differentiation of human promyelocytic cells, are usually used to examine the activities of synthetic retinoids. The heteroarotinoids have been shown to have remarkable activities in these standard assays (Dawson et al., 1984; Spruce et al., 1987). Preliminary studies also demonstrated that the heteroarotinoids (X=O, R=ET) supported growth and were active in reversing cornification of vaginal epithelial cells in ovariectomized vitamin A-deficient rats

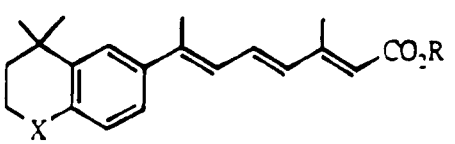


Basic Structure



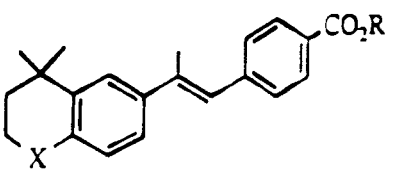
TTNPB (R-COOH)

Arotinoids



X = O, S
R = H, Et, etc.

Monoaryl Heteroarotinoids



X = O, S, NH, NMe, etc.
R = H, Me, Et, etc.

Diaryl Heteroarotinoids

Heteroarotinoids

Figure 2. Synthetic Retinoids

(Thorne et al., 1993). Compared with other retinoids, a diaryl heteroarotinoid (Figure 3), LWS-3 (X=O, R=H), was reported to be approximately equal in toxicity to *t*-RA but 630-940- fold less toxic than TTNPB. Thus, the discovery of retinoid nuclear-receptor-protein families and the identification of their cell specificities have promoted the search for receptor-selective retinoic acid analogs that would selectively bind to and then activate a particular receptor. Using 9-*c*-RA as a control, LWS-3 was reported to have affinity for the β,γ-receptors (Benbrook, 1995). It is appropriate to consider heteroarotinoids as a new selective ligand that can interact with specific receptors to regulate gene expression. The objective of the present study was to evaluate the effect of this heteroarotinoid, LWS-3, on induction of F9 cell differentiation.

CHAPTER III

EXPERIMENTAL METHODS

Preparation of Tissue Culture Plates

Before the F9 mouse teratocarcinoma cells were seeded, the tissue culture plates were coated with 0.1% sterile porcine skin gelatin solution. The 0.1% gelatin solution (Fisher) was autoclaved and allowed to cool to room temperature, and autoclaved again to destroy the possible fungal spores (Darrow et al., 1990). This sterile solution can be stored at 4 ^{o}C for several months. The sterile gelatin solution was added to tissue culture plates (Falcon), refrigerated for 2 hours at 4 ^{o}C, then the solution was removed by aspiration, and washed 3 times, using sterile distilled water. Treated plates can be stored at room temperature.

Maintaintance and Subculture of the Cells

The F9 mouse teratocarcinoma cell line was purchased from American Type Culture Company (ATCC CRL-1720). After thawing rapidly (within 40-60 seconds) in a 37 ^{o}C water bath, the cells were seeded at a density of 3 x 10^5 cells per 100 mm diameter cell culture plate and incubated in a 10% CO_2 incubator. Dulbecco's modified Eagle's medium (DMEM) containing 15% (v/v) fetal bovine serum and antibiotics (see Table 1) was used to maintain all F9 cell cultures. The medium was mixed well and sterilized through a 0.22 μm filter. After 3 days of growth, the cells were subcultured. The DMEM was removed by aspiration, 4-5 ml of fresh trypsin (0.25%)-EDTA (5 X 10^{-4} M) was added to rinse the cells for 1-2 minutes and then removed. The cell culture was kept at room temperature until

Table 1

Compositions of Cell Culture Medium for F9 Teratocarcinoma Cells

Stock solutions (Sigma)	Medium (1000 ml)
Dulbecco's Modified Eagle's Medium	850 ml
Fetal Bovine Serum	150 ml
Penicillin G (1575 units/mg)	63.4 mg
Streptomycin	100 mg
L-glutamine	0.584 g

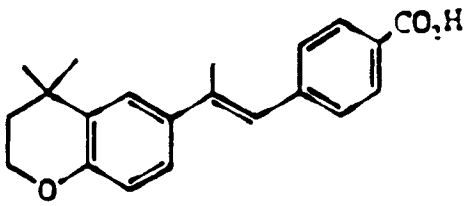
the cells detached from the plate entirely. Five milliliters of fresh DMEM was added and collected in a plastic conical centrifuge tube and centrifuged at about 300xg for 10-15 minutes. The pellet was resuspended in 2 ml of fresh medium. The culture was vigorously pipetted up and down using a 10 ml syringe with a 21.5 gauge needle. It was crucial to obtain a single cell suspension. A single cell suspension was seeded in 10 ml DMEM at a density of 3 x 10^5 cells per 100 mm diameter plate.

Preparation of Retinoic Acid and the Selected Heteroarotinoid

All-*trans*-retinoic acid, *t*-RA (Sigma), was stored as a powder at -20 ^oC in a lightproof container. It was dispensed in the dark and dissolved in 100% ethanol at a concentration of 3.0 mg/ml. The 3.0 mg/ml stock was diluted 1:10 in 100% ethanol and finally 1:100 in DMEM to prepare a 10^{-5} M stock solution. The heteroarotinoid *(E)*-P-[2-(4,4-dimethylchroman-6-yl)propeny]benzoic acid (LWS-3) was synthesized by Dr. Berlin's group in the Department of Chemistry, Oklahoma State University (Figure 3). A absolute ethanol used to dissolved the compounds has no effect on the morphological change of the F9 cells at the concentration below 0.1% (Benbrook, 1995). Like the preparation of retinoic acid, it was weighed in the dark to prepare a 10^{-2} M stock solution, diluted in 100% ethanol to get a 10^{-3} M and 10^{-4} M solution and then 1:100 in DMEM to prepare the 10^{-5} M and 10^{-6} M heteroarotinoid solutions. The maximum ethanol concentration in the culture medium was 0.1% with LWS-3 at 10^{-6} M with dilutions to 0.001% at 10^{-9} M. All the procedures after ethanol dilution were carried out under sterile conditions.

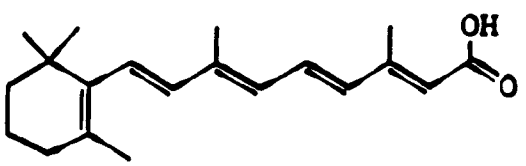
Induction of F9 Teratocarcinoma Cells

As a positive control for F9 cell differentiation into primitive extraembryonic endoderm, the cells were treated with 1 x 10^{-7} M *t*-RA at a density of 3 x 10^5 cells per 100 mm diameter plate. The heteroarotinoid (LWS-3) was tested on parallel plates using



LWS-3

(E)-p-[2-(4,4-dimethylchroman-6-yl)propenyl]benzoic acid



t-RA

All-*trans*-Retinoic Acid

Figure 3. The Structures of the Heteroarotinoid LWS-3 and All-*trans*-Retinoic Acid (*t*-RA)

concentrations from 10^{-6} M to 10^{-9} M. The F9 cells without *t*-RA or LWS-3 were used as negative control. After 84 hours incubation, the morphology of the F9 cell was investigated through a reverse-phase microscope.

RNA Isolation

Total RNA was isolated using the Chomezynski/Sacchi single step method (Chomezynski and Sacchi, 1987) with the following modification. The cell culture medium was removed by aspiration and the cells were rinsed with 10 ml of cold phosphate buffered saline (PBS). PBS (1 ml) was added to each plate and the cells were scraped off using a rubber policeman. The cells were resuspended and lysed by vortexing in 0.4 ml of guannidinium thiocyanate solution containing 4 M guannidinium thiocyanate (Sigma), 25 mM sodium citrate pH 7, 0.5% sarcosyl and 0.1 mM 2-mercaptoethanol. Twenty seven microliters of 3 M sodium acetate, pH 4.0 was added and mixed well by vortexing. Then the cells were extracted with an equal volume of water saturated phenol and chloroform:isoamyl alcohol (49:1). After vortexing for 10 seconds and cooling on ice for 10 minutes, the cells were centrifuged for 5 minutes at 14,000xg and the aqueous phase was collected carefully. The cells were precipitated with an equal volume of isopropanol and incubated for at least 2 hours at -20 ^{o}C. The mixture was centrifuged again for 10 minutes at 14,000xg. The pellet was carefully washed twice with 75 % ethanol and once with 100 % ethanol. The pellets were air dried and the RNA was dissolved in DEPC-treated water. The concentration of RNA was quantitated by measuring the absorbance at 260 nanometers.

SPARC Plasmid Purification

The plasmid containing SPARC gene was a gift kindly provided by Dr. Benbrook, Health Sciences Center, The University of Oklahoma. The plasmids were transformed into *E.* coli DH5 cells and screened by ampicilin resistance. The transformed DH5 cells, which

propagate plasmids at high copy numbers, grew in LB medium containing appropriate ampicilin overnight at 37 °C. For purification of the plasmid DNA, Promega Wizard™ (Promega) Miniprep kit was used according to the instructions of manufacturer, except that the DNA pellet was finally dissolved in 48 °C TE buffer. The concentration was measured by measuring absorbance at 260 nanometers.

Digestion of Plasmid DNA Containing the SPARC Gene

One microgram of purified plasmid DNA containing the SPARC gene was digested by the restriction endonuclease PvuII (GIBCO BRL) at 37 °C for 1 hour. The digestion products were then separated on a 1.5% agarose gel using TAE as a running buffer. The size of the fragment was estimated by comparison with the standard 1 Kb ladder. The digestion product expected at 415 bp was then excised from the agarose gel and extracted with QIAGEN gel extraction kit (QIAGEN) as described in the manufacture's guide.

Labeling of the Probe

The 415 bp fragment was then non-radioactively labeled using the Genius DIG DNA labeling kit (Boehringer Mannheim). A tube containing a 150 ng fragment of DNA was placed in a boiling water for 10 minutes, then transferred immediately to ice. The DNA was mixed well with sterile distilled water, 10X hexanucleotide mixture, dNTP mixture, and Klenow enzyme in the order listed. The labeled DNA was then incubated at 37 °C overnight. The next morning, the labeled DNA was extracted and dissolved in 50 µl of TE buffer. Just prior to completing the prehybridization, the probe was heated in boiling water for 10 minutes and diluted with cold prehybridization solutions.

Northern Hybridization

The 1.5 % agarose formaldehyde gel was prepared the day before running. The DEPC treated MOPS running buffer was used to cover the gel overnight. It was a very

efficient method to deactivate any RNase. RNA (10 μg/lane) was electrophoresed through a 1.5% agarose formaldehyde gel in MOPS running buffer. Two and half hours later, the gel was photographed under U.V. light and then blotted onto a nylon membrane (MagnaGraph) overnight at room temperature.

After blotting, the membrane was crosslinked for 2 minutes and prehybridized in prehybridization solution at 42 ^{o}C for at least 2 hours with agitation. After the prehybridization, the solution was discarded and the diluted SPARC gene probe was added to hybridize with the membrane at 42 ^{o}C overnight. After several washes with wash solution and maleate buffer, the membrane was incubated in 50 ml of Northern Blocking Solution for 2 hours at room temperature with agitation to stop the hybridization completely. Fifty mililiters of diluted anti-digoxigenin-alkaline phosphatase was incubated with the membrane for 30 minutes. After the membrane was equilibrated with genius buffer, the membrane was placed DNA side down on the top of one clear overhead sheet to which 0.4 ml lumi-phos 530 had been added. The membrane was moved up and down and side to side carefully to distribute the reagent over the surface. The membrane was then exposed to X-ray film (Kodak) at room temperature for 2 to 4 hours, depending on the strength of the signal.

Slot Blot Hybridization

A series of four 2X dilution of the same concentrations of RNA isolated from the F9 cells treated with different concentrations of drugs were performed. The original concentration of RNA was 5 μg/100 μl. RNA (100 μl) was denatured by adding 300 μl of 6.15 M formaldehyde in 10X SSC and heated at 65 ^{o}C for 5 minutes. The Nylon membranes were soaked in sterile distilled water, then in 10X SSC, and the slot apparatus was assembled. Samples of 400 μl were loaded into the slot, and aspirated through the Nylon membrane, washed with 400 μl 10X SSC and aspirated again. The Nylon membrane was UV crosslinked and baked at 80 ^{o}C in vacuum for 2 hours. The membrane

was hybridized with DIG-11-dUTP labeled SPARC DNA fragment following the procedures described for northern hybridization.

CHAPTER IV

RESULTS

Morphological Observations

To study the morphological changes of the F9 teratocarcinoma stem cell caused by the treatment of a selected heteroarotinoid (LWS-3), F9 cells were grown in the Dulbecco's modified Eagle's medium contained 15% FBS. As a negative control, the cells were grown in the cell culture without any drug or ethanol treatment. At the same time, the F9 stem cells grown in the culture presented with 10^{-7} M *t*-RA were used as positive control. All of the drugs used to treat F9 cells should be made fresh from the stock solution, since the retinoic acid is not stable in the medium.

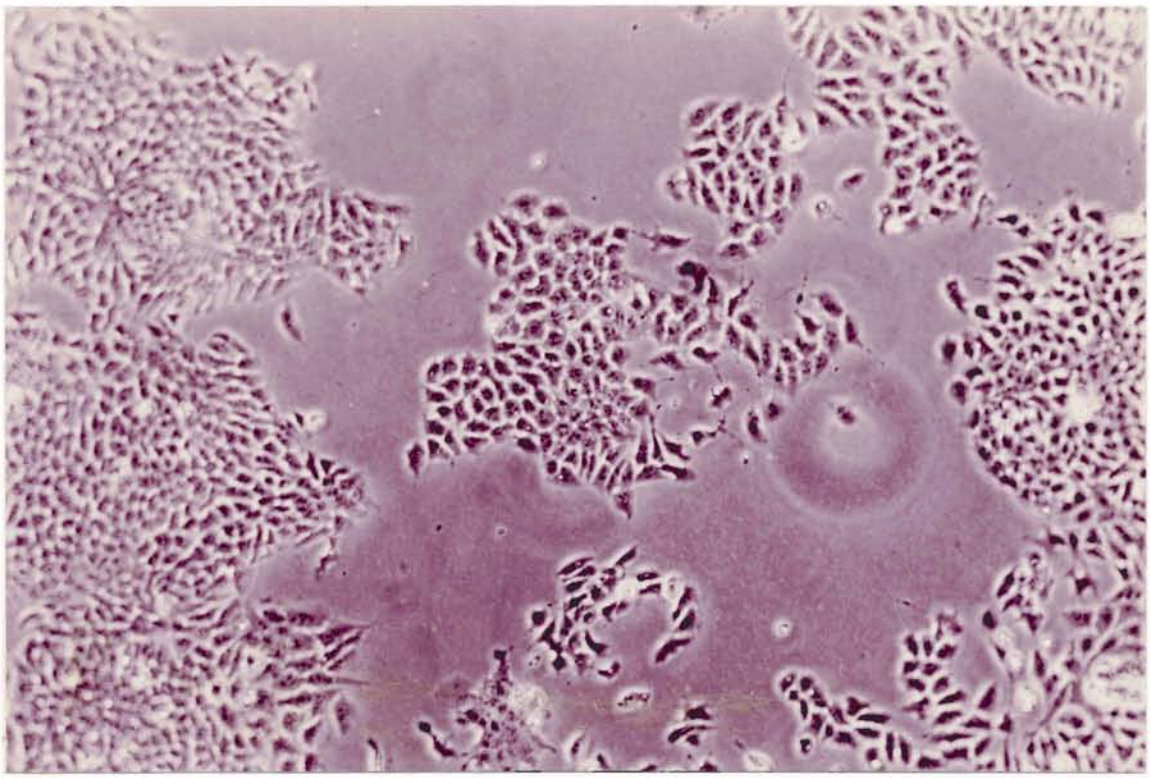
F9 stem cells grown in the culture without any drug treatment are closely packed colonies of embryonal carcinoma cells (Figure 4 A). The cell population appears predominately homogenous, although some cells were occasionally observed with different morphology. The positive control F9 embryonal carcinoma cells assume a flat morphology characterized by cytoplasmic granules and more cell colonies were triangular (Figure 4 B) after the 10^{-7} M *t*-RA was presented to the culture (Strickland and Mahdavi, 1978).

The F9 carcinoma cells were maintained in the DME medium which contained 10^{-6} M to 10^{-9} M concentrations of LWS-3. After incubation for 84 hours, the cells were photographed and the effect of LWS-3 on F9 cell morphology was investigated. The LWS-3 brought about morphological alterations of F9 stem cells to the differentiated state. After the cells were treated with the LWS-3 for 84 hours, the cells moved apart from one another, became larger, triangular, more spread out, and accumulated intracellular granules

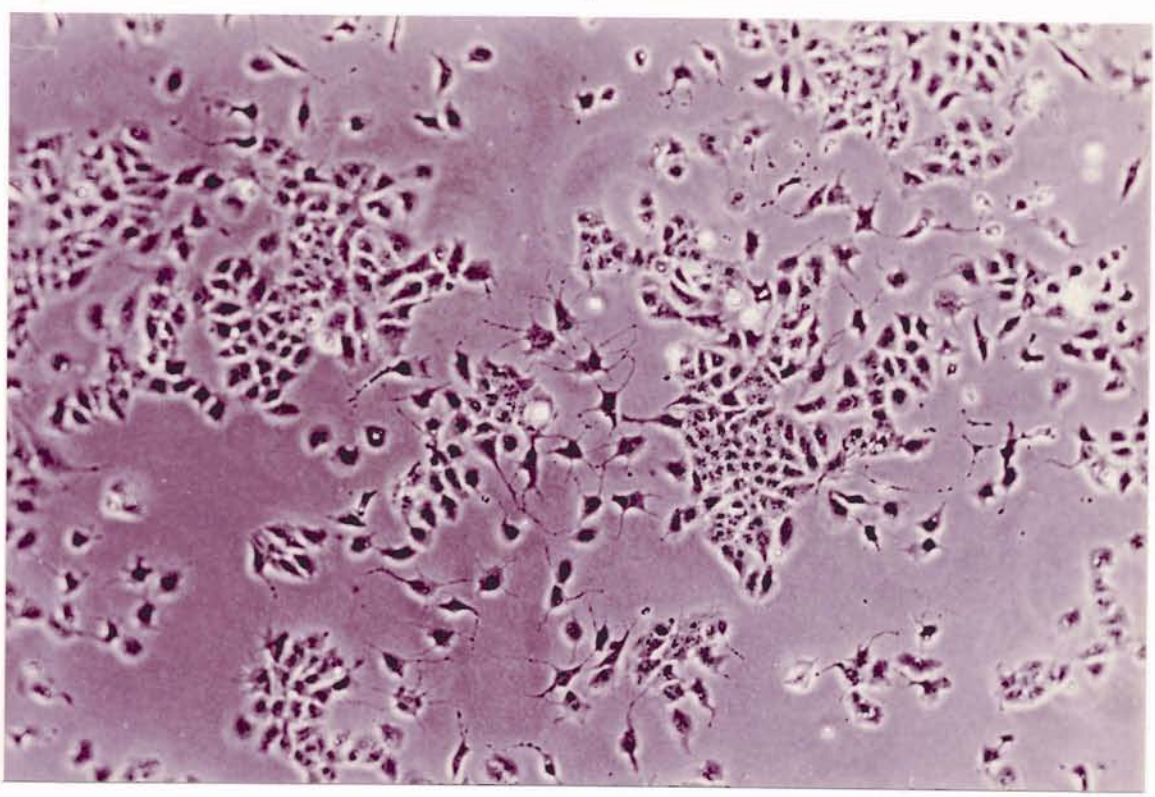
Figure 4.

Morphology of F9 Teratocarcinoma Cells after Treatment with LWS-3 for 84 Hours.

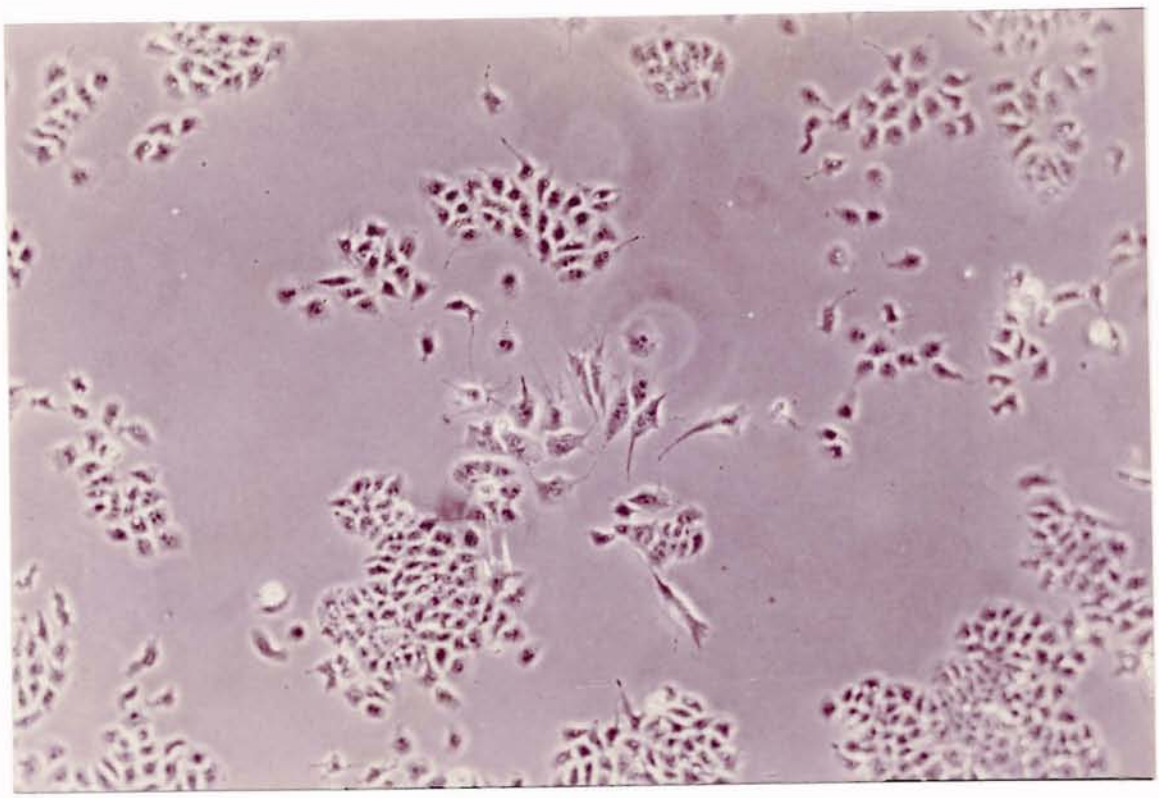
The F9 teratocarcinoma cells were seeded in the culture medium (Table 1) at a density of 3 x 10^5 cells per 100 mm diameter petri dish in the presence or absence of different concentrations of LWS-3 for 84 hours. (A) Negative control (no drug); (B) Positive control (10^{-7} M *t*-RA); (C) 10^{-6} M LWS-3; (D) 10^{-7} M LWS-3; (E) 10^{-8} M LWS-3; (F)10^{-9} M LWS-3.



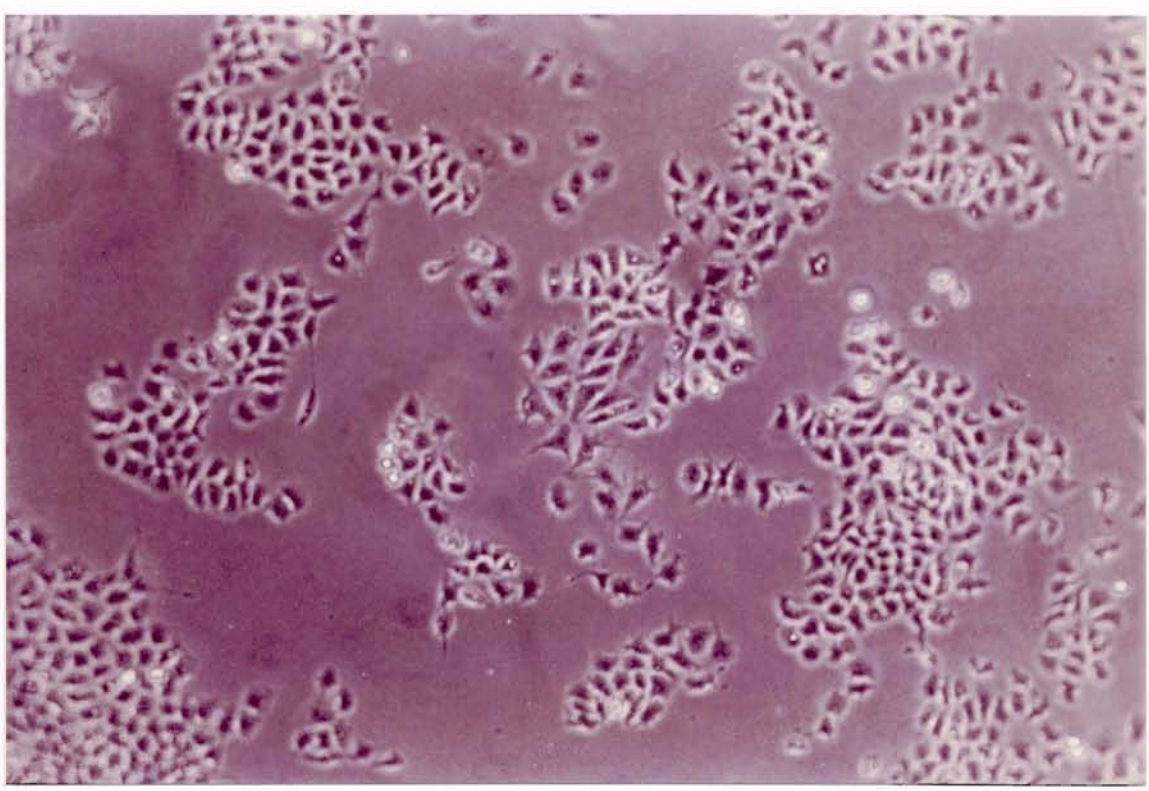
A: No drug treatment



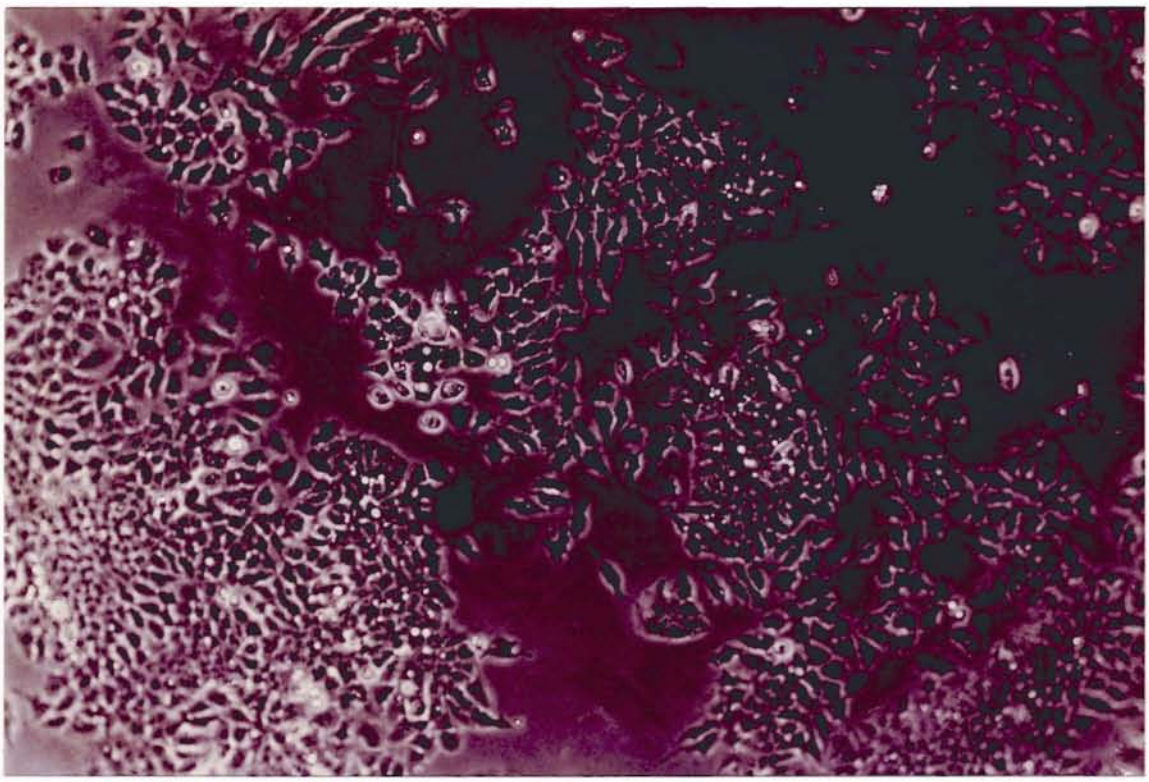
B: 10^{-7} M *t*-RA treatment



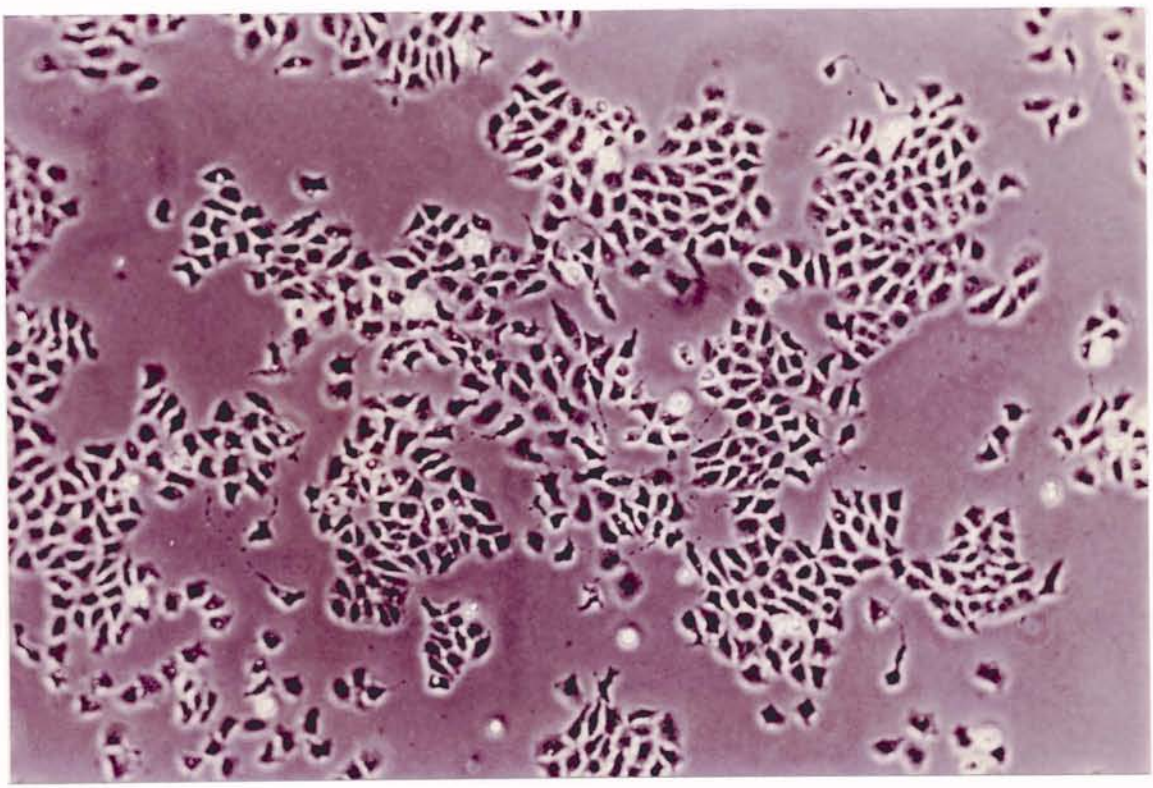
C: 10^{-6} M LWS-3 treatment



D: 10^{-7} M LWS-3 treatment



E: 10^{-8} M LWS-3 treatment



F: 10^{-9} M LWS-3 treatment

(Figure 4, C-F). The appearance of these cells closely resembles the presumptive endoderm derived from teratocarcinoma embryoid bodies (Martin and Evans, 1975). This evidence suggests that LWS-3 can induce the F9 stem cell to differentiate from embryonal carcinoma into endoderm carcinoma. However, it is difficult to distinguish the difference in morphology of the differentiated F9 cells treated with the different concentrations of LWS-3.

Isolation of RNA from the F9 Cell Culture

To prepare the RNA for northern blotting, the RNA was isolated from the F9 cell culture which had been treated with various concentrations of the heteroarotinoid, LWS-3, 10^{-7} M t-RA or no drug for 84 hours.

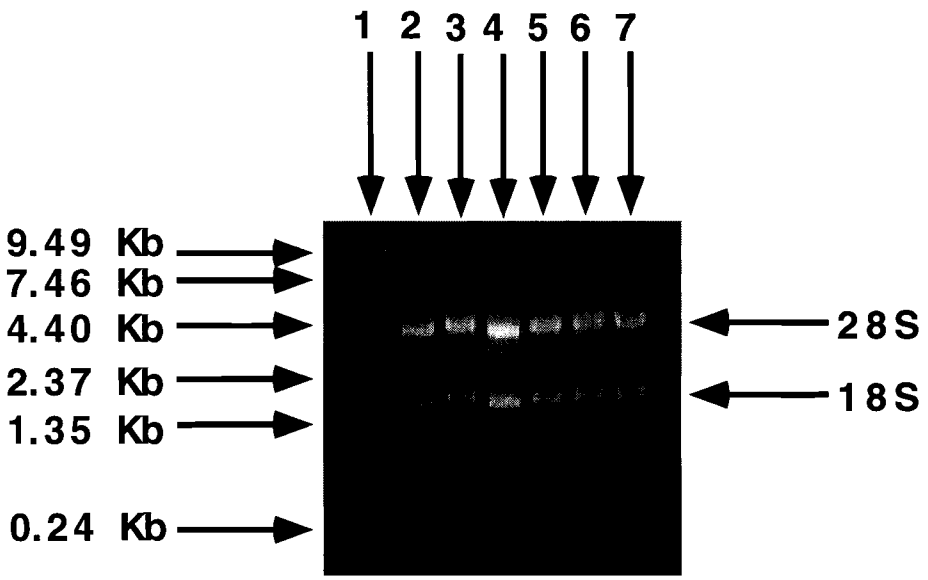
The RNA was isolated from the cell culture using modified single-step method as described. After purification, the RNA concentration was quantitated by measuring the absorbance at 260 nm and purity was checked by measuring the ratio of absorbance at both 260 nm and 280 nm and by agarose formaldehyde gel electrophoresis. Only the RNA with a ratio of 260 nm and 280 nm greater than 1.65 was used in the further hybridization (data not shown). To check whether the RNA isolated from the cell was pure enough for a northern blot, 5 μg of RNA was electrophoresed through 1.5% agarose formaldehyde gel. The results (Figure 5) showed that the two ribosomal RNA subunits of 18S and 28S could be clearly identified after the gel electrophoresis. This confirmed that no RNA degraded during the process.

However, as shown in the Figure 5, there was still some smear of the isolated RNA after the gel electrophoresis. Since high quality RNA is crucial for the northern blot, more phenol-chloroform was added for extraction to get higher quality RNA, but it was still not purified as expected after gel electrophoresis. Moreover, the RNA was isolated using TRIzol Total RNA Isolation Reagent from GIBCO BRL to get higher quality RNA. Although it was quick and easy to use, this reagent apparently was not reliable for F9 cell

Figure 5

1.5% Agarose Formaldehyde Gel of RNA Isolated from F9 Teratocarcinoma Cells in the Presence or Absence of LWS-3 for 84 hours.

F9 cells were plated at a density of 3 X 10^5 cells per 100 mm diameter petri dish in the medium (Table 1) in the presence or absence of LWS-3. After 84 hours incubation, RNA was isolated from F9 cells using the modified single-step method. RNA (10 µg) was then electrophoresed through 1.5% agarose formaldehyde gel at 100 V for 2 hours. Lane 1: RNA ladder; Lane 2: Negative control (no drug); Lane 3: Positive control (10^{-7} M t-RA); Lane 4: 10^{-6} M LWS-3; Lane 5: 10^{-7} M LWS-3; Lane 6: 10^{-8}M LWS-3; Lane 7: 10^{-9} M LWS-3.


1 2 3 4 5 6 7
9.49 Kb
7.46 Kb
4.40 Kb
2.37 Kb
1.35 Kb
0.24 Kb
28S
18S

RNA isolation (Data not shown). The isolated RNA was not pure enough and there was some DNA and protein that interfered. The ratio of 260 nm and 280 nm of isolated RNA was between 1.40 to 1.60. More seriously, a lot of sticky silk-like unknown substances existed in the RNA so that the RNA couldn't be dissolved well. So finally, the modified single-step method was chosen to isolate RNA because the smear of the RNA did not affect the result of northern hybridization as shown in Figure 10 and 11.

Probe Preparation for Hybridization

cDNA sequence of SPARC gene mRNA expressed after the F9 cells treated with retinoic acid was obtained by Mason et al in 1986 (Mason et al., 1986a). The cDNA sequence of the SPARC gene is shown in Figure 6. The restriction enzyme PvuII digested the sequence motifs from nucleotide 385 (CAG) to nucleotide 800 (CTG). The resulting 415 bp product was used as the probe. Figure 7 shows the result of PvuII digestion after the 1.5% agarose gel electrophoresis. The size of the DNA fragment after digestion was estimated according to the 1 Kb standard ladder. As shown on the Figure 7, one fragment of 415bp was separated by the agarose gel after electrophoresis as expected. Since the vector sequence was not known, it was very difficult to recognize the other three bands (band 1, 2, 3). These bands were probably the fragments of the vector digested by PvuII since there are only two cut sites of PvuII on the SPARC gene. But the 415 bp fragment was the exact product of PvuII digestion of the SPARC gene. To prepare the probe for the northern hybridization, the 415 bp product of PvuII digestion of the SPARC gene was extracted and then labeled using the random priming method described before for the further hybridization. The sequence of the probe was also shown in Figure 6 (385nt-800nt).

Using DIG nonradioactive labeling of the probe for the hybridization is a safe and easy method compared to using ^{32}P to label the probe. Furthermore, the probe can be reused several times and kept at -20 ^{O}C for over a year. However, one important

Figure 6.

The cDNA Sequence of SPARC Gene (Mason et al. 1986a)

50
GCATTCCTGCAGCCCTTCAGACCGCCAGAACTCTTCTGCCGCCTGCCTGCCTGCCTGCCTGCCTGTGC
100
CGAGAGTTCCCAGCATCATGAGGGCCTGGATCTTCTTTCTCCTTTGCCTGGCCGGGAGGGCCCTGGCA
150 200
GCCCCTCAGCAGACTGAAGTTGCTGAGGAGATAGTGGAGGAGGAAACCGTGGTGGAGGAGACAGGGG
250
TACCTGTGGGTGCCAACCCAGTCCAGGTGGAAATGGGAGAATTTGAGGACGGTGCAGAGGAAACGGTC
300
GAGGAGGTGGTGGCTGACAACCCCTGCCAGAACCATCATTGCAAACATGGCAAGGTGTGTGAGCTGGA
350 400
CGAGAGCAACACCCCCATGTGTGTGTGCCAGGACCCCACCAGCTGCCCTGCTCCCATTGGCGAGTTTG
450
AGAAGGTATGCAGCAATGACAACAAGACCTTCGACTCTTCCTGCCACTTCTTTGCCACCAAGTGCACC
500
CTGGAGGGCACCAAGAAGGGCCACAAGCTCCACCTGGACTACATCGGACCATGCAAATACATCGCCC
550 600
CCTGCCTGGATTCCGAGCTGACCGAATTCCCTCTGCGCATGCGTGACTGGCTCAAAAATGTCCTGGTC
650
ACCTTGTACGAGAGAGATGAGGGCAACAACCTCCTCACTGAGAAGCAGAAGCTGCGTGTGAAGAAGA
700 7
TCCATGAGAATGAGAAGCGCCTGGAGGCTGGAGACCACCCCGTGGAGCTGTTGGCCCGAGACTTTGAG
50 800
AAGAACTACAATATGTACATCTTCCCTGTCCACTGGCAGTTTGGCCAGCTGGATCAGCACCCTATTGAT
850
GGGTACCTGTCCCACACTGAGCTGGCCCCACTGCGTGCTCCCCTCATCCCCATGGAACATTGCACCAC
900 950
ACGTTTCTTTGAGACCTGTGACCTAGACAACGACAAGTACATTGCCCTGGAGGAATGGGCCGGCTGCT
1000
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1050
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1150
TCCGCCTGGGGATAAGGTGCTAACATAGATTTAACTGAATACATTAACGGTGCTAAAAAAAAAAAAAA
1200
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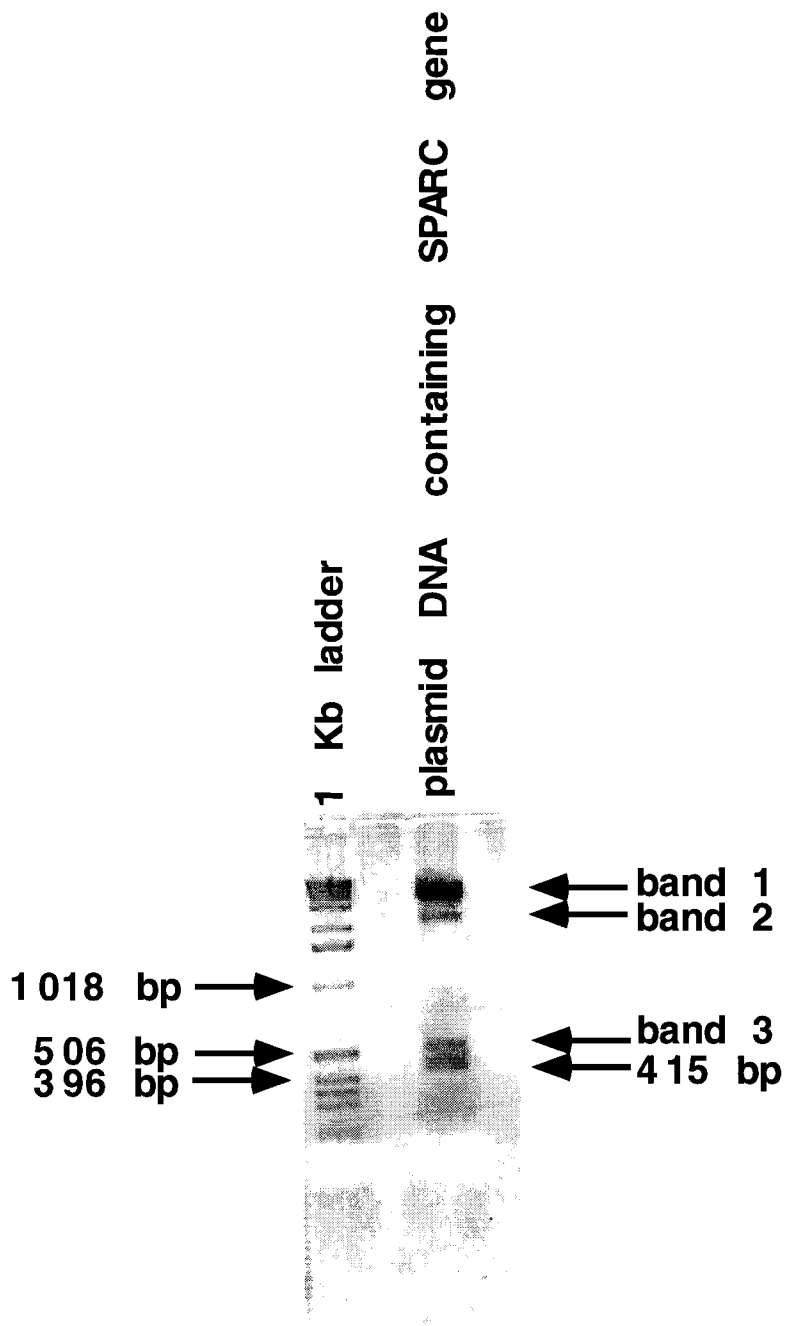
1250
CATCCACAGCCTCCTGGTCCCCTGCACTACCCAGTGTCTCACTGGCTGTGTTGGAAACGGAGTTGCAT
1300 1350
AAGCTCACCGTCCACAAGCACGAGATATCTCTAGCTTTCATTTCAATTTTGCATTTGACTCTTAACACT
1400
CACCCAGACTCTGTGCTTATTTCATTTTGGGGGATGTGGGCTTTTTCCCCTGGTGGTTTGGAGTTAGGCA
1450 1502
GAGGGAAGTTACAGACACAGGTACAAAATTTGGGTAAAGATACTGTGAGACCTGAGGACCCACCAGTC
1550
AGAACCCACATGGCAAGTCTTAGTAGCCTAGGTCAAGGAAAGACAGAATAATCCAGAGCTGTGGCAC
1600
ACATGACAGACTCCCAGCAGCCCGGGACCTTGCTGTCTTCTCGACTCTTCGGGCGTTTCTTTCCATGTT
1650 1700
TGGCTGTTGGTTTTAGTTTTGGTGAGCCATGGGTGGGCCAGAACATCACTCAACTGCAATTGGGCTTTC
1750
AGGTTCTTGCCGGGAGCTCTAGGCACTGGGAGGCTGTTTCAGGAAAGTGAGACTCAAGAGGAAGACAG
1800
AAAAGGTTGTAACGTAGAGGAAGTGAGAACTGGTGAATTGGTTTGATTTTTTTCACATCTAGATGGCTGT
1850 1900
CATAAAGTTTCTAGCATGTTCCCCCTCACCTCTCCCCACCCCCTGCCACTTGAAACCTTCTACTAATCA
1950
AGAGAAACTTCCAAGCCAACGGAATGGTCAGATCTCACAGGCTGAGAAATTGTTCCCCTCCAAGCATT
2000 2050
TCATGAAAAAGCTGCTTCTCATTAACCATGCAAACTCTCACAGCGATGTGAAGAGCTTGACAAGTCTTT

CAAAATAAAAAGTAACAACTTAGAAACGG

Figure 7.

Restriction Digestion of Plasmid DNA Containing the SPARC Gene.

One microgram of plasmid DNA containing the SPARC gene was digested with the restriction enzyme, PvuII. The digested plasmid DNA was electrophoresed on a 1.5% agarose gel at 90 V for 3 hours. The sizes (bp) of selected band of the plasmid DNA containing the SPARC gene and standards (1 Kb ladder) are shown. Lane 1: 1 Kb DNA ladder; Lane 2: Digested plasmid DNA containing the SPARC gene.


1 Kb ladder
plasmid DNA containing SPARC gene
band 1
band 2
band 3
415 bp
1018 bp
506 bp
396 bp

shortcoming of this method is that the yield of the probe DNA which has been labeled can not be detected directly after labeling and extraction. Attempts to detect the yield of the probe labeled through the agarose gel electrophoresis was unsuccessful. No bands of the probe were seen on the gel since the yield was too low (Data not shown). For this reason, it is possible that there was not enough probe for the detection of the hybridization, although the Genius detection kit is very sensitive.

Northern Hybridization

It is hard to judge the effect of LWS-3 on induction of F9 cells when depending only on the morphological change, since the alteration of the cell morphology was subtle. To examine the effect of induction of the F9 cells differentiation at the molecular level and more precisely, differentiation marker genes have been used to study gene transcription associated with F9 cell differentiation (Gudas et al., 1994). For these reasons, expression of the differentiation specific gene SPARC was chosen to investigate the effect of the heteroarotinoid LWS-3 on induction the F9 cell differentiation. The PvuII digestion product 415 bp fragment of the SPARC gene was labeled and was used as the probe with northern hybridization (Figure 7). RNA (10 μg) was isolated from the F9 cells treated with different agents and loaded on the gel. After electrophoresis through 1.5% agarose formaldehyde gel at 100 V for 2 hours, the RNA on the gel was then blotted onto Nylon membranes and the membranes were then hybridized with the probe overnight. The results were detected using the Genius Detecting Kit described before.

Figure 8 shows the differentiation specific SPARC gene expression profiles of F9 cells after drug treatment for 84 hours. As the negative control, the F9 cells were incubated in the normal medium without any drugs. After 84 hours incubation, the expression of the SPARC gene was at a very low level, almost not expressed (Lane 1). However in the presence of 10^{-7} M *t*-RA and 10^{-6} M of LWS-3, the SPARC gene expression increased

Figure 8.

Northern Hybridization of RNA Isolated from F9 Teratocarcinoma Cells in the Presence or Absence of 10^{-6} M LWS-3 with the SPARC gene.

F9 cells were plated at a density of 3 X 10^5 cells per 100 mm diameter petri dish in the medium (Table 1) in the presence or absence of LWS-3. After an 84 hour incubation, RNA was isolated from F9 cells using the modified single-step method. RNA (10 μg) was electrophoresed through 1.5% agarose formaldehyde gel at 100 V for 2 hours. The RNA on the 1.5% agarose formaldehyde gel was transferred onto a nylon membrane by northern blotting. The membrane was then hybridized with a labeled 415 bp SPARC gene probe. The size of bands was estimated by the position compared with 18S and 28S RNA subunits (Wang and Gudas, 1983). Lane 1: No drug; Lane 2: 10^{-7} M *t*-RA; Lane 3: 10^{-6} M LWS-3.

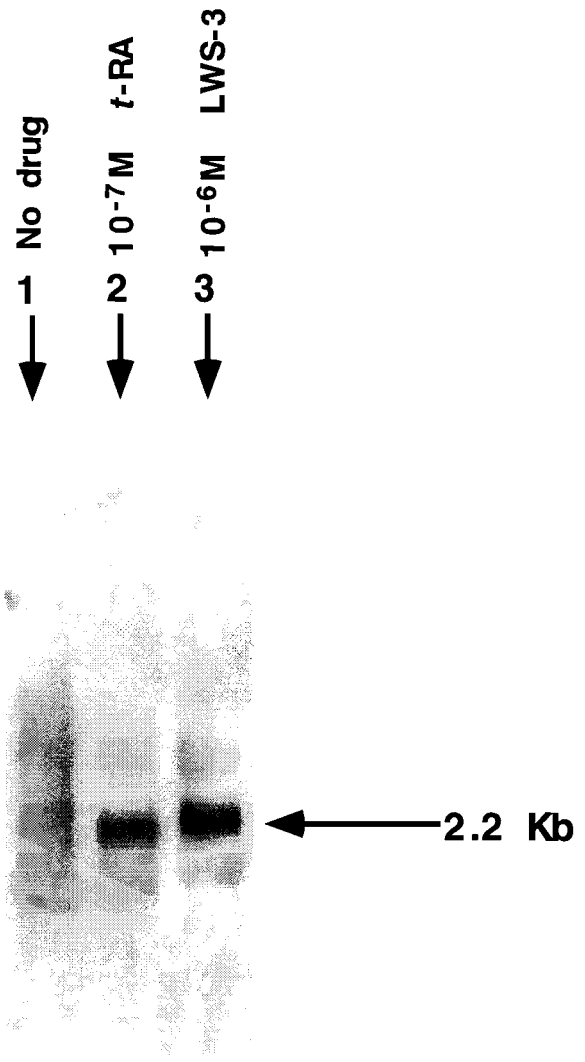
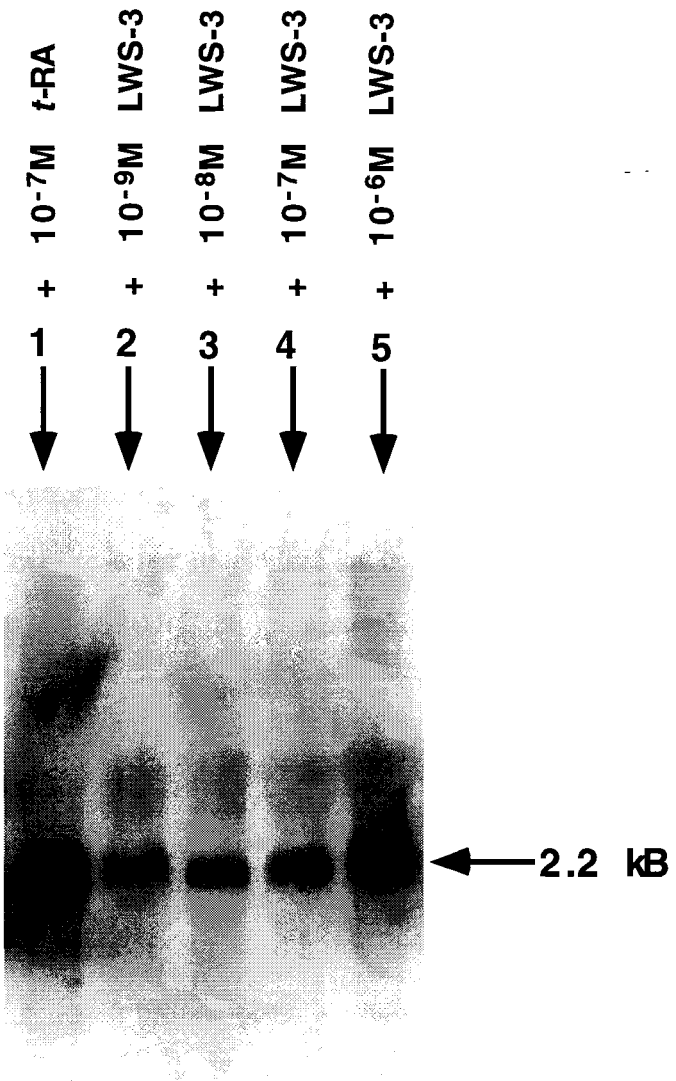

1 No drug
2 10-7M t-RA
3 10-6M LWS-3
2.2 Kb

Figure 9.

Northern Hybridization of RNA Isolated from F9 Teratocarcinoma Cells in the Presence of Different Concentrations of LWS-3 for 84 Hours with the SPARC Gene.

F9 cells were plated at a density of 3 X 10^5 cells per 100 mm diameter petri dish in the medium (Table 1) in the presence of different concentrations of LWS-3. After an 84 hour incubation, RNA was isolated from F9 cells using the modified single-step method. RNA (10 μg) was electrophoresed through 1.5% agarose formaldehyde gel at 100 V for 2 hours. The RNA on the 1.5% agarose formaldehyde gel was transferred onto a nylon membrane by northern blotting. The membrane was then hybridized with a labeled 415 bp SPARC gene fragment. The size of bands was estimated by the position compared with 18S and 28S RNA subunits (Wang and Gudas, 1983). Lane 1: 10^{-7} M *t*-RA; Lane 2: 10^{-6} M LWS-3; Lane 3: 10^{-7} M LWS-3; Lane 4: 10^{-8} M LWS-3; Lane 5: 10^{-9} M LWS-3.


1
2
3
4
5
+
+
+
+
+
10-7M t-RA
10-9M LWS-3
10-8M LWS-3
10-7M LWS-3
10-6M LWS-3
2.2 kB

dramatically. (Lane 2 and 3). Figure 9 shows the results of the SPARC gene expression after the F9 cells were treated with different concentrations of LWS-3 for 84 hours. As shown in Figure 9, with increasing concentrations of LWS-3, SPARC gene expression increased (Lane 2-5). The highest concentration of LWS-3 (10^{-6} M) caused the largest expression among the different concentrations of LWS-3 in this experiment (Lane 5).

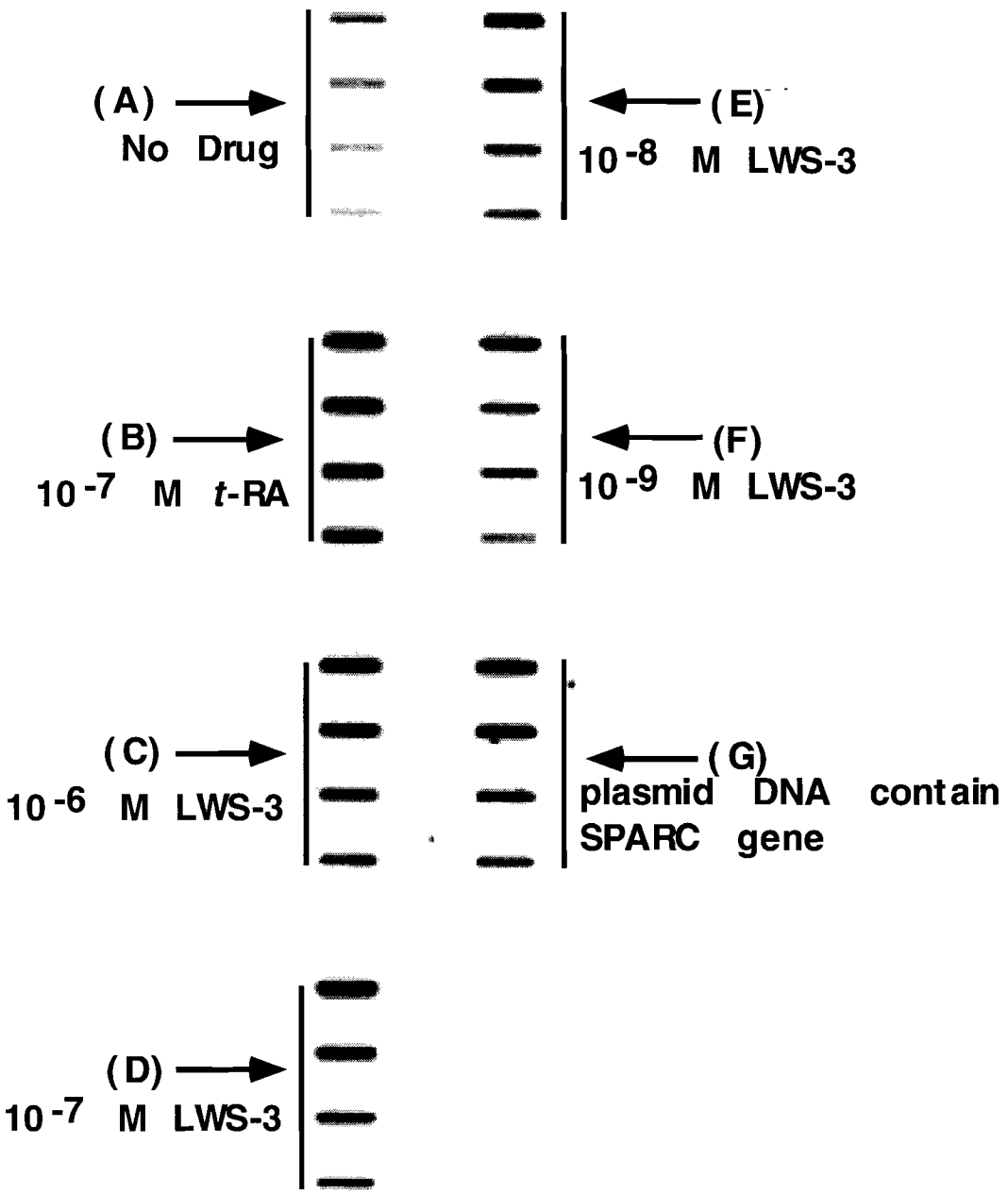
The size of the hybridized transcript can be estimated from its position relative to the 18S and 28S ribosomal RNA (Wang and Gudas, 1983). Mason et al. (1986b) indicated that the F9 cell induces a 2.2 kb SPARC mRNA expression after differentiation. Using this method, we estimated the size of the gene transcription after the F9 cells were treated with LWS-3. As shown in Figure 8 and 9, the expressed SPARC mRNA after the LWS-3 induction was 2.2 Kb. These results indicate that the heteroarotinoid (LWS-3) can induce the differentiation marker gene SPARC expression. Along with the observation of F9 cell morphology change, it confirmed the results that LWS-3 can induce F9 cell differentiation.

Slot Blot Hybridization

To quantitate the effects of different concentrations of LWS-3 on induction of SPARC gene expression, a series of four 2X dilutions of the same concentrations of RNA isolated from F9 cells treated with different concentrations of LWS-3 were blotted onto the Nylon membrane and the northern slot blot was performed. Figure 10 shows the results of slot blot hybridization after the cells were treated with the LWS-3 for 84 hours. The probe used in the slot blot was the same as that in the northern hybridization. As shown in Figure 10, the hybridization was detected as bands on the film. Incubation of LWS-3 for 84 hours (Pattern C, D, E, F) increased SPARC mRNA expression at the different level compared with the negative control (Pattern A). These data are consistent with the results of the northern hybridization and indicate that the heteroarotinoid can induce differentiation of SPARC gene expression. From densitomer scans of the film of Figure 10, the

Figure 10. Northern Slot Blot of RNA Isolated from F9 Teratocarcinoma Cells in the Presence or Absence of Different Concentrations of LWS-3 for 84 hours with the SPARC gene.

F9 cells were plated at a density of 3 X 10^5 cells per 100 mm diameter petri dish in the medium (Table 1) in the presence or absence of LWS-3. After 84 hours of incubation, RNA was isolated from F9 cells using the modified single-step method. A series of four 2 X dilution of concentration of 5 μg/100 μl RNA isolated from the F9 cells treated with the different drug were transferred onto a Nylon membrane through the slot apparatus. The membrane was hybridized with the SPARC gene probe. Pattern A: Negative control (no drug); Pattern B: Positive control (10^{-7} M *t*-RA); Pattern C: 10^{-6} M LWS-3; Pattern D: 10^{-7} M LWS-3; Pattern E: 10^{-8} M LWS-3; Pattern F: 10^{-9} M LWS-3.

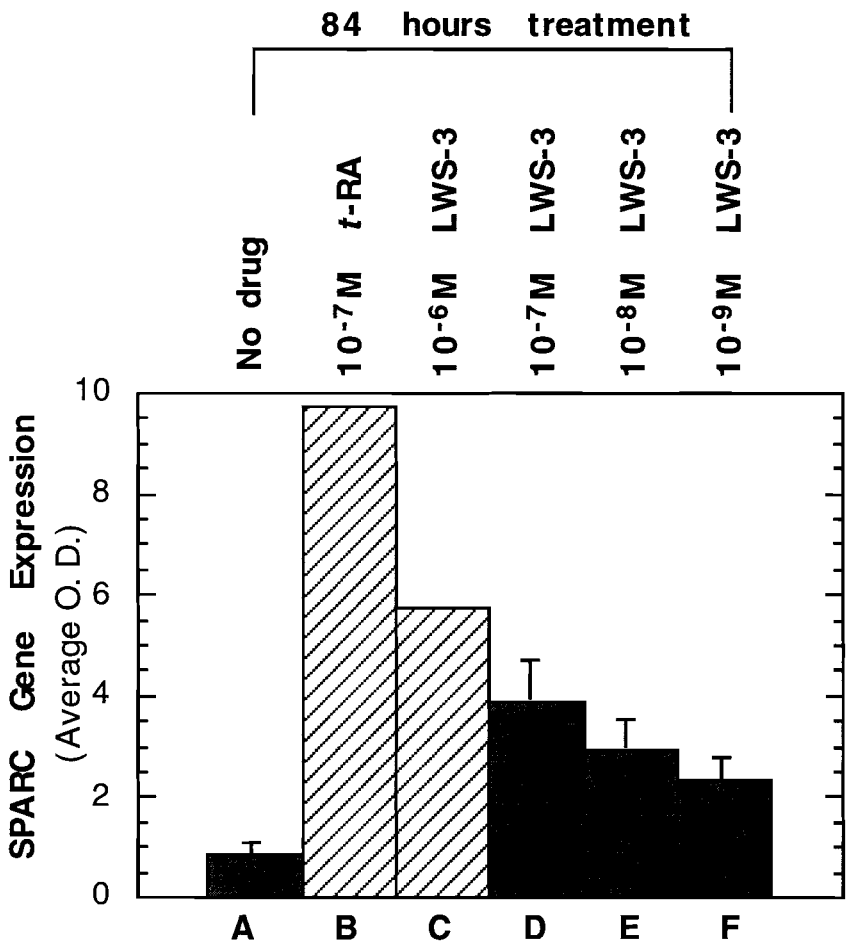

(A)
No Drug
(E)
10 -8 M LWS-3
(B)
10 -7 M t-RA
(F)
10 -9 M LWS-3
(C)
10 -6 M LWS-3
(G)
plasmid DNA contain
SPARC gene
(D)
10 -7 M LWS-3

levels of the SPARC gene expression can be quantitated. Figure 11 shows the results of SPARC gene expression level after the selected heteroarotinoid (LWS-3) treatment of F9 cells for 84 hours. The Optical Density of each band was measured by densitomer scaning and only those O.D. values in the linear range were selected and calculated. The SPARC gene expression level was represented by the average value of O.D. in the linear range multiplied by the factor of dilution. However, in the cases of treatment of 10^{-7} M t-RA and 10^{-6} M LWS-3, the gene expressions of the four dilutions were saturated (Pattern B and C). So the O.D. value of the most diluted concentration of the sample was selected and multiplied by a dilution factor of 8 to calculate the SPARC gene expression level. These results represented the least gene expression level after the F9 cells were treated with 10^{-7} M *t*-RA and 10^{-6} LWS-3. As shown in Figure 11, after F9 cells were treated with 10^{-7} M *t*-RA, the SPARC gene expression was increased at least 11 fold (Bar B) compared to the negative control (Bar A). Different concentrations of LWS-3 exhibited different effects on induction of SPARC gene expression. Among the four concentrations of LWS-3 tested, 10^{-6} M LWS-3 showed the strongest effect and increased SPARC gene expression at least 7 fold (Bar C). 10^{-9} M LWS-3 exhibited the weakest effect on induction of the cell differentiation and increased the SPARC gene expression about 3 fold (Bar F). This confirms the finding that the selected heteroarotinoid LWS-3 can induce differentiation in F9 cells. With increasing concentrations of LWS-3, the SPARC gene expression level was increased.

Figure 11

Effects of Different Concentrations of LWS-3 on Induction of SPARC Gene Expression after the F9 Cells Were Exposed to LWS-3 for 84 Hours.

The film of slot blot (Figure 10) was scanned by pdi densitomer Model DNA 35. SPARC gene expression level was represented by the average Optical Density values which were in the linear range after scanning. Crossed bars (Bar B and C) represented the least SPARC gene expression. Bar A: Negative control (no drug); Bar B: Positive control (10^{-7} M *t*-RA); Bar C: 10^{-6} M LWS-3; Bar D: 10^{-7} M LWS-3; Bar E: 10^{-8} M LWS-3; Bar F: 10^{-9} M LWS-3. See p39 for additional explanations.


84 hours treatment
No drug
10-7M t-RA
10-6M LWS-3
10-7M LWS-3
10-8M LWS-3
10-9M LWS-3
SPARC Gene Expression
(Average O. D.)
10
8
6
4
2
0
A
B
C
D
E
F

CHARPTER V

DISCUSSION

The property of certain anticancer drugs to induce cellular differentiation has made a great impact on the search for new and more effective strategies for cancer therapy, and has elicited many in vitro studies aimed at a better understanding of cell proliferation and differentiation (Egorin, 1988). Retinoids are among the drugs actually involved in clinical trials as anticancer drugs, since they regulate cell proliferation and differentiation, and cancer is associated with abnormal growth with loss of differentiation. However, the full therapeutic potential of this class of compounds has yet to be achieved because of the associated toxicity at pharmacological levels. The search continues for more effective drugs that can be used as chemopreventive agents of cancer.

Recently, heteroarotinoids, which are synthesized based on the structure of retinoids, have shown some potential anticancer function. Some heteroarotinoids have demonstrated an ability to support cell growth, reverse cornification of vaginal epithelial cells in ovariectomized vitamin A-deficient rats (Thorne, 1993) and inhibit some tumor cell growth (Benbrook, 1995). Furthermore, one selected heteroarotinoid, LWS-3, has shown toxicity equal to t-RA, but far less than synthesized arotinoids (Spruce and Berlin, 1994). The objective of the present study was to investigate the effect of a selected heteroarotinoid, LWS-3, on cell differentiation using embryonal carcinoma F9 cells as a model system. This study should help in our understanding of the role of newly synthesized heteroarotinoids in the control of cell differentiation as related to cancer treatment.

We have shown that the selected heteroarotinoid, LWS-3, brought about a morphological alteration of F9 stem cells into the endoderm differentiation stage (Figure 4). This resembles the differentiation of an endoderm-like cell induced by *t*-RA. The results show that LWS-3 induces differentiation in the F9 stem cell.

To determine whether the morphological change of F9 cells was the result of differentiation, the expression of a differentiation specific gene, SPARC, was examined. The transcription of the SPARC gene was increased after treatment by LWS-3 (Figure 8). Even at the lowest concentration, LWS-3 showed some effects on induction of SPARC gene expression. These results suggest that the selected heteroarotinoid, LWS-3, like *t*-RA, can act as a differentiation agent.

The SPARC gene was selected as a marker to study the level of transcription factor gene alteration during F9 cell differentiation. We have shown here that the SPARC gene fulfills this criterion. From the data in this study, there is little doubt that the overall increase in SPARC RNA levels is due, at least in part, to an increase in gene transcription after treatment of the F9 stem cell with the heteroarotinoid, LWS-3.

The SPARC gene has been studied as a differentiation marker gene for a long time. When the F9 stem cell was induced to differentiate from embroyl carcinoma to parietal endoderm carcinoma cell, SPARC gene expression increased about 20 fold. When F9 cells were induced to differentiate with *t*-RA, the parietal endoderm stage occurred after dibutyryl cAMP was subsequently added into the cell culture of the primitive endoderm F9 cells induced by the *t*-RA treatment. Treatment of the primitive endoderm F9 cell with *t*-RA alone at high density caused the F9 cell to aggregate and undergo differentiation to the visceral endoderm cell. In this situation, SPARC gene expression was found to have a transient decrease followed by an increase which plateaued after 4 days. However, there was no direct data to show the SPARC gene expression level after the F9 stem cells differentiated into the primitive endoderm stage by the induction of retinoic acid. In this study, we showed that the SPARC gene expression increased at least 11 fold when the F9

stem cell differentiated into the primitive endoderm stage induced by *t*-RA (Figure 11). These results support the finding that SPARC gene expression increased 20 fold when the F9 stem cell differentiated to parietal endoderm cell after the cell treated with *t*-RA and cAMP for 5.5 days.

In this project, the F9 stem cells were treated with different drugs for 84 hours without the presence of any cAMP in the cell culture. There are two reasons: 1. At a density of 3 x 10^5 cells per 100 mm diameter plate, the F9 cells started to aggregate when the cells were treated with *t*-RA and different concentrations of LWS-3 after 4 days incubation (Data not shown). 2. The main objective for this project was to study the effect of LWS-3 on induction of F9 cell differentiation, not to determine the exact differentiation stages of F9 stem cell induced by LWS-3. To evaluate the effect of LWS-3 on induction of F9 cell differentiation, the cells were compared to those cells only treated with *t*-RA in this project. No cAMP was used to treat the F9 cells. The results of the cell morphological change and increased level of differentiation marker gene expression strongly suggest that LWS-3 induces F9 stem cell differentiation. Even at the 10^{-9} M , the LWS-3 still showed some abilities to induce cell differentiation.

The effects of a selected heteroarotinoid, LWS-3, to induce F9 mouse teratocarcinoma cells differentiation have been studied in this project, and the changes in the patterns of SPARC gene expression between undifferentiated and differentiated cells have been well characterized. Following the LWS-3 treatment of F9 cells, one of the response genes is SPARC. The increased expression of the SPARC gene in LWS-3 treated F9 cells may be a potentially regulatory event in a gene cascade since the SPARC gene can induce the expression of type I plasminogen activation inhibitor (PAI-1) in cultured bovine aortic endothelial cells (Hasselaar et al, 1991), and can inhibit the binding of PDGF-BB and PDGF-AB complexes to the PDGF receptor on human dermal fibroblasts (Raines et al., 1992). Also, the SPARC gene encodes a calcium-binding glycoprotein that has been identified as a member of a group of proteins that exert anti-spreading effects on various

cell cultures. An understanding of the induction of SPARC gene expression by LWS-3 will help us to study the mechanism of regulation of other genes in the process of cell differentiation.

However, the actual molecular mechanism by which the heteroarotinoid modulates SPARC gene expression and cell differentiation is still obscure. Retinoids are thought to produce their biological effects by interacting with specific nuclear receptors for RA (Leid et al., 1992). These receptors are members of the steroid/thyroid superfamily of receptors and as such are ligand-dependent transcription factors (Evans, 1988). There are distinct ligand specificities of each of the receptor families, RAR and RXR. The RXR family of receptors binds 9-*c*-RA (Heyman et al., 1992) while the RARs can bind *t*-RA and 9-*c*-RA (Allenby et al., 1993). Thus, there are multiple retinoid receptors and multiple ligand pathways. Disruption of RARγ gene in the F9 cells does not affect the retinoic acid induction of SPARC gene transcription (Boylan et al., 1993). This result suggested that the RARγ is not directly responsive to retinoic acid for regulation of the SPARC gene expression. LWS-3 has been shown to have a binding affinity for the RARβ and RARγ receptors. If it is true that LWS-3 regulates SPARC gene expression through binding to the RARβ receptors, is the selected heteroarotinoid, LWS-3, a ligand for specific receptors? All of these predictable phenomena that occur during the differentiation of F9 cells remains to be shown.

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