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Involvement of gap junctional communication in the chemopreventive action of retinoids on in vitro carcinogenesis

Hossain, Mohammad Zahid, Ph.D.
University of Hawaii, 1991
INVolvEMENT OF GAP JUNCTIONAL COMMUNICATION IN THE
CHEMOPREVENTIVE ACTION OF RETINOIDS
ON IN VITRO CARCINOGENESIS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
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MAY 1991

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ABSTRACT

Retinoids (vitamin A derivatives) have been shown to reduce the incidences of several human cancers and to inhibit carcinogen-induced neoplasia. The mechanism for this action of retinoids has not been described. In the present study, the involvement of gap junctional communication (GJC) in retinoid action was explored. The rationale behind this study stems from previous observations linking retinoid-induced enhancement of GJC with the suppression of neoplastic transformation.

In the mouse fibroblast C3H/10T1/2 cell line, both the natural and synthetic retinoids significantly increased GJC among cells. This action of the tested retinoids was found to correlate strongly with their inhibitory effect on neoplastic cell transformation. Retinoids enhanced both the mRNA and the protein levels of connexin43. Besides that, retinoid-treatment elevated the phosphorylation of Cx43. Immunofluorescence studies documented the presence of extensive gap junction plaques after retinoid treatment. Tumor promoter, TPA, inhibited homologous GJC in 10T1/2 cells and antagonized retinoid-induced GJC. The existence of extensive heterologous GJC between normal and carcinogen-initiated 10T1/2 cells was observed in the
present study and it was postulated that through cell communication, normal cells suppressed the conversion of initiated cells into fully transformed phenotype. TPA was again inhibitory to the heterologous GJC.

Although retinoids were potent inhibitors of neoplastic transformation, they failed to inhibit the growth of fully transformed cells in coculture with normal cells and did not enhance heterologous GJC between these cell types. However, elevation of cellular cAMP resulted in the enhancement of normal:transformed heterologous GJC and decreased growth of the transformed cells. Retinoids antagonized cAMP action on both GJC and growth control. This inhibitory action of retinoids was not mediated through blocking cAMP generation or altering the levels of Cx43.

In this present study we demonstrate that the effects of retinoids on neoplastic transformation or on regulating cellular growth can be explained by their effects on GJC. The observed inverse relationship between GJC and neoplastic transformation suggests the involvement of growth regulatory signals which can traverse gap junctions. Future studies will be required to characterize these signals.
TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................ iii
ABSTRACT ..................................................... v
LIST OF TABLES .............................................. x
LIST OF FIGURES ............................................ xi
LIST OF ABBREVIATIONS ...................................... xiii
CHAPTER I. INTRODUCTION ................................ 1
  1.1. Retinoids ............................................ 1
      1.1.1. Structure and metabolism of retinoids .... 1
      1.1.2. Biological activity of the retinoids .... 7
      1.1.3. Chemopreventive action of retinoids ... 11
      1.1.4. Mechanism of retinoid action .......... 22
      1.1.5. Targets of retinoid action: Relationship with its antineoplastic activity .. 31
  1.2. Gap Junctional Communication ................... 32
      1.2.1. Structure of gap junctions ............... 33
      1.2.2. Protein components of gap junctions:
            The connexins .................................. 36
      1.2.3. Biological role of GJC .................... 44
      1.2.4. Role of GJC in modulating cellular growth:
            Relationship to cancer prevention ......... 46
      1.2.5. Enhancement of GJC leads to the suppression of neoplastic transformation . 50
  1.3. Aim of the study .................................. 52
CHAPTER II. METHODS .................................. 52
  2.1. Cell culture ...................................... 55
  2.2. Preparation of drugs ............................. 55
  2.3. Gap junctional communication assay .......... 57
  2.4. Coculture experiments ........................... 58
  2.5. Cyclic AMP assay ................................ 59
2.6. Northern blotting of connexin43 mRNA
2.6.1. Isolation of total mRNA
2.6.2. Agarose gel electrophoresis
2.6.3. Hybridization with connexin43 cDNA.

2.7. Western blotting of connexin43
2.7.1. Preparation of cell extracts.
2.7.2. Protein electrophoresis and western blotting

2.8. Immunofluorescent detection of gap junction plaques

CHAPTER III. RESULTS

3.1. Effect of retinoids on gap junctional communication (GJC) and its relationship to the inhibition of neoplastic transformation

3.1.1. Enhancement of GJC by retinoids in 10T1/2 cells
3.1.2. Time-course of GJC-induction by TTNPB
3.1.3. Dose-response of retinoids on GJC
3.1.4. Correlation between the effects of retinoids on neoplastic transformation and on cell-cell communication
3.1.5. Tumor promoter TPA inhibits retinoid-induced GJC
3.1.6. TPA inhibits normal:initiated heterologous GJC
3.1.7. Retinoids enhanced homologous GJC in transformed cells
3.1.8. Effects of retinoids on the cell area

3.2. Molecular mechanism of retinoid action:
   Effect on connexin43 expression

3.2.1. Effect of retinoids on Cx43 mRNA level
3.2.2. TTNPB-induced increase in Cx43 mRNA does not require any de novo protein synthesis
3.2.3. Retinoids enhanced Cx43 protein level
3.2.4. Retinoids enhanced the immunofluorescent localization of gap junction plaques
3.3. Modulation of heterologous GJC by retinoids and cAMP: Relationship with growth control

3.3.1. TTNPB inhibits heterologous GJC .... 120
3.3.2. Ro 20-1724 increases heterologous GJC through the activation of cAMP-dependent protein kinase (PK-A) .... 127
3.3.3. TTNPB does not interfere with the generation of cAMP .... 130
3.3.4. TTNPB-enhanced levels of Cx43 was not antagonized by Ro 20-1724 .... 135

CHAPTER IV. DISCUSSION .... 138

APPENDICES (Reprints of Publications)

Appendix A. Enhancement of gap junctional communication by retinoids correlates with their ability to inhibit neoplastic transformation .... 162

Appendix B. Retinoid-enhanced gap junctional communication is achieved by increased levels of connexin43 mRNA and protein. 168

BIBLIOGRAPHY .... 177
LIST OF TABLES

Table 1. Cell lines used in the present study . . . . 56
Table 2. Biological effects exerted by retinoids and TPA . . . . . . . . . . . . . . . . . . . . . . . . . . . . 87
LIST OF FIGURES

Figure

1. Structure of commonly used retinoids .................... 3
2. Metabolism of retinoids ................................. 6
3. Stages of chemical carcinogenesis ....................... 18
4. Schematic diagram of retinoic acid receptor (RAR) action .................. 29
5. Schematic diagram of gap junctions ....................... 35
6. Opening and closing of gap junctions ..................... 38
7. Proposed topographical model of connexins ............... 42
8. Effect of retinoids on homologous GJC in 10T1/2 cells .................. 71
9. Long-term effects of retinoids on 10T1/2 GJC ............ 73
10. Time-course of GJC-induction by TTNPB .................. 76
11. Dose-response of retinoid-induced GJC in 10T1/2 cells ............... 79
12. Dose-response of retinoid-induced GJC in H23 cells ............... 82
13. Correlation between transformation and junctional communication .................. 85
14. Effect of TPA on constitutive and TTNPB-induced GJC .................. 89
15. Effects of TTNPB and TPA on heterologous communication between 10T1/2 and initiated-10T1/2 cells .................. 92
16. Effect of retinoids on homologous GJC in 4B cells ............... 96
17. Effect of retinoids on homologous GJC in C1-4 cells ............... 98
18. Effects of retinoids on the size of 10T1/2 cells ........................ 101
19. Retinoid-induced increase of Cx43 mRNA in H23 cells .................. 106
20. Effect of cycloheximide on TTNPB-induced increase in Cx43 mRNA in 10T1/2 cells ........................ 109
21. Retinoids increased the Cx43 protein levels ........ 113
22. Immunofluorescent gap junction plaques in H23 cells after retinoid treatment .......... 116
23. Immunofluorescent gap junction plaques in Cl-4 cells ....................... 119
24. Effect of TTNPB and Ro 20-1724 on heterologous GJC and cell proliferation .... 123
25. Effect of TTNPB and Ro 20-1724 on homologous GJC and cell proliferation ........ 125
26. Effect of PK-A inhibitor (H-89) on Ro 20-1724-induced heterologous GJC between 4B and 10T1/2 cells .......................... 129
27. Effect of Ro 20-1724 on the generation of cAMP .......................... 132
28. Effect of TTNPB on the induction of cAMP .................. 134
29. Effects of TTNPB and Ro 20-1724 on the induction of Cx43 protein in 10T1/2 cells ... 137
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CRABP</td>
<td>cellular retinoic acid-binding protein</td>
</tr>
<tr>
<td>CRBP</td>
<td>cellular retinol-binding protein</td>
</tr>
<tr>
<td>Cx</td>
<td>connexin</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxy cytidine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GIT</td>
<td>guanidine isothiocyanate</td>
</tr>
<tr>
<td>GJC</td>
<td>gap junctional communication</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>kB</td>
<td>kilobases</td>
</tr>
<tr>
<td>kD</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>MCA</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>Na-PPi</td>
<td>sodium-pyrophosphate</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PK-A</td>
<td>cyclic AMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>RBP</td>
<td>retinol-binding protein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium saline citrate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TF</td>
<td>transformation frequency</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TTNPB</td>
<td>tetrahydrodextetramethylnaphthalenylpropenylbenzoic acid</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

1.1. RETINOIDs

1.1.1. Structure and Metabolism of Retinoids

The term "retinoid" includes both the naturally occurring compounds with vitamin A activity and the synthetic analogs, with or without biological activity, of retinol or retinoic acid. The existence of an essential fat-soluble factor in foods necessary for growth and vision was first reported by McCollum and Davis (1915). Later work by Karrer et al (1930) determined the structure of this factor as retinol (vitamin A). Other naturally occurring retinoids are retinaldehyde, retinoic acid, retinyl palmitate etc. (Fig 1). After the pioneering work of Kuhn and Morris (1937) to synthesize a retinoid, a great deal of effort has been put on the chemical and industrial synthesis of retinoids. These synthetic retinoids have been proved to be extremely useful in understanding the molecular mechanism of retinoid action (Bollag, 1984). Some of them are presented in Fig. 1.

Retinol is obtained in the diet from animal sources as preformed retinyl esters or as precursor carotenoids.
Figure 1. Structure of commonly used retinoids.

(a) Naturally obtained retinoids

(b) Synthetic retinoids
A. Naturally obtained retinoids

- all-trans-retinol
- 13-cis-retinaldehyde
- retinyl palmitate
- all-trans-retinoic acid
- retinyl acetate

B. Synthetic retinoids

- Tetrahydrotetramethylnaphthalenyl-propenylbenzoic acid (TTNPB)
- 3-methyl TTNPB
- Ethyl 3-methoxy-2-methyl-17-nor-1,2,3,4-tetrahydroretinoate (Etretinate)
from plant sources. β-Carotene is absorbed by the intestinal mucosa where it is split into retinaldehyde. Retinaldehyde is converted to retinol, which is esterified and absorbed into the body in the chylomicron fraction of lymph (Goodman et al, 1966). Most of the vitamin is stored in the liver as fatty acid esters, principally palmitate. Blood retinol level is regulated by the liver. In case of retinol deficiency, the stored retinol is deesterified and released as a complex with retinol binding protein (Goodman, 1980). Retinol binding protein (RBP)-retinol complex then delivers retinol into the target tissue (Rask and Peterson, 1976; Chen and Heller, 1977) where it interacts and forms a complex with another specific protein, the cellular retinol-binding protein (CRBP). CRBP has been located in a variety of organs. Since RBP does not enter the cell, the existence of membrane receptors was suggested (Rask and Peterson, 1976; Chen and Heller, 1977; McGuire et al 1981) which participate in the transfer of retinol across the membrane. However, recent studies by Noy and Xu showed that in in vitro model systems that do not contain any receptors, retinol readily dissociates and transfers across the membrane (Noy and Xu, 1990a, 1990b, 1990c).
Figure 2.
Metabolism of retinoids
\[ \text{β-carotene} \rightarrow \text{all-trans-retinaldehyde} \rightarrow \text{all-trans-retinoic acid} \]

- all-trans-retinyl esters
- all-trans-retinyl phosphate
- all-trans-retinyl ester
- all-trans-retinyl phosphate
- all-trans-retinaldehyde
- 11-cis-retinaldehyde
- all-trans-retinoic acid
Another prominent retinoid, retinoic acid is formed primarily from the irreversible oxidation of retinaldehyde (Frolik, 1984). This natural physiological metabolite of retinol and retinal (Fig. 2) has been detected in several organs. Unlike retinol, it is not stored in the liver but is rapidly excreted. Retinoic acid is predominantly found inside the cell where it binds to a specific cellular retinoic acid binding protein (CRABP). CRABP is distinct from CRBP and has been located in brain, skin, testes, eye, but not in liver (Mandel and Cohn, 1980). The role of these binding proteins in retinoid action will be described later.

1.1.2. BIOLOGICAL ACTIVITIES OF THE RETINOIDS

**Vision:** Vitamin A is required for normal growth, vision, reproduction and proper differentiation of tissues. The molecular mechanism of its action is poorly understood, except in the case of vision. Retinol is converted and then oxidized into 11-cis retinal which binds with the protein opsin to form the light sensitive pigment, rhodopsin (Bridges, 1984). Light converts 11-cis retinal to its all-trans isomer which is then reduced to retinol and re-enter the visual cycle. Retinoic acid is formed irreversibly from retinal (Frolik, 1984) and therefore is not active in this cycle (Dowling and Wald, 1960).
Role of retinoids in embryogenesis: Retinoic acid was found to be extremely important in embryogenesis. In the developing chick limb, the posterior part contains the zone of polarizing activity (ZPA) that significantly affects the development of anteroposterior pattern of vertebrate limbs (Saunders & Gasseling, 1968). Later work showed that all trans-retinoic acid closely mimics ZPA action (Tickle et al 1982, Summerbell, 1983). This resemblance raised an important question, whether ZPA action was mediated through retinoic acid. This was resolved by Thaller and Eichele (1987), who showed that chick limb buds contain endogenous retinoic acid. Retinoic acid forms a concentration gradient across the developing limb and is believed to be crucial for limb patterning (Eichele and Thaller, 1987). It was also shown that retinoic acid is not only necessary but also sufficient to specify the pattern developing and the presence of ZPA is not required for its action (Eichele, 1989). A synthetic retinoid was also shown to mediate similar effects when applied from a local source in the developing limb bud (Eichele et al, 1985; Eichele & Thaller, 1987). Recently from chick wing bud, another endogenous novel morphogen 3,4-didehydroretinoic acid, was isolated and shown to be equipotent to retinoic acid (Thaller & Eichele, 1990). Although much of the work has
been done in chick limb bud, a recent study showed that both retinoic acid and 3,4-didehydroretinoic acid may also be involved in the development of the central nervous system (Wagner et al, 1990) raising the possibility that signaling molecules involved in pattern formation in different embryonic tissues are conserved.

**Effects on cellular differentiation and teratogenicity:** The effect of retinoids on cellular differentiation has been studied in great detail. In 1953, Fell and Mellanby made the important discovery that addition of retinol or retinyl acetate to organ cultures of embryonic chick skin caused the ectoderm to differentiate into mucous-secreting epithelium, similar to that of normal nasal or tracheal mucosa. This study clearly showed that retinoids have a direct effect on cellular differentiation and supported the observation of Mori (1922) that in retinoid-deficient animals, normally mucous producing nasal trachea epithelium undergoes a change to a squamous keratinizing epithelium. Since then, numerous studies using organ culture as well as cell culture techniques have demonstrated similar effects of retinoids on cellular growth and differentiation (for a detailed list see Sporn and Roberts, 1984). The most striking examples of this phenomenon are the induction of terminal differentiation in
murine F9 teratocarcinoma cells (Strickland and Mahdavi, 1978) and human promyelocytic leukemia cell line, HL-60 (Breitman et al, 1980, 1981). However it should be noted that in many cell types, retinoids inhibited cell differentiation, indicating that retinoid effects are diverse in nature and are dependent on cell types.

The teratogenicity associated with hypervitaminosis A has been reviewed by Kamm et al (1985). Retinoic acid was shown to interfere with growth and developmental process of whole embryos, both in vitro (Steele et al, 1987) and in vivo (Kochhar et al, 1984). All-trans retinoic acid was found to be the active agent (Klug et al, 1989).

Role of retinoids in the immune response system:
The role of vitamin A in the immune response system was reported in as early as 1928 by Green and Mellanby. Numerous epidemiological studies have suggested a crucial involvement of vitamin A regarding the resistance to infection. Vitamin A deficiency increased the risk of respiratory infection and diarrhea (West et al, 1989). In animal studies, a similar profile was observed. In vitamin A-depleted rats, production of antibody was impaired after immunization with bacterial antigens (Pasatiempo et al,
Retinoids were shown to modulate T lymphocytes and macrophages (Shapiro & Edelson, 1985).

In addition to mediating these biological effects, retinoids have been shown to be required in reproduction (Thompson, 1970). Retinoic acid was reported to be capable of maintaining testosterone production (Appling and Chytil, 1981) and of inducing differentiation of spermatogonia in vitro (Haneji et al, 1984).

1.1.3. CHEMOPREVENTIVE ACTION OF RETINOIDS

Epidemiological evidences: One of the most exciting areas of retinoid research involves the role of retinoids as cancer chemopreventive agents. Bjelke (1975) demonstrated a negative association between vitamin A intake and the incidence of cancer. In a subsequent study, a similar inverse correlation between the consumption of green leafy vegetables (rich in vitamin A and its precursor β-carotene) and lung cancer was reported by MacLennan et al (1977). Other epidemiological studies showed that vitamin A intake was negatively correlated with the incidence of esophyssial cancer (Mettlin et al, 1981), bladder cancer (Mettlin and Graham, 1979) and laryngeal cancer (Graham et al, 1981). Analysis of plasma vitamin A level rather than dietary intake of vitamin A showed that in several human
cancers, the plasma vitamin A level was lower in cancer patients than that in the healthy population (reviewed by Bertram et al, 1987; Moon and Itri, 1984). Although vitamin A level was shown to be inversely correlated with cancer incidences in these studies, a positive association between this vitamin and the incidence of human cancer was also reported. In prostate cancer, dietary vitamin A intake was positively correlated with the cancer incidence (Kolonel et al, 1988 and Paganini-Hill et al, 1987). Despite these cases, overall epidemiological evidences suggest the involvement of vitamin A in cancer prevention.

Experimental animal studies: In order to get a better understanding of retinoid action, experimental animal studies were also performed. Vitamin A-deficient rats have been demonstrated to be more susceptible to chemically induced carcinogenesis of respiratory tract, bladder and colon (Nettesheim et al, 1975; Cohen et al, 1976; Newberne and Rogers, 1973). Furthermore, retinoid treatment consistently reduced the incidence of chemical carcinogen-induced tumors in several sites. These sites include mammary gland (Moon et al, 1976; McCormick et al, 1982), urinary bladder (Sporn et al, 1977; McCormick et al, 1982), skin in two-stage carcinogenesis (Verma et al, 1979; Gensler et al, 1987), respiratory tract (Cone and
Nettesheim, 1973), and lung (Port et al, 1975). Synthetic retinoids were also investigated for their ability to prevent experimental carcinogenesis. Bollag (1985) reported the antineoplastic activity of a series of synthetic retinoids in skin and mammary tumors in mice. In all the above mentioned studies, retinoids were unequivocally shown to inhibit carcinogen-induced neoplasia. However this effect was restricted to several organ sites. Retinoid-mediated anticancer effect was reversible and thus required continuous treatment (Thompson et al, 1979). The failure of retinoids to prevent tumor incidence or the enhancement of tumor incidence by retinoids were also reported (Hard and Ogiu, 1984; Birt et al, 1983; McCormick et al, 1987). As indicated by Moon and Itri (1984), retinoids significantly differ in their ability to prevent experimental carcinogenesis. For example, in rats 13-cis retinoic acid is a potent chemopreventive agent against bladder cancer whereas it is inactive against mammary cancer. The observed differences in the action of retinoids on carcinogenesis thus could be dependent on the type of retinoid used or on the site of cancer incidence. A similar suggestion has been made by Schroeder and Black (1980).

In vitro cell culture studies: As an alternative to animal studies, cell culture experiments were performed to evaluate the effect of retinoids. One of the most
extensively used in vitro cell culture model is the mouse embryo fibroblast C3H/10T1/2 cell line. This subtetraploid line was developed in the lab of the late Dr. Heidelberger (Reznikoff et al, 1973a, 1973b) and has achieved wide acceptance. This line was selected to exhibit a high degree of post-confluence inhibition of cell division, a major criterion of the nonmalignant phenotype in fibroblasts. The low spontaneous transformation frequency observed in this cell line was particularly beneficial for the usage of 10T1/2 cells in a carcinogen-induced transformation assay. In a typical transformation assay, cells in logarithmic growth phase were treated with a carcinogenic hydrocarbon, namely 3-methycholanthrene (3-MCA) for 24 hours. After that 3-MCA was withdrawn and cells were allowed to reach confluence. Foci of transformed cells were macroscopically visible after 5 weeks. The cultures were fixed and transformed foci were scored for determination of the transformation frequency.

In 1979, Merriman and Bertram showed that weekly treatment with retinyl acetate caused a dose-dependent inhibition of 3-MCA-induced neoplastic transformation in 10T1/2 cells. This action was reversible and after the withdrawal of the retinoid, transformed foci reappeared after 3-5 weeks of latent period. At the same time,
Borek's group demonstrated that retinoids inhibited radiation-induced transformation in the same cell line (Harisiadis et al, 1978). In a later study with a number of both natural and synthetic retinoids, Bertram showed that retinol, retinyl acetate and retinal were equally potent in inhibiting neoplastic transformation, but retinoic acid was ineffective (Bertram, 1980). The lack of activity of retinoic acid was due to its rapid metabolism by 10T1/2 cells (Rundhaug et al, 1988) and when added every three days instead of weekly treatment, retinoic acid exhibited antineoplastic activity (Hossain et al, 1989). Besides the fibroblast model, retinoids have been shown to be active in epithelial cells (Mass et al, 1984).

**Clinical intervention trials using retinoids:** The findings from the above mentioned epidemiological, experimental and in vitro cell culture studies strongly indicate an anticancer activity exerted by retinoids. Based on these evidence, a number of clinical trials to investigate the chemopreventive action of retinoids have already been completed. Skin cancer was the subject of most studies. Treatment with natural and synthetic retinoids caused significant regression of actinic keratoses and basal cell carcinoma (for a detailed list, see Bertram et al, 1987). Application of retinoic acid or isotretinoin

Process of carcinogenesis and points of retinoid action: To understand the molecular mechanism of retinoid action on carcinogenesis, the process of carcinogenesis needs to be described. The concept that carcinogenesis can be divided into stages derives from the pioneering work of Berenblum (1941). Using mouse skin, he showed that a subcarcinogenic topical application of carcinogen could be made carcinogenic by repeated application of croton oil, an irritant hyperplastic agent of plant origin. Subsequent studies with benzopyrene (BP) or dimethyl benzanthracene (DMBA) and croton oil reported similar findings (Boutwell, 1974; Bollag, 1972). It is now generally accepted that there are three major steps in chemical carcinogenesis (Fig 3), i.e., initiation, promotion and expression (Bertram, 1984).

In the initiation phase, within few hours of exposure, carcinogens covalently bind with DNA molecules and form
Figure 3.
Stages of chemical carcinogenesis.
Initiation Promotion Expression

Carcinogen → DNA Adducts → Normal Cell → Initiated Cell → Neoplastic Cell → Tumor

Death Repair
DNA-adducts. Prior to this reaction, most carcinogens require metabolic activation in order to exert their carcinogenic potential (Miller and Miller, 1976). A number of compounds, mainly environmental polycyclic hydrocarbons, have already been characterized as "initiators". After the formation of DNA-adducts, a requirement of cell division has been reported (Kakunaga, 1975). The current theory states that the chemical damage is corrected by most cells. In case of a lethal damage, cell death occur. In few cells, however the damage is converted to a stable biological lesion (mutation) during DNA replication. The identity of DNA-adducts and their interference with DNA replication have been reported (Jennette et al, 1977; Hsu et al, 1977). The nature of genetic damage is not well understood at present, but activation of ras oncogenes after carcinogen treatment has been described (Chen and Herschman, 1989; Fujiki et al, 1989). The genotypically altered "initiated" cells do not exhibit a transformed phenotype, but the genetic lesion is stable over long time periods. The stability of the mutations was demonstrated in studies where long latent periods were observed between carcinogen exposure and cancer incidence in humans (Doll and Peto, 1981). In animal studies, the initiation step can be separated by upto 1 year from the promotion step.
In the promotion phase, the above mentioned initiated cells undergo full transformation after the treatment or exposure with a second group of compounds known as "promoters" or "tumor promoters". 12-0-tetradecanoyl phorbol-13-acetate (TPA), isolated from croton oil by Hecker (1968), was the first promoter to be chemically characterized. Later work by Fujiki's group discovered other naturally available promoters, namely okadaic acid (Suganama et al., 1988), teleocidin, aplysia toxin, palytoxin etc. (Fujiki et al., 1987). Tumor promoting activity was detected in cigarette smoke (Loeb et al., 1984). Treatment with promoters alone did not result in neoplastic transformation (Roe, 1959). The effect of promoters appears to be epigenetic and requires continuous presence, but the mechanism is not yet elucidated. TPA was found to activate a Ca\(^{2+}\)-phospholipid dependent protein kinase, protein kinase C (Nishizuka, 1984) and to induce ornithine decarboxylase (Boutwell et al., 1985). Modulation of these two enzymes is believed to be important for the tumor-promoting action of TPA. Slaga et al. (1980) later showed that the promotional phase can be subdivided into two steps.

The third and last phase of carcinogenesis is the expression of the transformed phenotype. Transformed cells grow abundantly at this stage and give rise to transformed
foci (in cell culture) or tumors (in animals). In animal models and humans, transformed cells were often found to metastasize from their primary tumor sites and migrate to a different site where they can produce secondary tumors.

The inhibitory action of retinoids on in vitro neoplastic transformation was reported to be mediated by blocking the promotional phase of carcinogenesis (Bertram, 1988). The observation by Bertram's group that retinyl acetate inhibited cell transformation even when applied long after carcinogen exposure (Merriman and Bertram, 1979; Hossain et al, 1989), indicated that retinoids did not interfere with the initiation step. The progression of fully transformed cells was not inhibited by retinoids (Merriman and Bertram, 1979). The effect of retinoids was reversible and needed continuous treatment. All of this evidence indicate that retinoids are antipromoters. This suggestion was later experimentally shown in a putative 3-MCA-initiated 10T1/2 cell line. This cell line normally yields transformed focus after a 5-week latent period and the transformation frequency increased after TPA treatment (Mordan et al, 1982). Retinoid treatment completely blocked its spontaneous conversion into fully transformed phenotype and partially blocked TPA-induced transformation. The
The reversible nature of retinoid action was observed in mammary carcinogenesis in rats (Thompson et al., 1979). In the mouse skin model, pretreatment with retinoic acid antagonized TPA-induced tumor promotion (Verma et al., 1979).

In other experimental systems, retinoids were demonstrated to interfere with the activation of aflatoxin B₁, a potent liver and bladder carcinogen (Suphakaran et al., 1983). In mouse skin, retinoid treatment caused regression of papillomas (Bollag, 1972). Several in vitro studies showed that retinoid treatment inhibited growth of fully transformed cells (Gabbert et al., 1988). Apart from interacting with different phases of chemical carcinogenesis, retinoids were also demonstrated to have an antimetastatic potential (Schleicher et al., 1988).

1.1.4. MECHANISM OF RETINOID ACTION

Role of retinoid-binding proteins: The mechanism by which retinoids exert their diverse biological actions including their anticancer effects has been studied by many groups. The involvement of CRBP and CRABP in retinoid action was predicted by Chytil and Ong (1984). These two proteins bind with the respective retinoids after the retinoids enter the cell. Both the proteins (mol. wt. 15
kD) act as carriers of retinoids and were shown to deliver the retinoids to the nucleus. CRBP leaves the nucleus after delivering retinol (Takase et al, 1979; Liau et al, 1981), whereas CRABP was reported to remain in the nucleus (Sani and Donovan, 1979). The retinoids, either free or bound, were suggested to interact with the DNA and modulate gene expression (Chytil and Ong, 1984). Different studies have demonstrated a direct correlation between the ability of binding to either CRBP or CRABP and the biological activity of retinoids (Sani and Corbett, 1976; Jetten & Jetten, 1979). In a recent study, it was shown that the expression of CRABP is regulated during morphogenesis and differentiation of mouse limb and this differential expression of CRABP was suggested to be important in maintaining the retinoic acid gradients (Dolle et al, 1989). However, contrasting findings have also been reported. Studies with human myelocytic leukemia cell line HL-60, demonstrated that although differentiation took place in presence of retinoic acid, CRABP could not be detected in these cells (Breitman et al, 1981, Douer & Koeffler, 1982). A study with synthetic retinoids by Jetten et al (1987) also showed a lack of direct correlation between the presence of the binding proteins and the biological activity of retinoids.
The role of nuclear retinoic acid receptors: Retinoids enter the nucleus and modulate gene expression. This property of retinoids is very similar to that of steroid hormones. After entering the cell, steroid hormones bind with their receptors and the hormone-receptor complex then interacts with the DNA (Evans, 1988; O'Malley, 1990). A number of steroid hormone receptors have already been isolated and characterized. These receptors (mol. wt. 50 kD) contain three major regions of conserved amino acids. Region I is located within the interior of the molecule and comprises the DNA-binding domain. Eight of the conserved cysteins in this domain are thought to form two zinc fingers; it has been suggested that the first finger contains primary information for sequence specificity of binding while the second finger stabilizes binding of receptor to its DNA response element. Region II and III (located in the C-terminal side) have been described as the ligand (steroid hormones) binding domains. It is likely that the receptor makes multiple contacts with the ligand. The N-terminal region in the steroid receptor superfamily is less conserved. It is considered to be transcription modulators. Receptors for thyroid hormones also belong to this receptor superfamily. The present hypothesis about the steroid receptors is that after the ligand (hormone) binds with the receptor, the receptor undergoes a conformational
change and binds with a specific nucleotide sequence (usually located 5′-upstream to the gene regulated by the hormone) in the DNA molecule. This short nucleotide sequence is called the steroid response element. The receptor-response element binding event causes enhanced transcription of the target gene.

The requirement of such receptors for retinoid action was speculated by Roberts and Sporn (1984). Neither CRBP nor CRABP qualify for the receptors, because CRBP does not enter the nucleus and presence of CRABP is not required for mediating the effect of retinoic acid. In late 1987, two independent studies reported the existence of DNA-binding nuclear retinoic acid receptor (RAR) molecules in cells (Giguere et al, 1987; Petkovitch et al, 1987). These RAR molecules are homologous to steroid hormone receptors and bind with retinoic acid with high affinity. Subsequent studies showed that there are 4 types of RARs, namely a, β, t and s (Giguere et al, 1987; Petkovitch et al, 1987, Zelent et al, 1989, Ragsdale et al, 1989). Isoforms of these receptors were also described (Kastner et al, 1990; Giguere et al, 1990). Usage of specific antibodies revealed that, similar to steroid hormone receptors, both RARα and RARβ are 51 kD proteins (Gaub et al, 1989). A very recent report from Evans' group described the existence of another novel
retinoic acid receptor, RXR (Mangelsdorf et al, 1990). It had low sequence homology with the other RARs, yet showed considerable amount of reactivity towards retinoic acid and retinal. When compared with RARa, RXR demonstrated a different pattern of activity. Unlike other RARs, it was highly expressed in liver. It was proposed that another subfamily of retinoic acid receptors may exist to provide a second transduction pathway for the diversity of retinoid action (Mangelsdorf et al, 1990).

The expression of the RARs have been found to be organ specific. RARa was found to be expressed in most of the tissues examined, whereas RARβ transcripts were high in human kidney, prostate, cerebral cortex (de The et al, 1989) and in rat lung, liver, heart (Rees et al, 1989). RARt was predominantly expressed in skin (Zelent et al, 1989). RXR was found to be highly expressed in liver, kidney, lung (Mangelsdorf et al, 1990). Human RARa was encoded by chromosome 17 (Petkovitch, 1987) whereas the genetic loci for RARβ and RARt were chromosome 3 and 12 respectively (Brand et al, 1988; Ishikawa et al, 1990).

Although the RARs are closely related molecules, difference in their properties have been observed. Gene expression of RARβ, but not RARa or RARt, was induced by
retinoic acid (de The et al, 1989; Hu & Gudas, 1990, Martin et al, 1990). However cAMP analogs were reported to decrease the expression of all the RARs (Hu & Gudas, 1990). The difference in their retinoid binding affinity was also reported (Mangelsdorf et al, 1990; Hashimoto et al, 1990).

The mode of action of RARs is not well understood yet. It is predicted that they would act similar to steroid hormone receptors, e.g. the ligand-receptor complex will bind with a specific sequence of the target gene, known as the retinoic acid response element (RARE), which is crucial for the specificity of steroid/RA action (O’Malley, 1990; Evans, 1988; Wolf, 1990). In Fig 4, a schematic diagram of genomic action of retinoids (principally retinoic acid) is presented. So far two target genes that are inducible by RA have been reported to possess RARE sequence, the laminin gene (Vasios et al, 1989) and the RARβ gene (de The et al, 1990). Since RAR and the thyroid hormone receptor T₃R showed great homology in the DNA-binding domain (Giguere et al, 1987), it is possible that these two receptors can regulate the expression of overlapping networks of genes. Experimental proof for this suggestion was provided by Umesono et al (1988) and Graupner et al (1989), who showed
Figure 4.
Schematic diagram of retinoic acid receptor (RAR) action.
Reproduced from Wolf (1990).
that RAR can bind with the T\textsubscript{3}-responsive element and increase T\textsubscript{3}-specific gene expression. Glass et al (1989) observed that RAR can form a heterodimer with thyroid hormone receptor and this heterodimer is capable of regulating gene expression. Recently Schule et al (1990) showed that a response element in human osteocalcin gene is recognized by RARs as well as jun-fos oncoproteins.

Collins et al (1990) showed that in HL-60 cells, RA-induced differentiation is directly mediated through RAR\textalpha. In F9 cells, the expression of the receptors was found to be dependent on the state of cellular differentiation (Martin et al, 1990). The expression of different RARs have been correlated with mouse facial development (Osumi-Yamashita et al, 1990), mouse bone development (Noji et al, 1989), regenerating amphibian limb (Giguere et al, 1990) and mouse embryogenesis (Ruberte et al, 1990). Using antisense DNA to RAR\textalpha, Cope and Wille (1989) demonstrated the involvement of RAR\textalpha in the transduction of retinoid signal response. Recent observations indicate that the alteration of gene expression is mediated through RARs, whereas the retinoid-binding proteins are involved in maintaining the retinoid-gradient (Cope and Wille, 1989; Dolle et al, 1989).
1.1.5. TARGETS OF RETINOID ACTION: RELATIONSHIP WITH ITS ANTINEOPLASTIC ACTIVITY

It is presently apparent that most, if not all, of the biological effects produced by retinoids are mediated through RARs, which result into the alteration of gene expression. Retinoids have been reported to modulate a number of genes which may play crucial roles in the inhibitory effects of retinoids on neoplastic transformation. Among them are membrane and cytoskeletal proteins (Chytil, 1984), oncogenes (Roberts et al, 1985; Imiazumi and Breitman, 1988; Miller et al, 1990), growth factor and growth factor receptors (Miller et al, 1990; Roberts et al, 1984; Zheng and Goldsmith, 1990; Abbott et al, 1988). Retinoids inhibited PK-C and ODC (Cope and Boutwell, 1985; Verma et al, 1978) and platelet-derived growth factor (PDGF)-induced DNA synthesis (Mordan, 1989). In addition to modulating these diverse biomolecules, retinoids were recently reported to elevate gap junctional communication (Hossain et al, 1989; Mehta et al, 1989), which comprises part of the present dissertation. This cellular process has been demonstrated to be extremely important in the regulation of neoplastic transformation (Yamasaki, 1990; Mehta et al, 1986). Since modulation of gap junctional communication (GJC) is the topic of my
dissertation research, I wish to present a short description of GJC and its role in carcinogenesis in the following section.

1.2. GAP JUNCTIONAL COMMUNICATION

The interactions between cells in tissues or in the whole organism are extremely important in maintaining vital processes. Generally there are a number of ways a cell could receive or send signals (Loewenstein, 1981; Welsch, 1990), among them are hormones and growth factors, which are sent via the blood stream and act on distantly located target cells. Another way would be via signal molecules embedded in the membrane, which can directly interact upon cell contact. The most direct way of signal transfer is however through a channel between contacting cells. This is called gap junctional communication.

The first indication of direct cellular communication came from the work on heart muscle which was shown to be electrically coupled (Weidmann, 1952; Dewey and Barr, 1962). Later work by Kanno and Loewenstein (1964) demonstrated that large hydrophilic molecules such as fluorescein, can traverse the junctional pathway. This
knowledge distinguished the gap junctions from the ordinary ion channels.

1.2.1. STRUCTURE OF GAP JUNCTIONS

Gap junctions have been characterized as hydrophilic channels traversing connecting cell membranes (Fig. 5). They have an inner diameter of about 15-20 Å, wide enough for the passage of inorganic ions, metabolites, building blocks of biomolecular synthesis and the high energy phosphates. The pathway is leakproof to the exterior. They are nonspecific and allow for passive diffusion. The channel is made of two tightly joined hemichannels, each donated by the participating cell (Loewenstein, 1981). The involvement of gap junctions in intercellular communication was demonstrated by Loewenstein's group, by showing that communication-competence in cells goes hand in hand with the presence of gap junctions (Azarnia et al, 1974; Larsen et al, 1977).

Electron microscopy of gap junctions revealed that they are arranged in clusters, called gap junction plaques (Hertzberg, 1984; Ueda, et al, 1987). The channels were formed one channel-at-a-time (Chow and Young, 1987). X-ray diffraction showed that the particles are firmly bonded pairs (Makowski et al, 1977; Casper et al, 1977). The unit
Figure 5.

Schematic diagram of gap junctions.
constituted by a particle pair has been termed as a connexon (Casper et al., 1977). In electron microscopic rotational views, the particles of the arthropod gap junction exhibit a sixfold symmetry, suggesting that they are made of six subunits (Peracchia, 1973). Later work (Unwin and Zampighi, 1980; Zampighi and Unwin, 1980) reported that the six subunits are roughly rod-shaped, 2.5 nm diameter, 7.5 nm long. The existence of "open" and "closed" state of gap junctions was also reported by them. It was suggested (Fig 6) that the subunits slide on each other in a counterclockwise fashion to achieve the open state (Zampighi and Unwin, 1980). Later work demonstrated that the channels exist as either fully open or fully closed state (Verselis et al., 1986; Burt and Spray, 1988).

1.2.2. PROTEIN COMPONENTS OF GAP JUNCTIONS: THE CONNEXINS

A number of studies were performed on the components of the gap junctions. It was found that there are not just one but a number of proteins (Willecke and Traub, 1990) which share similar features. These proteins were named as connexins (Beyer et al., 1987) and they belong to the same family.

Connexin32: Hertzberg and Gilula (1979), Henderson et al. (1979), and Hertzberg (1984) purified a protein of 26-27
Figure 6.
Opening and closing of gap junctions.
Redrawn from Unwin and Zampighi (1980).
Rotation of subunits and sliding
kD as a major component from isolated liver junctional plaques. They also observed a high molecular weight band (46 kD), which was believed to be the dimeric form of the 27 kD. The presence of a 21 kD protein in the gap junction preparations were reported by Henderson et al (1979) and Nicholson et al (1987). Later work proved that 21 kD proteins are components of gap junctions and they are different from 27 kD protein (Traub et al, 1982, 1989). The involvement of 27 kD protein in gap junctions was shown by the immunocytochemical studies, where antibodies to this protein were localized to gap junction plaques (Dermietzel et al, 1987, Traub et al, 1982). The role of this protein in intercellular communication was established by several workers who showed that communication can be inhibited by microinjecting 27 kD antibody (Hertzberg et al, 1985, Warner et al, 1984). Dahl et al (1987) showed that by microinjecting mRNA for 27 kD protein in oocytes, cell-cell communication can be induced. After transfection with the cDNA for this protein, communication was established in communication-incompetent cells (Eghbali et al, 1990). Further evidence was provided by Young et al (1987) by showing that 27 kD protein alone is sufficient to establish communication in lipid bilayers.

The cloning and characterization of the 27 kD rat liver gap junction cDNA was first reported by Paul (1986)
and then by Kumar and Gilula (1986). Both the reports showed that the cDNA codes for a 32 kD protein. Subsequently the 27 kD protein was renamed as connexin32 (Cx32). The appearance of 27 kD, rather than a 32 kD protein band in SDS-gel electrophoresis was believed to be due to post-translational modification (Kumar and Gilula, 1986). However Green et al (1988) showed that the difference in molecular weights was due to their altered mobility in low percent acrylamide gels. Comparison of human liver connexin 32 cDNA with rat liver Cx 32 cDNA showed strong sequence homology in the protein coding region (Kumar and Gilula, 1986). The derived total amino acid sequence indicated that there are four transmembrane hydrophobic regions, of which the third transmembrane has amphiphilic property (Fig 7). Later studies supported this topological model (Cascio et al, 1990; Milks et al, 1988, Hertzberg et al, 1988). It has been suggested that the amphipathic region from six Cx 32 subunits may form the transmembrane channel (Willecke and Traub, 1990). Apart from Cx32, Cx26 (which corresponds to the 21 kD mouse and rat liver protein) has been characterized and found to have domains similar to Cx32 (Nicholson and Zhang, 1988).

**Connexin43:** In heart, a 28 kD protein was isolated from the gap junctions (Gros et al, 1983). Later work by
Figure 7.
Proposed topographical model of connexins.
Reproduced from Willecke and Traub (1990).
cytoplasm

extracellular

'gap'

membrane

hydrophilic region of amphiphilic helix

connexin (subunit of hemichannel)
Manjunath et al (1985) showed that it was a proteolytic product of 43-47 kD precursor. Cx 43, as it was named later, was found to be covalently cross-linked through disulfide bridges whose significance is yet to be known (Manjunath and Page, 1986). The cDNA for Cx 43 was isolated from rat heart by low stringency cross hybridization using rat liver Cx 32 cDNA (Beyer et al, 1987). Both Cx 32 and Cx 43 exhibited sequence homology and were suggested to belong to the same gene family (Kistler et al, 1988; Beyer et al, 1990). The role of Cx43 in establishing GJC was demonstrated by Werner et al (1989). They showed that microinjection of Cx43 mRNA into frog oocytes resulted into gap junctional competence. The proposed secondary structure for Cx 43 is quite similar to that of Cx 32, but has a longer cytoplasmic domain (Yancey et al, 1988). It was proposed that this cytoplasmic tail may regulate junctional permeability. Very recently, Beyer (1990) reported the presence of two more connexins, namely Cx42 and Cx45 in chicken heart. The expression of these two proteins is developmentally regulated although the significance is yet to be known. Apart from these connexins, the Xenopus homologs of Cx32 and Cx43 have been recently reported (Gimlich et al, 1988, 1990; Ebihara et al 1989).
**MP 28:** Another type of gap junction protein was found in eye lenses. They were called major intrinsic protein (MIP) and have a molecular weight of 26 kD. Later on they were termed as MP 28 (Johnson et al, 1988). They are immunologically different from the liver Cx 32 (Hertzberg et al, 1982). MP 28 cDNA was sequenced by Gorin et al (1984) and the deduced amino acid sequence was not similar to the known gap junction proteins thereby raising the possibility that it may belong to a separate class.

**Expression of connexins:** Presently it appears that the family of connexins are differentially expressed in different tissues. Cx 32 is expressed in liver, kidney, brain and stomach (Beyer et al, 1988), Cx 26 in liver, kidney and brain (Nicholson and Zhang, 1988), Cx 43 in heart, kidney, ovary, lens (Beyer et al, 1988). It was suggested by Willecke and Traub (1990) that Cx 43 may have had an ananchestral role for mammalian connexins and Cx 32 and Cx26 may have derived from Cx43 by different deletions and other genetic alterations.

1.2.3. BIOLOGICAL ROLE OF GJC

**Tissue homeostasis:** Gap junctional communication has been suggested to regulate a number of cellular events
(reviewed by Loewenstein, 1981; Pitts and Finbow, 1977; Caveney, 1985). As it was indicated by these authors, gap junctions provide a direct pathway for the transfer of various small molecules. These channels are present in nearly all animal tissues (For a detailed list, see Loewenstein, 1981). The most basic physiological role of them is the maintenance of tissue homeostasis, by transferring essential biological precursors, e.g., nucleotides, sugar phosphates, amino acids, inorganic ions, choline phosphate etc. (Pitts and Finbow, 1971). Due to the limitation of the channel diameter, large macromolecules such as DNA, RNA, proteins, glycoproteins are not transferred, thereby maintaining cellular identity.

**Embryogenesis:** During embryogenesis and tissue development, the role of gap junctional communication has been extensively studied (Caveney, 1985; DeHaan and Chen, 1990). It was seen that gap junctional communication is needed for normal oocyte growth and maturation (Eppig, 1982) and early embryonic development (Ducibella et al, 1975). The existence of different communication compartments in developing embryo was reported by many studies (Weir and Lo, 1984; Lo, 1988; van den Biggelaar and Serras, 1988). These compartments are a group of cells which are already destined to form a certain tissue (Weir and Lo, 1984). During the final stage of development,
tissue differentiation, gap junctions were found to be absent (Caveney, 1985; Kam et al, 1987). Further evidence for the importance of junctional transfer of growth regulatory signals in development was provided by Fraser et al (1987, 1988), where disruption of communication interfered with the patterning process in hydra. Similar findings were reported by Allen et al (1990) in the developing chick limb bud model. In the *Xenopus* embryo, differential regulation of connexin mRNAs was reported (Gimlich et al, 1990). Absence of cell-cell communication in regenerating liver (Dermietzel et al, 1986b) and its appearance after the cell proliferation during the regeneration of imaginal disc in *Drosophila* (Bryant and Fraser, 1988) suggest its regulatory mode of action.

1.2.4. ROLE OF GJC IN MODULATING CELLULAR GROWTH: RELATIONSHIP TO CANCER PREVENTION

Cancer cells are communication-incompetent: The involvement of gap junctions in regulating growth and differentiation has been originally suggested by Loewenstein and Kanno (1964). The hypothesis was that junctional communication serves as a passage for the growth regulatory signals from one cell to another. The importance of GJC in cancer was first demonstrated by Loewenstein and Kanno (1966) by showing that cancerous cells, which
presumably lost growth control, did not communicate, whereas their normal counterparts were capable of cell communication. Subsequent studies demonstrated that junctional competence and growth-control competence went hand in hand (Azarnia et al, 1977; Larsen et al, 1977).

These early findings established the foundation of later research involving the relationship between gap junctional communication and the development of cancer, since in cancer, cell proliferation controlling mechanism is lost. It was shown that the loss of intercellular communication in transformed cells correlates with their metastatic potential (Nicholson et al, 1988). Highly metastatic cells also showed less gap junctions (Ren et al, 1990).

**Tumor promoters inhibit GJC:** The ability of tumor promoters to inhibit junctional transfer of tracer dye (Fitzgerald et al, 1983; Enomoto et al, 1985a; Wade et al, 1986; Hossain et al, 1989) or metabolic transfer (Yotti et al, 1979; Davidson et al, 1985) indicated that blockade of cell-cell communication is necessary for tumorigenesis. This hypothesis was further substantiated by Rivedal et al (1985) who showed that a TPA-resistant cell line did not
transform or exhibit decreased communication after TPA treatment, whereas TPA-sensitive cells exhibited both. Since TPA was reported to activate protein kinase C (Nishizuka, 1984), this enzyme was postulated to be involved in the action of TPA. The inhibitory effect of diacylglycerol, the endogenous activator of protein kinase C (Enomoto and Yamasaki, 1985b) and the capability of PK-C to phosphorylate gap junction protein (Takeda et al, 1987) supported the above hypothesis. The observation that (a) TPA-induced inhibition of cellular communication was associated with a decreased number of gap junctions in Chinese hamster V-79 cells (Yancey et al, 1982), (b) the inhibition was rapid (Enomoto et al, 1981) and (c) reversible even in the presence of RNA or protein synthesis inhibitors (Yamasaki et al, 1983) suggest that phorbol esters affect the organization of functional gap junctions rather than inhibiting the expression of the protein components. Another class of tumor promoter, phenobarbital, was however shown to decrease the expression of Cx 32 in rat liver cells (Mesnil et al, 1988) indicating the existence of different pathways to inhibit gap junctional communication.

Oncogenes block GJC: Apart from the tumor promoters, several oncogenes have been demonstrated to inhibit gap junctional communication. Inhibition by src oncogene was
reported by Atkinson et al (1981) and Azarnia and Loewenstein (1984). The involvement of src gene product in this phenomenon was indicated by Chang et al (1985). Subsequent work confirmed that the activation of pp60V-src tyrosine protein kinase is correlated with the inhibition of cell communication (Azarnia et al, 1988). Recently Crow et al (1990) showed that in Rous sarcoma virus infected cells, tyrosine phosphorylation of Cx43 correlated with the loss of communication. Similar conclusions were reported by Hyrc and Rose (1990) who demonstrated that a 40-45 kD protein is heavily phosphorylated by v-src. Several Ha-ras transfected cell lines showed a similar loss of communication (El-Fouly et al, 1989; Bignami et al, 1988, Vanhamme et al, 1989). The molecular mechanism is yet to be known. myc oncogene transformed cells however did not show this lack in communication but they were not fully transformed either (Bignami et al, 1988). The expression of other oncogenes and protooncogenes was also shown to be involved in the modulation of intercellular communication (Beer et al, 1988; Atkinson and Sheridan, 1984).

Inhibition of GJC by other factors: Several other factors were also shown to inhibit GJC. Among them, the most prominent ones are the growth factors. In certain
studies they were demonstrated to promote carcinogenesis (Mordan, 1989; Hamel et al, 1988; Goustin et al, 1986) and inhibit junctional communication (Hamel et al, 1988; Madhukar et al, 1989). Testosterone, which was shown to facilitate the development of prostate cancer (Takizawa and Hirose, 1978), was recently shown to inhibit GJC in human cells (Kihara et al, 1990).

1.2.5. ENHANCEMENT OF GJC LEADS TO THE SUPPRESSION OF NEOPLASTIC TRANSFORMATION

Research findings in the above sections strongly suggest that loss of cell communication is required for the development of neoplasia. The other side of the story would be whether the incidence of tumorigenesis can be blocked by elevated communication. It was organelle proposed by Stoker that normal cells can regulate the growth of tumor cells (Stoker et al, 1966). In C3H/10T1/2 cell line, it was shown that when cocultured with normal cells, carcinogen-initiated cells did not undergo full transformation (Mordan et al, 1983). In vivo studies by Watanabe et al (1988) showed that unirradiated thyroid cells can inhibit carcinoma formation by radiation-initiated cells. Similar observations were made by Bignami et al (1988), Dotto et al (1988) and Herschman and Brankow (1986), where
transformation by myc or Ha-ras oncogene or by UV-radiation resulted into transformed phenotypes that were suppressed by surrounding normal cells. TPA, which is known to inhibit communication, released the transformed cells from the growth control (Bignami et al, 1988; Herschman and Brankow, 1987). In 10T1/2 system, fully transformed cells were also growth inhibited by normal cells (Bertram, 1977) and this growth inhibition was increased by cAMP-phosphodiesterase inhibitors (Bertram, 1979) or by adenyl cyclase activators (Bertram and Faletto, 1985; Mehta et al, 1986). The lack of any growth inhibition by the drugs in absence of normal cells suggested that cellular cAMP is involved in promoting cellular communication between the normal and transformed cells. cAMP was reported to elevate GJC in many systems (De Mello and van Loon, 1987; Spray and Burt, 1990; Veld et al, 1985). This action of cAMP was shown to be mediated through enhanced expression and subsequent phosphorylation of connexins (Saez et al, 1986a; Traub et al, 1987). Later work demonstrated that in 10T1/2 model, cAMP-induced growth control was indeed highly correlated with the increase in communication (Mehta et al, 1986; Hossain and Bertram, 1990). Similar effects were reported by Yamasaki's group where transformed phenotype was completely reversed by cAMP in presence of normal cells and it was associated with an induction in heterologous
communication between these cell types (Yamasaki and Katoh, 1988). All these evidence point out that establishment of intercellular communication between normal and transformed cells can allow the passage of growth regulatory signals from normal to the transformed cells causing the growth arrest of the latter.

1.3. AIM OF THE STUDY

The main focus of my dissertation research is to understand the molecular mechanism of the inhibitory action of the retinoids on neoplastic transformation in the 10T1/2 mouse fibroblast model reported previously (Merriman and Bertram, 1979; Bertram, 1980; Hossain et al, 1989). However, retinoids were found to be completely ineffective in suppressing the growth of transformed cells in coculture with normal cells (Merriman and Bertram, 1979; Mehta et al, 1986). From the upper sections, it is evident that gap junctional communication plays a crucial role in the inhibition of neoplastic transformation, or in the suppression of transformed cell growth. In my study, I wanted to investigate whether these diverse effects of retinoids can be explained by the modulation of gap junctional communication. The major questions that were asked in the present study are presented below.
(1) Since elevation of GJC was suggested to be required in the inhibition of neoplastic transformation, is retinoid-induced inhibition of neoplastic transformation associated with an increase in GJC?

(2) If GJC is indeed increased by retinoids, what is the time course of induction and the dose-response [profile of retinoid action?]

(3) Are these two cellular events (i.e., inhibition of neoplastic transformation and induction of GJC) related?

(4) Do retinoids affect GJC in other 10T1/2-derived cell lines?

(5) Is the observed antagonism between the effects of TPA and retinoids reflected in their effects on GJC?

(6) 10T1/2 cells were shown to inhibit the spontaneous transformation of the initiated cells (Mordan et al, 1983). Is there any existence of heterologous GJC between these two cell types? What are the effects of retinoids and TPA on this heterologous GJC?

(7) There is evidence that retinoids enhance the gene expression of Cx43, a major gap junction protein in 10T1/2 cells (Rogers et al, 1990). Is this effect restricted to only the tested synthetic retinoid or to only the normal cells?

Retinoid treatment was found to increase the phosphorylation of Cx43. Can this phenomenon be demonstrated in
other cell lines using different retinoids? What is the cellular localization of Cx43?

(9) Although very effective in inhibiting carcinogen-induced-cell transformation, retinoids failed to inhibit the growth of transformed cells in coculture with normal cells. Is this action of retinoids related to their effects on normal:transformed heterologous GJC? Retinoids antagonized the growth inhibitory action of cAMP; is this effect of retinoids mediated via the blockade of cAMP-induced heterologous GJC?

(10) What are the other possible ways through which retinoids antagonize cAMP-induced GJC?
CHAPTER II
METHODS

2.1. CELL CULTURE

The parental C3H/10T1/2 CL8 and other derived cell lines were cultured in basal medium Eagle's with Earle's salts (GIBCO), supplemented with 5% fetal calf serum (Irvine Scientific or HyClone Laboratories) and 25 ug/ml gentamicin sulfate and were incubated at 37°C in 5% CO2 in air as described previously (Reznikoff et al, 1973). In Table 2, the sources and description of the cell lines used are given. Unless otherwise mentioned, cells were seeded onto plastic culture dishes and grown to confluence with weekly medium change. Drug treatment started after atleast two days of last medium change. Since some transformed cell lines rapidly lift off from the culture dish after attaining confluence, in some experiments they were treated either 3 days after seeding or when they were subconfluent. Cell counts were done with an electronic Coulter counter.

2.2. PREPARATION OF DRUGS

All the drugs used in the study were dissolved in acetone. Concentrations of retinol and retinoic acid (Sigma) were determined by their UV absorbance and from published extinction coefficients (Ito et al, 1974). The
TABLE 1

Cell lines used in the present study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transforming Agent</th>
<th>Derivation</th>
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<tbody>
<tr>
<td>C3H/10T1/2</td>
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<td>C3H Mouse Embryos. Reznikoff et al (1973)</td>
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<tr>
<td>Initiated 10T1/2</td>
<td>3-MCA</td>
<td>10T1/2 cells. Mordan et al (1982)</td>
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<td>(H23)</td>
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<tr>
<td>4B</td>
<td>3-MCA</td>
<td>10T1/2 cells. Mehta et al (1986)</td>
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synthetic benzoidal derivative of retinoic acid, tetrahydrotetramethyl naphthalenyl propenylbenzoic acid (TTNPB) and the cyclic AMP-phosphodiesterase inhibitor, Ro 20-1724 were kind gifts from Hoffman-La Roche (Nutley, NJ). The cAMP-dependent protein kinase inhibitor, H-89 was a generous gift from Dr. Hidaka, Nagoya University, Japan. Cultures were mildly agitated after the addition of the drugs. The final concentration of acetone was 0.5%. For experiments of longer than 3-4 days of duration, cultures were treated twice weekly with the retinoids since previous work have shown that 10T1/2 cells rapidly metabolize retinoic acid (Rundhaug et al, 1987).

2.3. GAP JUNCTIONAL COMMUNICATION ASSAY

Confluent cultures were microinjected with the fluorescent tracer dye Lucifer Yellow CH (Sigma). This dye was reported to travel through gap junctions and was utilized in the previous studies (Mehta et al, 1986; 1989; Hossain et al, 1989). Micropipettes were prepared from glass capillary tubes with the aid of a Flaming-Brown micropipette puller, model P-80/PC (Sutter Instrument Co., Novata, CA) and they were back filled with a 10% solution of lucifer yellow (in 0.33 M LiCl). The fluorescent dye was microinjected at constant pressure into cells (duration of
microinjection, 2-3 seconds) with an Eppendorf
Microinjector. The total number of fluorescent neighboring
cells was counted after 10 minutes and served as an index
of junctional communication. The communicating networks
were digitized using a video camera and stored in a
computer for later photography.

For heterologous junctional communication
(communication between two different cell types) assay,
fluorescent bead labeled cells (see section D) was
microinjected with lucifer yellow and the passage of this
dye into adjacent non bead labeled cells was quantitated.
Along with the number of fluorescent cells, a different
index was used for heterologous GJC, i.e., communication
frequency, which is the percent of injected bead labeled
cells that communicated with their non labeled neighbors.

2.4. COCULTURE EXPERIMENTS

Coculture experiments were performed according to a
previous study (Mehta et al, 1986). For microinjection and
3H-Thymidine labeling studies, cells to be overlayed were
incubated for 90 minutes at 37°C with fluorescent
microspheres (Polyscience, Warrington, PA) diluted 1:100
with sterile PBS, pH 7.4. After that, the cells were
washed twice with sterile PBS, trypsinized, centrifuged and

58
the cell pellet was washed 3 times with PBS to get rid of any unincorporated free beads. These bead labeled cells were then suspended in medium and were seeded on either confluent 10T1/2 cells (heterologous cultures) or dense cultures of the same cell lines (homologous cultures) at a density of 10,000 per 60 mm dish. Drug treatment started 4 hours later. After two days, dishes were probed for junctional communication as described in section 2.3. Parallel dishes were incubated with $^{3}$H-Thymidine (1 uCi/ml) for 2 hours at 37°C and then were fixed with methanol:acetic acid (3:1, v/v) for 30 minutes. These cultures were then thinly coated with a photographic emulsion (Kodak), exposed for 1 week in dark box and then developed using D-19 developer (Kodak). To quantitate the labeling index, fluorescent bead labeled cells were first identified under a Nikon epifluorescence microscope and then they were examined for positive thymidine labeling indicated by dark nuclei. In the present study, labeling index is thus the percent of bead labeled cells that were $^{3}$H-Thymidine labeled.

2.5. CYCLIC AMP RADIOIMMUNE ASSAY

Cyclic AMP was extracted from the cell culture medium as well as from the cells by the procedure of
Bertram and Faletto (1985) with minor modification. 10T1/2 and 4B cells were grown in 100 mm culture dishes. The former line was treated after reaching confluence while the latter was treated at subconfluent stage. For the extraction of medium cAMP, 200 ul of medium was removed and added immediately to 800 ul of cold acid:ethanol (1 ml N HCl in 100 ml absolute ethanol) and left on ice for 5 min, after which they were centrifuged at 10,000 g for 5 minutes. The supernatant was collected and stored at -70°C. Cells were briefly washed with ice-cold PBS and then, 1 ml of acid:ethanol was added to the dishes. After 5 minutes, cells were removed by scraping and centrifuged at 10,000g for 5 min. The pellet was reextracted with 500 ul of acid ethanol and the supernatants were pooled. The supernatants from cell and medium samples were frozen at -70°C and then were lyophilized and stored at -20°C. Samples were reconstituted for assay with 200-500 ul of 50 mM sodium acetate, pH 6.2.

cAMP was assayed following the protocol of a commercial radioimmune assay kit (New England Nuclear, Boston, MA). In the assay tubes, 100 ul of cell extracts were pipetted and were acetylated with 5 ul of freshly made acetylating reagent (acetic anhydride:triethanolamine, 1:2, v/v). They were then added with 100 ul of diluted succinyl cAMP tyrosine methyl ester-[125I] and 100 ul of
diluted cAMP antiserum (Kew Scientific, Ohio). Tubes were vortexed and incubated overnight at 4°C. On the next day, 1 ml of ice-cold charcoal solution [activated charcoal (2 mg/ml)/bovine serum albumin (2.5 mg/ml) in 50 mM Na-acetate buffer] was added to each tube. They were vortexed and centrifuged at 2,200 rpm for 40 minutes at 4°C. The supernatants were decanted and counted in a gamma counter for 1 minute. The concentration of cAMP in the samples were determined by comparing them with the standard curve prepared by using known concentrations of cAMP.

2.6. NORTHERN BLOTTING OF CONNEXIN43 mRNA

Isolation of total RNA, agarose gel electrophoresis and northern blotting, cDNA labeling were performed according to Maniatis et al (1982) with minor modifications. The cDNA/RNA hybridization was done following a protocol by Fregien et al (1983).

2.6.1. ISOLATION OF TOTAL RNA

For each sample, 1.5 - 2 x 10^7 cells were used. Dishes were washed with cold PBS/10 mM EDTA and cells were removed from the dish by scraping in the same solution. After centrifugation at 3,000 rpm for 10 minutes, the obtained
cell pellets were dissolved in 8 ml of guanidine isothiocyanate (GIT) buffer (4 M GIT, 0.3 M Na-acetate, 0.8% B-mercaptoethanol) and this lysate was overlaid onto 4 ml cesium chloride and centrifuged at 32,000 rpm for 21 hours at 20°C. The RNA pellet was dissolved in proteinase K buffer (0.01 M NaCl, 0.01 M EDTA, 0.1% SDS in 0.01 M Tris, pH 7.5) and extracted three times with phenol/chloroform followed by a cold ethanol precipitation for 2-3 hours. After centrifuging the samples at 10,000g for 30 minutes, RNA was pelleted and washed twice with 70% ethanol, vacuum dried and dissolved in 15-20 ul of sterile water. 1 ul of this solution was then diluted with 700 ul of sterile water to quantitate and determine the purity of total RNA by UV spectroscopy at 260/280 nm. In some later studies, total RNA was isolated following the protocol of a commercially available kit (Cinna/Biotecx, Friendswood, Tx).

2.6.2. AGAROSE GEL ELECTROPHORESIS AND NORTHERN TRANSFER

10 ug of total RNA was mixed with TE buffer (1 mM EDTA in 10 mM Tris, pH 7.4) and loading buffer keeping the final volume 10-12 ul. The samples and standard RNA markers (BRL, Gaithersburg, MD) were then loaded on a 1% agarose/16.5% formaldehyde gel and run for 4 hours at 100 V. After that the gel was cut, the RNA markers were stained in Ethidium
bromide and the rest was partially hydrolyzed in 50 mM NaOH/10 mM NaCl for 45 minutes, followed by a neutralizing wash in 0.2 M Tris, pH 7.4 for 45 minutes and then soaked in 20x SSC (1x SSC = 0.15 M NaCl, 0.015 Na citrate, pH 7.0) for 60 minutes. The gel was then inverted and blotted overnight onto a 0.22 um nitrocellulose membrane (Schleicher and Schuell, Keene, NH) by capillary action using 20x SSC buffer. After transfer, the blot was washed for 10 minutes in 20x SSC and vacuum baked at 80°C for 2 hours.

2.6.3. HYBRIDIZATION WITH CONNEXIN43 cDNA

The blot was washed three times in a plastic seal-a-meal bag; the first 30 minute wash was with 4x SET (1x SET = 0.015 M NaCl, 0.002 M EDTA, 0.03 M Tris, pH 7.4) at room temp. The second 3 hour wash was with 4x SET/50% deionized formamide/0.1% SDS/10x Denhardt’s (100x denhardt’s = 2% BSA, 2% Ficoll 400,000 MW, 2% Polyvinylpyrrolidone 360,000 MW) at 42°C, the third wash (30 minutes at 42°C) contained 10 ug/ml poly adenylic acid/250 ug/ml denatured salmon sperm DNA/0.1% Na-pyrophosphate along with the materials present in the second wash. During the washes, a 1.5 kB cDNA clone of connexin 43 (kind gift from Dr. E. Beyer, Harvard University, Boston, MA) was radiolabeled with a 32P
dCTP (ICN Biochemicals, Costa Mesa, CA) using a commercial labeling kit (Pharmacia, Piscataway, NJ) and the labeled cDNA was purified from unincorporated radioactivity by passing it through a commercially available column (Schleicher and Schuell). This labeled cDNA was boiled at 100°C for 5 minutes, mixed with hybridization solution at a concentration of $10^6$ cpm/ml and the blot was hybridized overnight at 42°C.

On the next day, the blot was washed twice with 4x SET/10x Denhardt's/0.1% SDS/0.1% Na-PPi/10 μg/ml Poly A first at room temperature and then at 55°C for 30 minutes. The next three washes were with 4x SET/0.1% SDS/0.1% Na-PPi for 20 minutes at 55°C followed by two stringent washes with 0.5x SET/0.1% SDS/0.1% Na-PPi at 65°C for 20 minutes. The final post-hybridization wash was with 4x SET at room temperature for 20 minutes after which the blot was gently wiped with paper towel, encased in plastic wrap and autoradiographed for 48-72 hours in Kodak X-Omat AR film at -70°C using double intensifying screens.

2.7. WESTERN BLOTTING OF CONNEXIN43

2.7.1. PREPARATION OF CELL LYSATES

Cells were grown in 100 or 150 mm dishes for this experiment and unless otherwise mentioned, they were
treated after reaching confluence. Dishes were washed with ice-cold PBS containing 1 mM NaF and 1 mM PMSF and cells were scraped off with the same solution. After the centrifugation at 3000 rpm for 10 minutes, cell pellets were either frozen at -70°C or lysed in 30-100 μl of lysis buffer (1% NP-40, 0.05 M iodoacetamide, 10 mM PMSF, 1 mM EDTA, 1 μM leupeptin, 2 μg/ml aprotinin, 0.7 μg/ml pepstatin in borate buffer, pH 8.0) for 1-2 hours at 4°C in microfuge tubes. Lysates were then centrifuged at 10,000 g for 10 minutes and the supernatant was used as the source of connexin 43 and assayed for protein content done by a commercially available kit (Pierce Chemical Co., Rockford, Ill).

2.7.2. PROTEIN ELECTROPHORESIS AND WESTERN BLOTTING

Cell lysates containing equal amount of protein were mixed with either 4x or 2x SDS sample buffer with a final 5% or 10% concentration of β-mercaptoethanol and they were electrophoresed on a 10% SDS-polyacrylamide minigel (Bio-Rad, Richmond, CA) at 200 V according to Laemmli (1970). Then the proteins were transferred to immobilon membrane (Millipore, Bedford, MA) at 4°C for 30-35 minutes and the membrane was blocked with blotto (5% non-fat dry milk in PBS) either overnight or for 1 hour at 4°C. The blot was
then reacted with a rabbit polyclonal antibody (raised against a 15-mer synthetic polypeptide located in the C-terminal domain of rat heart connexin 43) diluted 1:200 in blotto:borate (1:2, v/v). After this 1 hour incubation at room temperature, the blot was washed with excess borate buffer for 45 minutes with 3-4 changes of the buffer. $^{125}$I-protein A (a kind gift from Dr. A. Benedict, Department of Microbiology, University of Hawaii) was diluted in blotto:borate to achieve a concentration of $10^6$ cpm/10 ml of solution and the blot was incubated with it for 1 hour at room temperature, followed by a 45 minute wash in borate buffer. The blot was then gently pressed against absorbent paper to get rid of excess liquid, wrapped in plastic foil and exposed on Kodak X-Omat AR film with double intensifying screen.

2.8. IMMUNOFLUORESCENT DETECTION OF GAP JUNCTION PLAQUES

Cells were seeded on Permanox plastic slides (Nunc Inc., Illinois) for fluorescent microscopy since conventional plastic dishes autofluoresce and interfere with the FITC signal and cells when grown on glass have a different morphology from that on plastic. Cells were treated after reaching confluence or in case of fully transformed cells at subconfluence. After 4 days of
retinoid treatment, slides were briefly washed in warm PBS, then were fixed in -20°C methanol for 2 minutes followed by a 10 minutes incubation in cold PBS. Slides were blocked for an hour in 5% BSA/0.2% Na-azide in PBS at 4°C. They were then washed twice for 10 minutes in cold PBS with slow rocking after which they were briefly dipped in blotto. Primary anti-connexin43 antibody was diluted 1:20 with blotto:borate and applied on the slides which were incubated at 4°C for 30 minutes. Slides were rinsed in cold PBS for 10 minutes, in high salt PBS (0.5 M NaCl in PBS) for 20 minutes followed by another wash in PBS. FITC-conjugated second antibody (goat anti rabbit IgG F(ab)² fragment in blotto:borate, diluted 1:40) was added to slides and incubated for 30 minutes at 4°C. After this, slides were washed as above and mounted in 50% glycerol in PBS (pH 7.2) containing 100 μg/ml p-phenylenediamine HCl to retard photobleaching (Johnson and Araujo, 1981). Cover-slips were put on the slides, sealed with clear nail polish and epifluourescent microscopy was performed using a Zeiss Axioplan microscope. Gap junction plaques were identified by fluorescent dots in contacting cell membranes as observed by others (Rogers et al, 1990; Musil et al, 1990; Dermietzel et al, 1987b). Images of both phase and fluorescent microscopy were digitized and stored in the computer for future photography.
3.1. EFFECT OF RETINOIDs ON GAP JUNCTIONAL COMMUNICATION (GJC) AND ITS RELATIONSHIP TO THE INHIBITION OF NEOPLASTIC TRANSFORMATION

Overview:

Retinoids, both natural and synthetic, have been demonstrated to be very potent in inhibiting carcinogen-induced neoplastic cell transformation in C3H/10T1/2 system (Merriman and Bertram, 1979; Bertram, 1980). Among the retinoids tested, retinyl acetate, hydroxyphenyl retinamide exhibited high inhibitory activity whereas retinoic acid was inactive (Bertram, 1980). Later work showed that retinoic acid was rapidly metabolized by 10T1/2 cells (Rundhaug et al, 1987) and thus were not available to exert any activity. In a modified transformation assay (Hossain et al, 1989), where retinoids were administered every third day instead of previous once per week treatment protocol, retinoic acid was shown to be equipotent to retinol. The mechanism by which retinoids act as anti-neoplastic agents is yet to be known. As it was reported by many groups that gap junctional communication between
adjacent cells play crucial role in the inhibition of neoplastic cell growth (Mehta et al, 1986; Yamasaki and Katoh, 1988), the possible involvement of gap junctional communication in retinoid-mediated inhibition of neoplastic transformation was explored. In this section, effects of retinoids on both homologous GJC (communication between similar cell types, i.e., normal:normal) and heterologous GJC (communication between two cell types, i.e., normal:carcinogen-initiated) were studied.

3.1.1. ENHANCEMENT OF GJC BY RETINOIDS IN 10T1/2 CELLS

In this study, 10T1/2 cells were treated similarly to the modified transformation experiment (Hossain et al, 1989), i.e., retinoids were given every third day with a weekly medium change and the experiment was continued for 5 weeks. This is a typical time-course for a transformation assay (Merriman and Bertram, 1979; Bertram, 1980). The cultures were not previously exposed to 3-MCA to avoid heterogeneity in the cell monolayer. The GJC assay was started 6 days after cell seeding when cells were about to reach confluence to ensure cell-cell contact. The minimum concentrations required to completely inhibit neoplastic transformation were used for the three retinoids tested in this experiment. These concentrations were $10^{-6}$ M for retinol and retinoic acid and $10^{-9}$ M for the highly potent
Figure 8.

Effect of retinoids on homologous GJC in 10T1/2 cells

10T1/2 cells were seeded at 2 × 10^4/dish and treated with retinoids on day 1 post-seeding and 3 days thereafter. GJC was indexed by the number of cells to which Lucifer Yellow was transferred 10 minutes after injection into a test cell. A. acetone (0.2%); B. TTNPB (10^-8 M); C. Retinol (10^-6 M); D. Retinoic acid (10^-6 M).
Figure 9. Long-term effects of retinoids on 10T1/2 GJC

Cells were seeded and treated identically to figure 8. The cultures were probed for GJC at intervals shown. Cultures were probed at intervals shown. Data points are the mean ± SE of at least 10 microinjection trials in each of two dishes.
synthetic retinoid, TTNPB (Hossain et al, 1989). In figure 8, it is shown that 6 days after the first treatment, all three retinoids at their equipotent doses significantly enhanced GJC (8-10 fold) in 10T1/2 cells. This increased GJC in all retinoid-treated cultures was of similar magnitude and it was sustained over the 5-week observation period (Fig 9). As seen previously, 10T1/2 cultures (both treated and control) exhibited increased GJC with age (Mehta et al, 1989). At a 1000-fold lower concentration than that of the natural retinoids, TTNPB was capable of producing comparable enhancement in GJC.

3.1.2. TIME-COURSE OF GJC-INDUCTION BY TTNPB

To determine the minimum time required for the retinoids to cause a significant increase in intercellular communication, confluent 10T1/2 cultures were treated with 10^{-9} M of TTNPB or with acetone control (0.2%). GJC was assayed at the time points indicated in figure 10. It was observed that the enhancement of GJC by TTNPB required several hours of treatment. After 18 hours, GJC in TTNPB-treated cultures were significantly higher than the control (p < 0.001) and continued to increase thereafter, whereas enhancement was not detectable after 6 hours. This delay in the action of TTNPB suggests the involvement of gene
Figure 10.

Time course of GJC-induction by TTNPB.

Confluent 10T1/2 cultures were treated with either acetone (0.2%) as control or TTNPB (10^{-9} M). The cultures were probed at indicated time points. Results represent the mean ± SE of 20 trials.
expression rather than opening of pre-existing gap junction channels.

3.1.3. DOSE-RESPONSE OF RETINOIDS ON GJC

To provide a stronger relationship between the inhibitory effect of retinoids on neoplastic transformation and the inducing effect on GJC, the next study was designed to determine whether the rise in GJC was dependent on the doses of retinoids. In the transformation assays the inhibitory effects of the retinoids was highly dose-dependent (Merriman and Bertram, 1979, Hossain et al, 1989).

10T1/2 cells: Three previously mentioned retinoids were tested using doses ranging from $10^{-10}$ M to $10^{-6}$ M. Among them, TTNPB demonstrated the most potent activity. At $10^{-9}$ M, it elevated GJC significantly and this increase was greater at higher concentrations (Figure 11). Both retinol and retinoic acid were ineffective in causing any significant rise in GJC at low concentrations ($10^{-10}$-$10^{-8}$ M) but at higher doses they exhibited GJC-enhancing activity similar to TTNPB. It is interesting to note that in the transformation assay, $10^{-9}$ M TTNPB treatment resulted in the complete inhibition of cell transformation, whereas both retinol and retinoic acid required a 1000-fold higher concentration ($10^{-6}$ M). The inverse
Figure 11.

Dose-response of retinoid-induced GJC in 10T1/2 cells.

Cultures were seeded and treated as in figure 9. They were probed for GJC 2 days after reaching confluence. Data points represent mean ± SE of two experiments each involving 20 micrinjections. Linear regression analysis results in \( r^2 \) values of 0.99, 0.96 and 0.98 for TTNPB, retinol and retinoic acid respectively. Dose-response relationships were statistically significant. P values were 0.002 for TTNPB, and 0.03 for retinol. We excluded the zero dose group for retinoic acid, since 10\(-10\) M retinoic acid inhibited GJC. The resulting dose-response was highly significant (P= 0.003).
relationship between these two biological effects exerted by retinoids becomes more evident when at \(10^{-10}\) M, retinoic acid blocked GJC and increased neoplastic transformation (Hossain et al, 1989). Dose-response relationships were found to be statistically significant.

**Carcinogen-initiated 10T1/2 (H23) cells:** Retinoids have been suggested to block the promotion step of a two-stage carcinogenesis model, i.e., the conversion of carcinogen-initiated cells into fully transformed cells (Fig 3). This hypothesis makes initiated cells the prime target for retinoid action. A dose response study was performed on a putative initiated 10T1/2 cell line (Mordan et al, 1982). This cell line (H 23) maintains normal phenotype in the presence of retinyl acetate but becomes neoplastically transformed and tumorigenic after a 3-4 weeks of retinoid removal.

All three retinoids tested in this study exhibited induction of GJC in H23 cells (Fig 12) and the induction was quite similar to the previous dose response study on 10T1/2 cells (Fig 11). H 23 cells however showed higher basal level of communication. TTNPB was again the most potent retinoid, it significantly enhanced intercellular communication at \(10^{-9}\) M and it continued to increase GJC thereafter. Retinol also elevated GJC, but this retinoid
Figure 12:

Dose-response of retinoid-induced GJC in H23 cells.

Cells were treated and probed as in figure 10. Statistical analysis yielded significant dose-response of the retinoids on GJC-induction. P values were 0.004 for TTNPB, 0.08 for retinol and 0.002 for retinoic acid. Similar to figure 10, the zero dose group was not taken for the analysis of retinoic acid data.
achieved the maximum level of inducing effect at $10^{-6}$ M. Retinoic acid exhibited a similar but less potent activity than retinol. Consistent with the prior observation in 10T1/2 cells, retinoic acid dramatically inhibited GJC at low concentrations ($10^{-10}$ M). This inhibitory action of retinoic acid once again established the inverse relationship between GJC and neoplastic transformation. As with the parental 10T1/2 cells, the dose response of retinoids was statistically significant in H23 cells.

3.1.4. CORRELATION BETWEEN THE EFFECTS OF RETINOIDS ON NEOPLASTIC TRANSFORMATION AND ON CELL-CELL COMMUNICATION

To validate our hypothesis that induction of GJC results in the inhibition of neoplastic transformation, the enhancement of junctional communication was plotted against the inhibition of cell transformation by retinoids published recently (Hossain et al. 1989). GJC data were pooled from 10T1/2 and H23 cells (Fig 11 and 12). Both the parameters were expressed as the percentage of the control group, which is carcinogen-only control treated for the transformation data and acetone control for the GJC data. In figure 13a and 13b, it is shown that both of these retinoid-modulated phenomenon were highly correlated in both the cell types. Analysis of the data showed a strong negative correlation coefficients ($-0.86$ for 10T1/2 and
Figure 13

Correlation between transformation and junctional communication. A. 10T1/2 cells, B. H23 cells

GJC data were taken from figure 11 and 12. Transformation data was taken from a recently published report (Hossain et al, 1989). The Pearson correlation coefficient was -0.86 for A and -0.89 for B, indicating a strong negative association between the two events.
-0.89 for the carcinogen-initiated cells) between the two parameters, which was statistically significant for both initiated and normal cells.

3.1.5. TUMOR PROMOTER TPA INHIBITS RETINOID-INDUCED GJC

Tumor promoter, TPA inhibits cell-cell communication (Yotti et al, 1979; Enomoto and Yamasaki, 1985a). Studies by Yamasaki’s group demonstrated that TPA-induced blockade of GJC was of transient nature and the inhibition was down-regulated by repeated treatments (Enomoto and Yamasaki, 1985b). It was indicated that the inhibition of GJC may play a crucial role in the TPA-induced cell transformation (Yamasaki, 1990). Retinoids and TPA have been shown to antagonize each other in many ways, including neoplastic transformation (Table 2). In H23 cells, it was shown that this cell line rapidly undergoes full transformation after TPA treatment, whereas retinoid treatment prevents this process (Mordan et al, 1982). Our next aim was to study the effects of these two agents on GJC in 10T1/2 cells. In Fig 14, it is shown that after the first treatment, TPA decreased both the basal as well as TTNPB-induced level of GJC in a dose-dependent manner. When these cells were retreated with TPA, basal level of GJC was not affected. However, TTNPB-induced level of GJC was blocked and it was brought down to the basal level. This pattern continued.
<table>
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<th>TPA</th>
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Figure 14.

Effect of TPA on constitutive and TTNPB-induced GJC.

10T1/2 cells were treated on day 8 post seeding with TTNPB (10^{-5} M) or acetone control. 24 hours later they received TPA (0.1 or 0.01 ug/ml) and were probed after an additional 48 hours later. Cultures were retreated with TTNPB on days 15, 22 and 29 immediately after medium change; then they were retreated with TPA after 24 hours and were probed for GJC after an additional 48 hours. Open bars, acetone control; hatched bars, TPA (0.01 ug/ml); solid bars, TPA (0.1 ug/ml). Symbols over bars: #, significantly different from acetone control; *, significantly different from TTNPB-treated cultures. GJC data was obtained as in figure 9.
for the rest of the experimental period. The specific inhibitory action of continued TPA treatment on TTNPB-induced GJC suggests that the tumor promoter and retinoids utilize a common pathway for the modulation of GJC. The antagonistic effect of TPA on retinoid action is in accord with its effect on cell transformation (Mordan et al., 1982). The progressive rise in communication with culture age, both basal and retinoid-induced, is consistent with our previous observation (Fig 9).

3.1.6. TPA INHIBITS NORMAL:INITIATED HETEROLOGOUS GJC

The importance of normal:initiated cell interaction became evident when Mordan et al (1983) showed that the transformation frequency of the initiated cells can be significantly decreased by coculturing them with the normal 10T1/2 cells. Similar results were observed by Herschman’s group where UV-transformed cells were growth inhibited by the surrounding normal cells (Herschman and Brankow, 1986, 1987).

In our next study we wished to investigate the existence of any heterologous GJC between normal 10T1/2 and initiated 10T1/2 (H 23) cells and whether this GJC was modulated by retinoids or TPA. The cocultures of H 23 and 10T1/2 cells were probed for GJC after 72 hours of treatment. In Fig. 15, we demonstrate that unlike fully
Figure 15.

Effects of TTNPB and TPA on heterologous communication between 10T1/2 and initiated-10T1/2 cells.

Initiated-10T1/2 (H23) cells were labeled with fluorescent beads and then overlaid onto confluent monolayers of 10T1/2 cells or unlabeled H23 cells. After 4 hours they received TTNPB and/or TPA and were probed after 72 hours. Open symbols, acetone (0.2%); Closed symbols, TPA (0.1 ug/ml). Data points represent the number of communicating cells plotted as means ± SE of three separate experiments, error bars smaller than than symbols if not shown. The numbers in parentheses above each datum point represents the communication frequency.
transformed cells (described later), carcinogen-initiated H23 cells maintained considerable level of GJC with normal cells (communication frequency 63%). Treatment with TTNPB did not alter the heterologous GJC, although the homologous GJC assayed in an identical protocol was enhanced. The failure of TTNPB to upregulate heterologous GJC was consistent with the previous studies using retinol and retinoic acid (Mehta et al, 1986). TPA treatment significantly inhibited both homologous and heterologous GJC. The existence of extensive heterologous GJC between the initiated and normal cells supports a recent model (Noner and Loewenstein, 1989) where growth regulatory signals were proposed to travel via gap junctions and are possibly responsible for the observed decrease in transformation frequency (Mordan et al, 1983). The inhibitory effect of TPA can explain the observation of Herschman and Brankow (1986) where TPA treatment released the UV-transformed cells from the growth-suppressive control of the normal cells.

3.1.7. RETINOIDS ENHANCED HOMOLOGOUS GJC IN TRANSFORMED CELLS

In this study, two fully transformed cell lines namely, 4B and C1-4 (derived from 10T1/2 cells after 3-MCA treatment) were used. The aim of this investigation was to
deduce whether the effect of retinoids on GJC was universal and not limited to only normal or initiated cells.

Since after reaching confluence, the transformed cells rapidly detach themselves from the petri dish, in this experiment, they were probed for GJC prior to confluence. The treatment protocol was identical to fig 11 and fig 12. As seen with the normal and carcinogen-initiated cells, the transformed cells also showed a similar dose-response to the tested retinoids suggesting that the GJC-enhancing effects of the retinoids were not dependent on a particular phenotype. In Figure 16, 4B cell GJC was markedly enhanced by TTNPB resulting into a consistent rise in GJC throughout the entire dose-range. Retinol and retinoic acid-induced GJC was also of similar magnitude. At the maximum dose (10\textsuperscript{-6} M) GJC was induced 10-15 fold by all retinoids. Cl4 cells however demonstrated a slightly different dose-response profile (Figure 17). TTNPB caused major induction (30 fold) in GJC which peaked at 10\textsuperscript{-8} M, and at higher doses TTNPB decreased GJC. This was the first instance where at higher concentrations, TTNPB was inhibitory to GJC. Similar phenomenon was observed by a recent study where at 10\textsuperscript{-5} M, TTNPB inhibited GJC significantly in both in normal and transformed fibroblasts (Mehta et al, 1989). This inhibitory action was not due to cytotoxicity
Figure 16.

Effect of retinoids on homologous GJC in 4B cells.

Cells were seeded and treated as in figure 11. GJC was assayed before the cells reached confluence because, soon after reaching confluence these transformed cells rapidly detach from the petri dish. The numbers represent the means ± SE of 20 microinjection trials.
Figure 17.

Effect of retinoids on homologous GJC in C14 cells.

Cell seeding and treatment was identical to that in figure 16.
and its reason is currently unknown. In this instance a possible explanation would be the enlargements of cell area which are consistently observed after retinoid treatments. If the injected tracer dye is detectable only within a certain network size, enlargement of cell size would result into fewer cells in that network and thus would appear as inhibited GJC since extent of GJC is quantitated by counting the number of the tracer dye positive cells. Retinol and retinoic acid were inactive at lower concentrations ($10^{-10}$-$10^{-8}$ M), but at $10^{-7}$ and $10^{-6}$ M, they elevated GJC significantly. Both the transformed cell lines exhibited significantly lower level of basal GJC (less than 1 cell per injection trial) compared to their normal and initiated counterparts which was in accordance with previous reports (for a detail list, see Yamasaki, 1990).

3.1.8. EFFECTS OF RETINOIDS ON THE CELL AREA

It was shown by Mordan and Bertram (1983) that retinoids decrease saturation density of 10T1/2 and initiated 10T1/2 (H23 cells) but they did not affect the growth rate of these cells. The inhibitory effect was observed only when the cells were about to be confluent. This finding suggests that the effect of retinoids on the cell saturation density is probably mediated through GJC by an enhancement of growth control. That is why the effect is
observed when the cells are in contact and thus are capable of establishing junctional communication with the adjacent cells. However, there is an alternate explanation. It was seen that retinoid-treated confluent cells were larger in area, and one can argue that retinoids are affecting the cell size, i.e., they are making the cells larger. Although the growth rate is not decreased after retinoid treatment, but fewer cells will be needed to fill the culture dish thereby resulting into a lower saturation density. To examine the latter possibility, next experiment was performed.

10T1/2 cells were seeded at a lower density (10,000/60 mm dish) and treated with retinoids for 3 days. After that, individual dishes were observed under microscope and cell area was determined by digital image analysis. Parallel cultures were allowed to grow near confluence and then treated identically. Cell area was calculated by counting the number of cells per dish and dividing the total dish area with that number. In figure 18a it is shown that in the growing cells there was no significant effect of retinoids on the cell area. Even the most potent retinoid TTNPB, failed to exhibit any change. In the confluent cells, retinoids showed marked increase on cell area of 10T1/2 cells (Fig 18b). TTNPB was able to exert its effect
Figure 18.

Effects of retinoids on the size of 10T1/2 cells
A. Growing 10T1/2 cells, B. Confluent 10T1/2 cells

10,000 cells were seeded in 60 mm dishes. In 18. A, cells were treated with retinoids on the next day. 3 days after treatment, single cells were identified under microscope and their images were digitally stored in the computer. Later these images were utilized to determine the cell area using a software from Loates Inc., MA. Each datum point represents the mean ± SE of 30-40 cells. In 18. B, cells were allowed to reach confluence and then treated with retinoids for 3 days. Cells were then trypsinized and counted. To determine the cell area, total area of the culture dish was divided with the number of cells. The numbers represent the mean ± SE of three identically treated dishes.
at a lower concentration \((10^{-9} \text{ M})\) than that of retinol and retinoic acid \((>10^{-7} \text{ M})\). It is worthwhile to point out that these were the concentrations of the respective retinoids to induce GJC and to inhibit neoplastic transformation described above. This study rules out the possibility that retinoids decrease saturation density by increasing cell area and our data supports the involvement of gap junctional communication in retinoid-induced growth control reported previously (Mehta et al, 1989).

All the above mentioned results indicate that retinoid-modulated growth control and inhibition of neoplastic transformation strongly correlates with retinoid-induced GJC. In the next section, I intended to elucidate how retinoids are modulating GJC at the molecular level.

3.2. MOLECULAR MECHANISM OF RETINOID ACTION: EFFECT ON CONNEXIN43 EXPRESSION

Overview:

The induction of homologous GJC by retinoids was found to require a minimum treatment period (18-20 hours). This observation indicates that retinoids exert their effects on GJC by altering gene expression of gap junction components rather than rapid activation of pre-existing junctions. It
was recently demonstrated that in 10T1/2 cells, connexin43 (Cx43) is the major gap junction protein (Rogers et al, 1990). This protein was first described in rat heart (Beyer et al, 1987) and was shown to form functional gap junctions (Fishman et al, 1990). Treatment with a synthetic retinoid, TTNPB resulted into an increased expression of Cx43 mRNA and protein in 10T1/2 cells. The time course of the induction showed that Cx43 level increased significantly after 48 hours of treatment, which substantiated our GJC observation (Fig 10). In my study, 3 retinoids were tested on three cell lines derived from 10T1/2 fibroblasts, i.e., carcinogen-initiated (H 23) and fully transformed cells, to show whether the observed retinoid-induced GJC in these lines is associated with a similar increased expression of Cx43.

3.2.1. EFFECT OF RETINOIDS ON Cx43 mRNA LEVEL

In this experiment, only the initiated 10T1/2 (H 23) cells were tested. Fig. 19 demonstrates that in H 23 cells, TTNPB and retinyl acetate treatment increased Cx43 mRNA level significantly. The 3.1 kB mRNA was identified by utilizing a cDNA probe specific for Cx43 mRNA (Beyer et al, 1987). Retinyl acetate was highly active in inhibiting neoplastic transformation (Merriman and Bertram, 1979) and is routinely used to prevent spontaneous transformation of
Figure 19.

Retinoid-induced increase of Cx43 mRNA in H23 cells.

Confluent H23 cells (2 X 10^7) were treated with TTNPB (10^{-8} M), retinyl acetate (10^{-6} M) or with acetone control for 4 days. 10 ug of total RNA was loaded per lane, electrophoresed and transferred to nitrocellulose. Hybridization with Cx43 cDNA was performed as described in the method section.
ACETONE
TTNPB
RET. ACETATE

- 3.1 kb
initiated cells (Mordan et al, 1982). Induction of Cx43 mRNA by this retinoid proves that apart from the highly potent synthetic retinoid TTNPB, a naturally obtained retinoid also demonstrates the same effect.

3.2.2. TTNPB-INDUCED INCREASE IN Cx43 mRNA DOES NOT REQUIRE ANY DE NOVO PROTEIN SYNTHESIS

After the discovery of nuclear retinoic acid receptors (Giguere et al, 1987; Brand et al, 1987) it has been proposed that retinoids exert their effects through forming complexes with these receptors. Later studies shown that the expression of at least one of these receptors (RAR-β) was enhanced by retinoic acid (Hu and Gudas, 1990). In the following experiment I wanted to explore whether the induction of Cx43 gene expression by TTNPB was directly mediated or whether prior activation of other gene(s) was required.

Confluent 10T1/2 cells were treated with $10^{-8}$ M TTNPB along with 5 ug/ml of cycloheximide, a potent inhibitor of protein synthesis. Cells were harvested after 6 and 10 hours of treatment. mRNA for Cx43 was quantitated by Northern blot analysis. In Fig 20a, it is shown that cycloheximide did not decrease the RNA level of Cx43. Since the concentration of cycloheximide was less than what
Figure 20.

Effect of cycloheximide on TTNPB-induced increase in Cx43 mRNA in 10T1/2 cells.

Confluent cultures of 10T1/2 cells were treated with acetone control, TTNPB (10⁻⁸ M) or with TTNPB plus cycloheximide (5 ug/ml or 10 ug/ml). Cells were harvested after 6 or 10 hours of treatment for the isolation of total RNA. Remaining protocols were identical to that in Fig 19.
A. Cycloheximide (5 μg/ml)

B. Cycloheximide (10 μg/ml)
was used in some studies (Mitchell et al, 1985), this experiment was repeated using a higher concentration of cycloheximide (10 ug/ml). Fig 20b demonstrates that even at a higher dose, the protein synthesis inhibitor failed to cause any decrease of the TTNPB-induced level of Cx43 mRNA. It was seen that the intensity of the mRNA band was somewhat increased after cycloheximide treatment, which may be due to the stabilization of the mRNA observed previously (Mitchell et al, 1985; Müller et al, 1984). This study thus proves that it is likely that TTNPB-induced Cx43 gene expression was directly mediated and prior de novo protein synthesis was not required.

3.2.3. RETINOIDS ENHANCED Cx43 PROTEIN LEVEL

For the analysis of Cx43 protein levels, cells were treated with the indicated retinoids for 4 days. Retinoic acid was readministered after 2 days of first treatment since it is rapidly metabolized by 10T1/2 cells (Rundhaug et al, 1987). Cell lysates were reduced with 5 or 10% B-mercapto ethanol prior to SDS-PAGE, because Cx43 was reported to form disulfide bridges (Manjunath and Page, 1986) and appeared as high molecular weight dimers (Rogers et al, 1990).
In confluent H 23 cells, TTNPB, retinol, retinyl acetate and retinoic acid increased Cx43 protein level (Fig 21a). These retinoids elevated homologous GJC at the used concentrations (Fig 12). Similar to the previous observation in the 10T1/2 cells (Rogers et al, 1990), retinoid treatment resulted into the appearance of a 45 kD protein (Cx45) in the initiated-10T1/2 cells. This protein band was found to be the phosphorylated form of Cx43 and believed to be responsible for junctional competence. The level of Cx43 and Cx45 increased with the dose-increase of TTNPB as seen in 10T1/2 cells (Rogers et al, 1990). The relative Cx45:Cx43 ratio was higher in these cell line after retinoid treatment when compared with that in 10T1/2 cells (Fig 21 b). This ratio maybe responsible for the higher GJC observed in H23 cells (Fig 12).

In fully transformed cells, Cx43 and Cx45 levels were also elevated by retinoid treatment (Fig 21c and 21d). In these cases, a high molecular weight protein band (65 kD) were seen. This band was also seen in 10T1/2 cells (Rogers et al, 1990) and was explained as the dimeric form of Cx43. The identity of two protein bands (38 kD and 40 kD) in untreated 4B cells which disappear after retinoid treatment is currently unknown.
Figure 21.

Retinoids increased the Cx43 protein levels.

A. Initiated-10T1/2 (H23) cells. B. 10T1/2 cells
C. Cl-4 cells. D. 4B cells

Cells were treated with the indicated retinoids for 4 days. After that they were lysed. The cell extract was solubilized and reduced with β-mercaptoethanol. Equal amounts of protein (40-75 ug) were loaded in each lane of SDS-PAGE. Gel electrophoresis, western transfer and detection of Cx43 were performed according to the methods described in section 2.7

21. A.
lane 1. acetone
2. TTNPB (10⁻¹⁰ M)
3. TTNPB (10⁻⁹ M)
4. TTNPB (10⁻⁸ M)
5. Retinol (10⁻⁶ M)
6. Ret. Acid (10⁻⁶ M)
7. Ret. acetate (10⁻⁶ M).

21. B.
lane 1. acetone
2. TTNPB (10⁻⁸ M)
3. Retinol (10⁻⁶ M)
4. Ret. Acid (10⁻⁶ M)

21. C.
lane 1. acetone
2. TTNPB (10⁻¹⁰ M)
3. TTNPB (10⁻⁹ M)
4. TTNPB (10⁻⁸ M)
5. Retinol (10⁻⁶ M)
6. Ret. Acid (10⁻⁶ M)

21. D.
lane 1. acetone
2. TTNPB (10⁻⁸ M)
3. Retinol (10⁻⁶ M)
4. Ret. Acid (10⁻⁶ M)
3.2.4. RETINOIDs INCREASED THE IMMUNOFLOuRESCENT LOCALIZATION OF GAP JUNCTION PLAQUES

Connexins are integrated into the membrane and form clusters of gap junctions (gap junction plaques). This gap junction plaques can be identified by double antibody immunofluorescence techniques. Previous studies have shown the presence of gap junctions using this method (Dermietzel et al., 1987b; Rogers et al., 1990). The question asked in this study was whether retinoid-induced levels of Cx43/Cx45 was reflected in the assembly of membrane-associated gap junctions.

H23 and Cl4 cells were grown onto Permanox plastic slides. After reaching subconfluence, they were treated with retinoids identical to the above mentioned western blot study. When fixed and stained with antibodies to the C-terminal domain of Cx43, control untreated H23 cells showed no fluorescent plaques in the regions of cell-cell contact (Fig 22). After 96 hours of retinoid treatment, dramatic appearance of punctated fluorescent plaques was observed. The linear arrangement of these plaques clearly defined the region of intercellular contact which was not obvious in phase microscopy due to high flattening of retinoid-treated cells. The presence of extensive gap junction plaques in cells after treatment with retinoids
Figure 22.

Immunofluorescent gap junction plaques in H23 cells after retinoid treatment. A and C. Phase micrographs; B, D, E, and F. Fluorescence micrographs.
A and B. acetone controls; C and D. TTNPB $(10^{-8} \text{ M})$
E. retinol $(10^{-6} \text{ M})$ and F. retinoic acid $(10^{-6} \text{ M})$.
Gap junctional plaques are indicated with arrows.
suggests that the cells are within a vast communicating network observed previously (Fig 12).

In Cl4 cells, retinoid-treatment exhibited similar effect on gap junction plaques (Fig 23). In this transformed cell line, the increase in the brightness and number of junctional plaques appeared to be less than that in H 23 cells (Fig 22). This finding is in accord with the Western analysis data (Fig 21) where Cl4 cells showed lesser elevation in the amount of Cx43 and Cx45 after retinoid treatment.

The findings in this section provide strong evidence that retinoids induce GJC in 10T1/2 and other cell lines derived from 10T1/2 by increasing the expression of the gap junction protein, Cx43. It is also demonstrated that the action of retinoid is direct and does not require any prior gene activation. Cx43 was found to be phosphorylated after synthesis. Immunofluorescence studies showed that retinoid-treated cells exhibited the presence of gap junction plaques which was absent in untreated control cultures.
Figure 23.
Immunofluorescent gap junctional plaques in Cl-4 cells.
Experimental protocols were identical to that of Fig 22.
3.3. MODULATION OF HETEROLOGOUS GJC BY RETINOIDS AND cAMP: RELATIONSHIP WITH GROWTH CONTROL

Overview:

Previous studies showed that the establishment of heterologous GJC between normal and transformed cells was highly correlated with the growth suppression of the latter phenotype (Mehta et al, 1986). This type of GJC was achieved by elevated cellular cAMP. Retinoic acid and retinol were found to be inhibitory to the heterologous GJC, which explained why retinoids failed to suppress transformed cell growth in coculture with normal cells (Merriman and Bertram, 1979). In this section I wanted to study this apparently opposing action of retinoids on GJC. TTNPB was the only retinoid tested in the following experiments.

3.3.1. TTNPB INHIBITS HETEROLOGOUS GJC

In a recent study, TTNPB was demonstrated to antagonize cAMP-mediated growth suppression of a transformed cell line by normal 10T1/2 cells (Hossain and Bertram, 1990). In this reconstitution experiment, TTNPB alone did not significantly alter the number of transformed colonies, whereas Ro 20-1724 (inhibitor of cAMP-phosphodiesterase and elevates cAMP level) dramatically reduced
the colony numbers. When cocultures were treated with Ro 20-1724 and TTNPB, there was a dose dependent rise in the colonies. Whether the observed antagonistic activity of TTNPB was related to its effect on heterologous GJC was explored in the next study.

To understand the nature of heterologous GJC better, more than one cell combinations were used. One initiated and two fully transformed cell lines were labeled with fluorescent beads and seeded on confluent 10T1/2 monolayer, treated and probed for GJC 48 hours later. Parallel cultures were labeled with $^3$H-Thymidine to determine the degree of cell proliferation. In fig. 24, it is shown that there was an inverse relationship between the level of heterologous GJC and the proliferation of the overlaid cells. The initiated H 23 cells maintained heterologous GJC with normal cells and showed low labeling index compared to the transformed cells which communicated poorly with 10T1/2 cells. Ro 20-1724 elevated heterologous GJC and decreased thymidine labeling. TTNPB failed to alter the basal level of heterologous GJC and cell proliferation, except in H 23:10T1/2 cocultures, where it decreased cell growth without significantly increasing GJC. This finding will be discussed later. When the cocultures were treated simultaneously with Ro 20-1724 and TTNPB at their highest
Figure 24.

Effect of TTNPB and Ro 20-1724 on heterologous GJC and cell proliferation.

A. Initiated 10T1/2: 10T1/2 coculture
B. 4B: 10T1/2 coculture
C. Cl4: 10T1/2 coculture

Both the initiated and fully transformed cells were labelled with fluorescent microspheres and then overlaid on confluent 10T1/2 cultures. The dishes were treated 4 hours later and probed for heterologous GJC 48 hours after treatment. Parallel sets of cultures were labeled with $^3$H-thymidine for 2 hours.

GJC data (solid bars) are the mean ± SE of 20 micro-injection trials. Labeling index (hatched bars) are the percent of fluorescent cells that were positive for thymidine incorporation. These values are the mean ± SE of 400-800 cells.
Figure 25.

Effect of TTNPB and Ro 20-1724 on homologous GJC and cell proliferation.

A. 10T1/2 cells; B. H23 cells; C. 4B cells; D. C14 cells.

Cells were identically labeled as in Fig 24. They were overlaid on confluent or dense cultures of similar cells. GJC and cell proliferation were determined as described in Fig 24.
doses, the retinoid antagonized the upregulation of heterologous GJC by Ro 20-1724. This decreased GJC was associated with a rise in cell proliferation indicating that these two phenomenon were related as suggested by previous studies (Mehta et al, 1986). It is interesting to note that, in Cl4:10T1/2 cocultures, TTNPB did not completely inhibit the effect of Ro 20-1724 and accordingly cell proliferation was not induced.

To explore whether the fluorescent bead labeling protocol interfered with GJC, bead labeled cells were seeded on dense cultures of similar cells types and treated similarly and were probed for homologous GJC and for cell proliferation. In Fig 25, it is demonstrated that as previously observed, TTNPB increased homologous GJC in all the cell lines tested. The induced GJC was again associated with a decreased cell proliferation. Ro 20-1724 did not significantly modulate either homologous GJC or cell growth. In the combined treatment group, GJC was found to be increased and thymidine labeling was low.

From the above two studies, it can be safely concluded that (a) elevated GJC (either homologous or heterologous) was associated with lower cell proliferation, (b) TTNPB increased homologous and inhibited heterologous GJC and (c) the inhibition was not due to the lack of
responsiveness to the cell pairs tested since homologous GJC in all the cells was increased by TTNPB.

3.3.2. Ro 20-1724 INCREASES HETEROLOGOUS GJC THROUGH THE ACTIVATION OF cAMP-DEPENDENT PROTEIN KINASE (PK-A)

Ro 20-1724 inhibits the breakdown of cAMP thereby elevating the level of the latter (Sheppard and Wiggan, 1971). cAMP has been reported to increase GJC in previous studies (De Mello and Van Loon, 1987; Spray and Burt, 1990). This action was reported to be mediated through the activation of protein kinase A (Saez et al, 1986a) and subsequent phosphorylation of connexin molecules (Saez et al, 1986a). The role of PK-A in our observed elevation of heterologous GJC was studied in the next experiment.

Cocultures of 4B and 10T1/2 cells were pretreated with a recently described PK-A inhibitor, H-89 (Chijiwa et al, 1990) and treated with Ro 20-1724. GJC was assayed at different time points. In this short time course study, it was seen that Ro 20-1724 elevated heterologous GJC about 4 hours after treatment which reached the maximum level after 8 hours (Fig 26). Pretreatment with H-89 completely abolished the induction of heterologous GJC, suggesting that PK-A plays crucial role in modulating cAMP-induced GJC.
Figure 26.

Effect of PK-A inhibitor (H-89) on Ro 20-1724-induced heterologous GJC between 4B and 10T1/2 cells.

4B cells were labeled with fluorescent beads and overlaid on confluent 10T1/2 cells. After 12 hours, some dishes were treated with 30 μM of H-89. After 45 minutes, dishes were treated with acetone or Ro 20-1724 (10^-4 M) and heterologous GJC was assayed at indicated time points.
0-0 acetone

- Ro 20-1724 (10^{-4} M)

- H-89 and Ro-20 1724
3.3.3. TTNPB DOES NOT INTERFERE WITH THE GENERATION OF cAMP

To explain the observed antagonizing effect of TTNPB on Ro 20-1724 action, we asked the question whether TTNPB was interfering with the generation of cAMP by Ro 20-1724. To study the responsiveness of the cells to this phosphodiesterase inhibitor, 10T1/2 and transformed 4B cells were treated with Ro 20-1724 and cAMP levels were determined at different time points. In fig. 27A, 10T1/2 cells showed elevation of both medium and cellular cAMP levels after Ro 20-1724 treatment. Although the difference in cAMP concentrations between treated and control cultures was visible after 12 hours of treatment, the elevation was maximum after 48 hours. The transformed 4B cell line did not show any increase in cAMP levels after Ro 20-1724 treatment (Fig 27B). These findings are consistent with previous observations (Matsukawa and Bertram, 1988; Bertram and Faletto, 1985).

To investigate the effect of TTNPB, only 10T1/2 cells were utilized since 4B cells failed to respond to Ro 20-1724. Cofluent cultures were treated with TTNPB for 3 days and then with Ro 20-1724 (10^{-4} M). cAMP was assayed 48 hours later. In this study, TTNPB failed to antagonize the effect of Ro 20-1724 to increase medium and cellular cAMP levels (Fig 28). Both the basal and induced levels of cAMP
Figure 27.

Effect of Ro 20-1724 on the generation of cAMP in (A) 10T1/2 and (B) transformed (4B) cells.

Confluent or dense cultures were treated with acetone or Ro 20-1724 (10^{-4} M). cAMP was extracted from the medium or from the cells at indicated time points.

Triangles: Medium cAMP; Circles: Cellular cAMP
Open symbols: acetone; Closed symbols: Ro 20-1724.
A. 10T1/2 Cells

B. 48 Cells

HOURS

medium cAMP pmol/liter/10^6 cells

CAMP (p mole/10^6 cells)
Figure 28.

Effect of TTNPB on the induction of cAMP levels.

Confluent 10T1/2 cultures were treated with TTNPB for 3 days and then with Ro 20-1724 (10^{-4} M) for 2 days. Medium and cellular cAMP were assayed as in Fig 27.

Symbol legends are similar to that in Fig 27.
were unaffected by pretreatment with TTNPB. Although there was a slight decrease in the induced level of cAMP in 10^{-8} M TTNPB treated cultures. But total cAMP concentration was still high and thus can not explain the observed complete blockade of Ro 20-1724 action on heterologous GJC and cell proliferation.

3.3.4. TTNPB-ENHANCED LEVELS OF Cx43 WAS NOT ANTAGONIZED BY Ro 20-1724

Since GJC in 10T1/2 and other cell lines were found to be closely related to the induction of Cx43 protein, we wanted to investigate whether the observed antagonistic activity of TTNPB on Ro 20-1724 action due the differential modulation of Cx43 gene expression. For this study, 10T1/2 cells were treated identically to Fig 28. After the treatment, cells were harvested and Cx43 was separated by SDS-PAGE and identified by Western blot analysis. Fig 29 shows that, TTNPB caused a dose-dependent rise in Cx43 and an increased appearance of Cx45, the phosphorylated form of Cx43. Ro 20-1724, either alone or given after TTNPB, did not cause any major alteration in the levels of these proteins, suggesting that these two drugs (i.e., TTNPB and Ro 20-1724) did not mediate their opposing action on GJC through modulating Cx43 gene expression or its posttranslational phosphorylation.
Figure 29.

Effect of TTNPB and Ro 20-1724 on the induction of Cx43 protein in 10T1/2 cells.

Confluent cultures of 10T1/2 cells were treated identically to Fig 28. Cx43 was separated by SDS-PAGE and identified by reacting with specific antibody. Experimental protocols described in section 2.7.

Lanes:
1. acetone + Ro 20-1724 (10^{-5} M)
2. acetone + acetone
3. acetone + Ro 20-1724 (10^{-4} M)
4. TTNPB (10^{-10} M) + acetone
5. TTNPB (10^{-10} M) + Ro 20-1724 (10^{-4} M)
6. TTNPB (10^{-9} M) + acetone
7. TTNPB (10^{-9} M) + Ro 20-1724 (10^{-4} M)
8. TTNPB (10^{-8} M) + acetone
9. TTNPB (10^{-8} M) + Ro 20-1724 (10^{-4} M)

Molecular weight standards are expressed in kDa.
CHAPTER IV
DISCUSSION

The importance of the dynamics of drug action is well established in the worlds of biochemistry, pharmacology and other branches of biomedical science. It is not sufficient to know the overall effect of a drug, its molecular mechanism of action is equally important for a variety of reasons. The knowledge involving the total pathway by which an agent produces an effect helps us to design more effective drugs as well as enables us to recognize the vital steps which are crucial for the development of disease and are modulated by the drug.

Retinoids have been demonstrated to be very effective in the chemoprevention of cancer in humans, experimental animals and in cell culture models and are currently being used in clinical intervention trials (Bertram et al, 1987). They are also widely used in dermatological abnormalities (Ganguly, 1989). Although numerous studies presented evidence for chemopreventive effects of retinoids, so far there are no reports elucidating the mechanism of the chemopreventive action of these compounds.

In vitro cell culture models are often utilized in biomedical research in studying the mechanism of action of
any drug. They can be well controlled and interactions involving other organs can be avoided. The 10T1/2 mouse fibroblast system (Reznikoff et al, 1973a, 1973b) has been demonstrated to closely correspond the in vivo models and it is widely used in cancer research. Retinoids were shown to inhibit neoplastic transformation in this cell line (Merriman and Bertram, 1979; Bertram, 1980; Harishiadish, 1978) and thus this cell line is quite suitable for the study of the mode of action of retinoids. 10T1/2 cell line offers another advantage; that is both carcinogen-initiated and fully transformed lines have been isolated from the parental line. One can thus study the effect of retinoids on different stages of chemical carcinogenesis.

The goal of our study was to investigate the role of gap junctional communication in the antineoplastic actions of the retinoids. Two natural and one synthetic retinoids which inhibited neoplastic transformation in 10T1/2 cells were found to induce gap junctional communication in the same cell system. In a 5-week treatment study similar to the transformation assay protocol, all three retinoids maintained the elevated state of the junctional communication. A dose-response study showed that among the tested retinoids, TTNPB was the most potent inducer. This synthetic benzoidal derivative of retinoic acid was also found to be most active in the transformation assay. The
strong negative correlation between these two biological effects suggests that retinoids may inhibit the transformation frequency through elevated junctional communication. A similar inverse relationship was observed in the carcinogen-initiated 10T1/2 cell line. The relationship is strengthened by the fact that at a low dose (10^{-10} M), retinoic acid increases transformation and decreases cell communication.

Apart from the similarity in the dose-response of retinoids, the reversibility of retinoid action was also exhibited by both the induction of GJC and the inhibition of neoplastic transformation. Merriman and Bertram (1979) reported the requirement of continuous presence of retinoids for their antineoplastic action. After the withdrawal of retinoids, microscopic foci of transformed cells appeared after a latent period of 2-3 weeks. On the other hand, Mehta et al (1989) showed that within 2 days of retinoid removal, the retinoid-induced GJC dropped significantly. The fact that a decrease in elevated GJC after retinoid withdrawal preceded the appearance of transformed foci strongly suggest that retinoid-mediated inhibition of neoplastic transformation was dependent on the elevation of GJC by the same drug.
In the literature, there are several reports on the effect of retinoids on junctional communication. Yamasaki's group demonstrated that in BALB/c 3T3 cells, retinoic acid can upregulate cell-cell communication (Yamasaki and Enomoto, 1985) and through this effect, retinoic acid can not only inhibit cell transformation but also reverted the transformed phenotype to normal phenotype (Yamasaki and Katoh, 1988). 13-cis-retinoic acid was shown to increase metabolic cooperation in V79 Chinese hamster cells (Shuin et al, 1983). Electron microscopic studies in tumor and skin tissues showed the increase in the gap junction area after long term retinoic acid treatment (Prutkin, 1975; Elias et al, 1980).

The inhibitory action of retinoids on junctional communication was reported by Pitts et al (1986) and Davidson et al (1985). On the other hand, Morel-Chaney et al (1986) did not observe any effect of retinoic acid. However, these studies either used high concentrations of retinoids or the treatment time was short. Both of these factors would greatly influence the action of retinoids as we have shown that at least 20 hours of treatment is needed to observe any significant effect. At high concentrations (>10^{-5} M), retinoids were shown to be inhibitory to GJC (Mehta et al, 1989).
The discovery of the upregulation of GJC by retinoids presents a mechanism by which these compounds modulate growth and inhibit cell transformation. It was suggested by many authors that gap junctions provide the most direct pathway for the transfer of growth regulatory signals (Loewenstein, 1981; Yamasaki, 1990; Mehta et al, 1986). In our model which was originally suggested by Nonnar and Loewenstein (1989), in a population of cells, there are few cells acting as the signalling source. In the control situation, due to low intercellular communication, only a small number of cells will be under their control. After retinoid treatment, the size of the communicating network of cells increases, thus bringing more cells under the control of the signalling cell. It is assumed that these communicating networks overlap and the growth of every cell is controlled. The end result would be a decreased cell saturation density after retinoid treatment observed by Mordan and Bertram (1983) and by Mehta et al (1989). Similar to the present study, fully transformed cells were also shown to follow the model (Mehta et al, 1989). It is particularly interesting to note that in a v-mos-transformed cell line retinoids inhibited GJC and as predicted by the model, saturation density was increased.

In case of cell transformation, this model is also valid. What is being controlled here would be the
conversion of carcinogen-initiated cells into fully transformed cells. It was observed that unlike transformed cells, initiated cells maintain high communication with the normal cells (Fig. 15) and thus have the potential to be strongly influenced by the latter. In this regard the distribution of the initiated cells is important. If there are small clusters of initiated cells in the midst of normal cells, due to extensive communication they will be part of the normal cell network. In this case their conversion to transformed phenotype would be inhibited. But if the initiated cells are in big clusters, cells in the center will be insulated from the control of normal cells and thus will transform. Experimental proof for this model was provided by Mordan et al (1983), by showing that transformation frequency indeed depended on the size of initiated cell clusters. Transformation frequency was zero, in case of small clusters (<10 cells) but was 70% when the clusters were big (>2000 cells).

The involvement of normal: initiated cell interaction in inhibiting neoplastic transformation was demonstrated by other studies. Dotto et al (1988) reported that a putative initiated keratinocyte cell line can be inhibited from forming papillomas when co-injected with a large number of normal keratinocytes. The existence of GJC in skin was
demonstrated by Kam and Pitts (1988) and the possibility of retinoids increasing skin cell GJC is indicated by Prutkin (1975) who showed an increased gap junctional area in skin tumors after treated with retinoids. Similarly, normal unirradiated thyroid cells were shown to inhibit carcinoma formation by radiation-initiated thyroid cells (Watanabe et al., 1988). Herschman's group isolated an UV-transformed 10T1/2 cell line which will produce transformed foci when grown alone, but when grown in presence of normal 10T1/2 cells, this cell line failed to yield transformed foci (Herschman and Brankow, 1986). Existence of GJC between normal and a myc-oncogene-transformed cell line was reported (Bignami et al., 1988), this phenomenon was associated with a suppression of neoplastic phenotype by the normal cells. These above mentioned studies suggest that normal cells are capable of suppressing neoplastic transformation through direct cell-cell communication as observed in our study.

Tumor promoter TPA was shown to inhibit junctional communication in many studies (Yotti et al., 1979; Wade et al., 1986; Fitzgerald et al., 1983) and this blockade of GJC was suggested to be important for the tumor promoting action of TPA. TPA action was described to be mediated through the activation of PK-C (Nishizuka, 1984; Blumberg, 1988) and this suggestion was substantiated by the observed
inhibition of GJC by diacylglycerol, the endogenous ligand for PK-C (Yada et al., 1985; Enomoto and Yamasaki, 1985). The later discovery of diacylglycerol as a potent tumor promoter (Verma, 1988) further substantiates that blockade of GJC is necessary for the tumor promoting activity. The transient nature of TPA-mediated inhibition of GJC (Enomoto et al., 1981) may be due to the down-regulation of PK-C in the continuous presence of TPA (Rodriguez-Pena and Rozengurt, 1984). In 10T1/2 cells, TPA caused a dose-dependent blockade of GJC (Fig 14); both the basal and TTNPB-induced GJC were blocked. However repeated TPA treatment resulted in the decrease in the induced GJC only. The specific antagonistic effect of TPA on TTNPB-induced GJC was similar to its antagonistic action on many biological events modulated by retinoids (Table 2).

In 10T1/2 cells, TTNPB failed to protect the elevated GJC from the inhibitory action of TPA. This finding is in accord with with a previous report, where treatment with retinyl acetate did not completely inhibit the TPA-induced conversion of the carcinoogen-initiated cells into fully transformed cells (Mordan et al., 1982). In BALB/c cells, however quite opposite effect was observed. In this cell line, TPA caused a dramatic decrease in GJC which was blocked by retinoic acid (Yamasaki and Enomoto, 1985).
a rat tracheal epithelial cell line, cigarette smoke condensate, which was reported to possess tumor promoting activity (Loeb et al, 1984), inhibited GJC. Treatment with retinol fully antagonized this effect of TPA (Rutten et al, 1988). The reason why retinoids were not effective in fully blocking the effect of TPA in 10T1/2 cells remains unclear at present.

The mechanism of TPA effect on retinoid-induced GJC is also not known. Since retinoids did not protect PK-C from TPA-induced downregulation (Rundhaug, 1989), it was postulated that there may be more than one junction involved in the 10T1/2 system (Hossain et al, 1989). Very recently, Beyer (1990) demonstrated the presence of 3 connexins under the subfamily of Cx43, the major gap junction protein in 10T1/2 cells. This discovery supports our speculation and it is possible that the constitutive and induced junctions consist of different types of connexins; one is sensitive to PK-C downregulation (constitutive) and the other (retinoid-induced) is regulated via a different pathway.

TPA also inhibited initiated: initiated homologous and initiated: normal heterologous GJC (Fig 15) and thus presumably released the initiated cells from the control of
normal cells. The unregulated initiated cells can now undergo full transformation. Bignami et al (1988) and Herschman and Brankow, 1987) experimentally proved that TPA treatment would inhibit the growth suppressive action of normal cells on either oncogene- or radiation-induced transformed cells.

From the above mentioned studies, it can be safely argued that retinoids exert their inhibitory actions on neoplastic transformation and on cellular growth via upregulating junctional communication. Since the induction of GJC required a minimum time period after retinoid treatment (16-20 hours), it was predicted that retinoids may elevate the gene expression of gap junction components. The most obvious target would be the connexins, proteins of gap junctions (Beyer et al, 1987). 10T1/2 cells were shown to express connexin43 (Cx43), a gap junction protein first described in rat heart (Beyer et al, 1987). In a recent study, Rogers et al (1990) showed that TTNPB increased gene expression of Cx43. The 3.1 kb Cx43 mRNA showed a time-dependent rise after TTNPB treatment and this rise was quite similar to the time course of induction of GJC after by the same retinoid. The role of Cx43 in establishing GJC was demonstrated by Werner et al (1989) and Fishman et al, (1990). They showed that introduction of Cx43 cDNA into
frog oocytes or hepatoma cells resulted into junctional competence in these cells.

In carcinogen-initiated.10T1/2 cells, TTNPB and a natural retinoid, retinyl acetate exhibited induction of Cx43 mRNA level (Fig 19). The latter drug was the first retinoid demonstrated as an inhibitor of chemical carcinogenesis in 10T1/2 cells (Merriman and Bertram, 1979). In the initiated cell line, retinyl acetate successfully blocked spontaneous transformation (Mordan et al, 1982) and is routinely used for maintaining the initiated phenotype in culture. According to the previously described model, retinyl acetate is thus highly likely to facilitate GJC and the Northern analysis demonstrates the validity of this prediction. The induction of Cx43 mRNA level by both natural and synthetic retinoids indicate that they act through a common pathway, i.e., increased Cx43 gene expression. Activation of other genes by retinoids have been reported (Ann et al, 1988; LaRosa and Gudas, 1988).

The modulation of gene expression by retinoids have been suggested to be mediated through a class of recently discovered nuclear proteins, named retinoic acid receptors or RARs (Wolf, 1990; O'Malley, 1990). So far 4 RARs have been reported (Giguere et al, 1987; Brand et al, 1988; Zelent et al, 1989; Ragsdale et al, 1989). They strongly
bind with retinoic acid and with other retinoids but with lesser affinity. Similar to steroid/thyroid hormone receptors, these proteins bind with their ligands (retinoids) and then interact with genomic retinoic acid response elements (RARE). This DNA-RAR interaction is believed to cause altered gene expression. The existence of RARE has been demonstrated in two genes (Vasios et al, 1989; de The et al, 1990) that are induced by retinoids. It remains to be shown whether Cx43 gene also contain the RARE sequences.

Whether retinoid modulated Cx43 gene expression is directly mediated or required prior activation of other genes was also addressed. The possibility of the involvement of de novo protein synthesis stems from the observation of Hu and Gudas (1990). They described that in F9 teratocarcinoma cells, retinoic acid increased the expression of RARβ gene, which unlike RARα or RARt is expressed at a low level. The time course of this induction was similar to that of the induction of GJC in our study, suggesting that elevation of Cx43 mRNA or GJC may be dependent on the induction of RARs. Pretreatment with the protein-synthesis inhibitor, cycloheximide, did not decrease Cx43 mRNA induction by TTNPB (Fig 20). A slight increase in Cx43 mRNA was rather seen, which indicates the
stabilization of the message by cycloheximide observed by others (Mitchell et al, 1985; Müller et al, 1984). The failure of cycloheximide to block Cx43 mRNA induction suggests that prior activation of other genes (e.g., RARB) is not required for TTNPB action on Cx43 gene expression. Similar findings were reported for the induction of Era-1 mRNA by retinoic acid (LaRosa and Gudas, 1988). TTNPB thus induced Cx43 gene expression possibly through interacting with existing RARs. In this regard, RARa is a likely candidate because it was reported to be constitutively expressed at a higher level (Hu and Gudas, 1990) and TTNPB showed high affinity towards RARa (Mangelsdorf et al, 1990). It is not quite known how other retinoids modulate gene expression. They showed lower affinity towards the reported RARs. Future studies will presumably describe the existence of receptors specific for their action.

Since TTNPB can directly induce Cx43 gene expression, it is not clear why a significant increase in Cx43 mRNA level was observed after 6-12 hours of treatment (Rogers et al, 1990). In macrophages, increased transcription of tissue transgluminase was seen within 15 minutes of retinoic acid treatment (Chiocca et al, 1988). The possibility of a slow cellular uptake of TTNPB may appear relevant, although studies with other retinoids demonstrated that quite a large amount of retinoids were
detected in 10T1/2 cells after 4-5 hours of treatment (Rundhaug et al, 1987).

Western blot analysis revealed that both natural and synthetic retinoids increased Cx43 protein levels in normal, carcinogen-initiated and fully transformed 10T1/2 cells. Most dramatic effect was however the appearance and increase of a 45 kD protein band (Cx45). These findings are consistent with the dose-dependent rise in Cx43 and Cx45 in 10T1/2 cells after TTNPB-treatment (Rogers et al, 1990). Cx45 was reported to be the phosphorylated form of Cx43. Phosphorylation of Cx43 has been observed in embryonic chick lens (Musil et al, 1990) and in cultured vole cells (Crow et al, 1990). Since a recent study demonstrated the existence of 2 more connexin genes in chicken heart (i.e., Cx42 and Cx45), one could argue that the upper band in the western blots is the indigenous Cx45, not the phosphorylated Cx43. However, the disappearance of Cx45 band and a simultaneous increase in Cx43 after digestion with alkaline phosphatase (Rogers et al, 1990; Musil et al, 1990) indicates that Cx45 is truly the phospho-form of Cx43. Moreover, the rabbit polyclonal antibody used in the present study was developed against a 15-mer synthetic peptide sequence located in the carboxy-terminal region of Cx43; this sequence is absent in chicken Cx45. Thus our
antibody would only recognize Cx43 or its phosphorylated form. Posttranslational phosphorylation resulted into a shift in the apparent molecular weight of Cx43. This observation is in contrast with Cx32, the only other connexin reported to be phosphorylated, which did not exhibit any shift in mol. wt. (Saez et al, 1986a; Traub et al, 1987). The appearance of high molecular weight protein bands (67-70 kD) in transformed cells possibly represent the dimeric forms of Cx43, which was not completely reduced. Cx43 has been shown to be extensively cross linked through covalent disulphide bridges (Manjunath and Page, 1986).

The biological significance of the phosphorylation of Cx43 is not known. In previous studies with the liver gap junction protein Cx32, Saez et al (1986a) showed that cAMP elevated GJC by phosphorylating Cx32. Conversely inhibition of GJC by pp60^V-src in vole cells was associated with the presence of phosphotyrosine residues in addition to phosphoserine residue on Cx43 seen in uninfected cells (Crow et al, 1990). Cx43 carries sites for Ser/Thr phosphorylation as well as Tyr phosphorylation in the cytoplasmic region (Musil et al, 1990) and thus can be regulated in both positive and negative ways. Retinoid-induced phosphorylation seems to be necessary for the elevation of GJC. This suggestion is supported by the
following evidences, (a) fully transformed cells expressed Cx43 but they did not communicate, (b) retinoids increased GJC and the level of Cx45 in these cells and (c) Cx45 was the major gap junction protein in extensively communicating chick lens (Musil et al., 1990) and in a Ha-ras-transfected 10T1/2 cell line (unpublished observation).

The mechanism of retinoid-induced phosphorylation of Cx43 is not yet elucidated. Nor do we know which enzymes are involved in this phenomenon or the site of phosphorylation. cAMP-dependent protein kinase (PK-A) has been shown to phosphorylate Cx32 (Saez et al., 1986a) and retinoic acid was found to alter PK-A activity (Hohman and Greene, 1990). PK-A thus appears to be a likely candidate for retinoid action. However, retinoids may increase the phosphorylated Cx43 level by reducing the dephosphorylation of this protein. In 10T1/2 cells, retinoids were shown to decrease protein phosphatase activity (Rundhaug, 1989). This enzyme may be important for the turnover of the phosphorylated Cx43, since it was reported to dephosphorylate Cx45 in vitro (Crow et al., 1990).

Immunofluorescence studies provided conclusive evidence that retinoid treatment caused enhanced assembly of functional gap junctional plaques (Fig 22 and 23). The
appearance of punctated gap junctional plaques in the region of cell-cell contact was typical of a communicating cell population (Yancey et al, 1988; Musil et al, 1990; Dermietzel et al, 1987b). Whether the incorporation of Cx43/Cx45 was facilitated by retinoid treatment remains unknown. The role of other proteins in stabilizing the gap junctional assembly and whether they are also modulated by retinoids is yet to be answered.

Although retinoids are potent upregulators of homologous GJC, they failed to elevate heterologous GJC between normal and transformed cells (Mehta et al, 1986). The interaction between normal and transformed cells is usually very poor (Enomoto and Yamasaki, 1984; Yamasaki et al, 1987; Mehta et al, 1986), but elevated cellular cAMP caused an increase in heterologous GJC (Mehta et al, 1986). This elevated GJC was strongly correlated with the observed cAMP-induced growth inhibition of the transformed cells by the surrounding normal cells (Bertram and Faletto, 1985, Bertram, 1979). In order to explain a recent study where TTNPB caused a dose-dependent inhibition of the cAMP-induced growth suppression of transformed cells (Hossain and Bertram, 1990), modulation of homologous and heterologous GJC by cAMP and TTNPB was investigated. This study demonstrated that TTNPB increased homologous GJC and this phenomenon was always associated with a decrease in
cell proliferation. The inverse relationship between GJC and cell growth was reported by Mehta et al (1989), who used cell saturation density as the index of cell growth.

Normal:transformed heterologous GJC was elevated by the cAMP-inducer, Ro 20-1724. This drug also decreased thymidine labeling of transformed cells in coculture with normal cells, which suggests direct growth suppressive action of normal cells. The association between increased heterologous GJC and decreased transformed cell growth was further substantiated by the action of TTNPB. This retinoid did not affect the basal level of either thymidine labeling or GJC but it antagonized the cAMP-induced GJC and growth suppression. The activity of TTNPB differs from an observation of Yamasaki and Katoh (1988), where retinoic acid increased heterologous GJC between normal and transformed cells and caused the reversal of the transformed phenotype to normal phenotype. In contrast to transformed cells, initiated cells maintained heterologous GJC with 10T1/2 cells. TTNPB did not increase this GJC but decreased cell proliferation. This apparent lack of association between GJC and growth inhibition can be explained by TTNPB-induced homologous GJC in 10T1/2 cells. Since initiated cells are already in communication with normal cells, increased homologous GJC in 10T1/2 cells will
place the initiated cells under the control of a bigger communicating network. Thus the initiated cell will receive more growth regulatory signals which will result into decreased cell proliferation as observed.

In order to explain the role of heterologous GJC in regulating cell growth, Mehta et al (1986) suggested that normal cells produce growth inhibitory signals which are lacking by transformed cells. In the control situation, poor heterologous GJC thus insulates the transformed cell from the regulatory action of normal cells. Once GJC is established between these two cell types, these signals travel through gap junctions and will inhibit transformed cell growth. The observed growth inhibition of transformed cells indicates that although these cells can not produce the growth inhibitory signals, they are still capable of responding to the signals. An alternative suggestion also made by Mehta et al (1986) describes that in transformed cells, growth stimulatory factors are produced. Establishment of heterologous GJC allows the transport of these signals from the transformed cell to surrounding normal cells. This event will decrease the signal concentration below a threshold value which is postulated to be required for tumor cell growth. The end result would also be a decreased growth of transformed cell growth. The difference between these two models lies in the signal type
and in the signalling source. Which of these models represent the true mechanism of suppression of transformed cell growth is not yet known. However, when normal and transformed cells were hybridized, hybrid cells usually assumed the normal phenotype (Harris, 1988; Stanbridge et al, 1982). The dominance of normal cell phenotype over tumor cells indicates the presence of a growth inhibitory signal. Enhanced heterologus GJC observed in the present study may work as a mini-hybridization of two types of cells.

The existence of growth regulatory signals that travel through gap junctions and regulates cell growth has been suggested by many authors (Loewenstein, 1981; Noner and Loewenstein, 1989; Yamasaki, 1990; Mehta et al, 1986). The molecular identity and mode of action of these hypothetical signals are completely unresolved. The constraints of gap junctional pore size and the expectation that signals should not be membrane permeable predicts a water soluble, charged ion or molecule of less than 1000 daltons. These criteria rules out the involvement of macromolecules, e.g., proteins or mRNAs exhibiting growth inhibitory properties (Lumpkin et al, 1986; Feltham et al, 1987). There are several small molecules namely, cAMP, calcium and inositol triphosphate, which have been reported to travel through
gap junctions (Fletcher et al. 1987; Saez et al. 1989) and they were shown to possess the physicochemical properties of the predicted growth regulatory signals. cAMP has been demonstrated to modulate cell growth (Matsukawa and Bertram, 1988).

Our observation that a synthetic inhibitor of PK-A, H-89 completely abolished the inducing effect of Ro 20-1724 on heterologous GJC strongly suggests that cAMP (generated by Ro 20-1724) elevated GJC by activating PK-A. This recently reported PK-A inhibitor was demonstrated to specifically inhibit for PK-A at the concentration utilized in the present study (30 μM) but not the other kinases (Chijiwa et al. 1990). H-89 was shown to antagonize the effects of forskolin (activator of adenylate cyclase), on neurite growth. The role of PK-A in enhancing GJC was reported (Saez et al., 1986a; Wiener and Loewenstein, 1983). The role of PK-A substrates in GJC has not been characterized in 10T1/2 system. Phosphorylation of Cx43 seems unlikely since the presence of a high molecular weight connexin band was not seen after treatment with Ro 20-1724 (Fig. 29).

The differential effect of retinoids on homologous versus heterologous GJC is not understood. All the cell lines showed increased homologous GJC after retinoid
treatment, but mixed cultures failed to show any inducing effect of TTNPB on heterologous GJC. This ineffectiveness of TTNPB on heterologous GJC indicates that there is a qualitative difference between homologous and heterologous gap junctions. Miller and Goodenough (1986) demonstrated that in chick lens, high CO₂-treatment inhibited intraepithelial dye transfer but not fiber-to-epithelium dye transfer. The observed antagonism between the actions of TTNPB and Ro 20-1724 was not reflected either in the generation of cAMP or in the induction of Cx43 and Cx45. It can be suggested that TTNPB may exert its inhibitory action through modulating PK-A. Hohman and Greene (1990) reported that retinoic acid inhibited type I PK-A while increased type II PK-A. This differential modulation of PK-A may explain the difference in retinoid action. The involvement of other membrane proteins in the establishment of heterologous junctions is relevant. The fact that although both normal and transformed cells express Cx43 they do not communicate, indicates that other proteins may facilitate proper "docking" of the hemichannels to establish a functional channel. The docking is possibly more important in the heterologous GJC since there are large differences in the morphology of normal and transformed cells. Cell adhesion molecules (CAM) have been reported to be associated with gap junctions and shown to be crucial for
intercellular communication (Mege et al, 1988; Keane et al, 1988; Rutishauser et al, 1988). Retinoic acid has been shown to upregulate one of these molecules (Husmann et al, 1989).

Even though the molecular mechanism of retinoid action is not yet completely understood, the differential modulation of gap junctional communication can explain much of the chemopreventive activity of retinoids. The increase or decrease in GJC by retinoids were always in accord with their effect on cell growth, neoplastic transformation and the suppression of transformed phenotypes. Usage of a synthetic retinoid, which was shown to be highly potent in the present study, offers a better alternative than the natural retinoids with considerable side effects as chemopreventive agents. Our GJC data explains why retinoids are more effective in inhibiting the process of neoplastic transformation than inhibiting the expression of the transformed phenotype observed by Merriman and Bertram (1979). This finding clearly establishes that retinoids are chemopreventive not chemotherapeutic agents. This information is extremely useful for designing clinical trials, several of which are already in progress (Bertram et al, 1987). 10T1/2 cell system has been shown to closely represent the in vivo system and we can thus expect that the observations of this study reflects the whole animal

160
models. Future studies are needed to determine the effect of retinoids on gap junction associated proteins, to elucidate the identity and mode of action of the predicted growth regulatory signals and whether these signals are modulated by retinoids.
APPENDIX A

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Enhancement of gap junctional communication by retinoids correlates with their ability to inhibit neoplastic transformation

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Introduction

Retinoids, the natural and synthetic analogs of vitamin A, are presently undergoing widespread clinical trials as cancer chemopreventive agents (1). Except for the role of retinol in vision (2), the mode of action of retinoids is unknown. Recent discoveries of a class of DNA-binding proteins, homologous to thyroid and steroid receptors, with ligand-binding sites for retinoic acid, are an important breakthrough (3,4). The next stage is to identify genes whose activity is regulated by these DNA-binding proteins. A potential target area are genes regulating gap junctional communication as is suggested by recent studies reporting that retinoids enhance gap junctional communication and stabilize the initiated state of the cell (7). We investigate here the hypothesis that retinoids act to stabilize the initiated state of the cell (7). We have previously demonstrated that growth control can be re-established in neoplastic cells when they are in heterologous junctional communication with growth-inhibited, non-transformed 10T1/2 cells (10). In this paper, we report that retinoids enhance homologous junctional communication in 10T1/2 cells and in initiated cells. Heterologous communication in these cells was extensive and was unchanged by retinoid treatment.

Materials and methods

Lucifer Yellow CH, retinoic acid, retinol and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co., St Louis, MO. Transhydromethylcholanthrene propionylnitric acid (TTNPB) was a gift from Hoffmann-La Roche, Switzerland.

Cells and culture conditions

The 10T1/2 cell line (10T1/2) and the chemically initiated C3H/101/2 INT (INT/10T1/2) were used throughout the study. Studies

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on junctional communication were performed on cells cultured in basal Eagle's medium (BEM) in 5% fetal calf serum and gentamicin (25 μg/ml) in 60-mm Petri dishes.

Transformation assays using 3-methylcholanthrene (MCA) were performed essentially as previously described (7, 12, 13), with the important exception that treatment with retinoids, which as before confirmed 7 days after carcinogen removal, was performed every 3 days in a dose volume of 10 μl acetone/dish.

The first treatment was immediately after addition of fresh medium, the second was given without medium change; the third immediately after medium change 6 days later, etc. until termination of the experiment. This more frequent treatment protocol was adopted after we became aware of the rapid metabolism of retinoic acid by 10T1/2 cells (15). Cultures were fixed and stained with Giemsa after 35 days and the number of type II and III foci scored.

Junctional permeability was probed with Lucifer Yellow CCl (10 μl in 0.23 M LiCl). This fluorescent dye was microinjected at constant pressure into cells of confluent cultures, with the aid of an Eppendorf microinjector. The total number of fluorescent neighbors of the injected cells was scored 10 min after the end of the injection (duration of injection, ~2 s) and served as an index of junctional communication. The fraction of microinjection trials that resulted in fluorescent first-order neighbors (communication frequency) served as a further index in some experiments.

In studies of heterologous communication between initiated and 10T1/2 cells, the former cells were labeled for a 90-min period with fluorescent microspheres, mean diameter 0.60 μm (Polyscience, Warrington, PA). These injected beads did not influence the viability of the cells and permitted their unambiguous identification in mixed cultures (10).

Statistics
A weighted least-squares linear regression was performed to investigate the dose-response relationship between retinoid concentration and cell communication. The mean number of fluorescent cells within the treatment groups was used as the dependent variable. Each mean value was assigned a weight inversely proportional to its variance, in order for means of higher precision to carry more weight in the analysis. The weights were scaled so that the sum of the weights was equal to the unweighted number of data points, thereby providing correct power for the test of significance. Our model assumed that the number of fluorescent neighbors was linearly related to the duration of drug exposure. When replicate experiments were performed, indicator variables were entered into the model to account for inter-experimental differences (16). The test was used to compare pairs of independent means.

The Pearson correlation coefficient (r) was used to evaluate the association between the number of communicating cells and the transformation frequency.
The enhancement of gap junctional communication by retinoids

Fig. 3. Comparative responses of IOT1/2 cells and INIT/1OT1/2 cells to retinoid induced communication. Cultures were seeded and treated as in Figure 2. They were probed for junctional communication 2 days after reaching confluence. (A) TINPB. (B) retinol; (C) retinoic acid. Closed symbols, IOT1/2: open symbols, INIT/1OT1/2. Data points represent mean ± SE of two experiments each involving ~20 microinjections in two separate cultures. Linear regression analysis results in r values of 0.99, 0.96 and 0.98 for TINPB, retinol and retinoic acid respectively. Dose–response relationships were statistically significant for the following groups: TINPB, IOT1/2: P = 0.002; TINPB, INIT: P = 0.004; retinol, IOT1/2: P = 0.03; retinol, INIT: of borderline significance at P = 0.08. We excluded the zero dose group for the retinoic acid group, since 10^-10 M retinoic acid inhibited communication. The resulting dose–response relationships were statistically significant: IOT1/2: P = 0.0001; INIT: P = 0.002. In the case of TINPB, initiated cells were significantly more responsive than were IOT1/2 cells (P < 0.05).

Fig. 4. Correlation between transformation and junctional communication. Data taken from Figures 1 and 3A–C. A—A, retinoic acid; D—D, 10^-4 M TINPB. (A) IOT1/2 cells; (B) initiated cells. The Pearson correlation coefficient was -0.86 and -0.89 for (A) and (B) respectively, indicating a strong negative association between the two events. This was highly statistically significant in both cases: P = 0.001 and 0.007 respectively.

10T1/2 cells in parallel experiments. Retinoic acid also enhanced junctional transfer at concentrations > 10^-8 M, but it inhibited at 10^-10 M (Figure 4C). This inhibition of communication correlated with the enhancement of transformation found with this dose (Figure 1). Initiated cells had higher basal levels of communication than did 10T1/2 cells, and this difference was maintained across treatment groups.

Because junctional transfer as measured is a function of both the junctional permeability and the loss of fluorescent dye due to the permeability of non-junctional cell membrane (6), enhancement of junctional transfer, in principle, could result from an increase in junctional permeability or a decrease in non-junctional membrane permeability. It was previously shown that the non-junctional membrane permeability of 10T1/2 cells is not significantly changed by retinoids (5). Thus, the present enhancements of junctional transfer represent enhancements of junctional permeability.

The transformation—communication correlation

In general, the enhancement of junctional permeability correlated with the inhibition of transformation, and vice versa. Figure 4 shows this relationship in normal and initiated 10T1/2 cells. The transformation frequency of the retinoid-treated cells (expressed as a percentage of the transformation frequency of carcinogen- only treated control cells) was plotted against the junctional transfer (expressed as a percentage of junctional transfers of the controls); the plots pool the data for all retinoids and retinoid concentrations. Analysis of the data showed a strong negative correlation between the two parameters (Pearson correlation coefficients of -0.86 and -0.89 for initiated and normal cells respectively), which was statistically highly significant for both initiated and normal cells.

Effects of TPA

To determine the effects of TPA, 10T1/2 cells were grown to confluence, then treated with TTPNB (10^-9 M), followed after 24 h with TPA (10 and 100 ng/ml). Communication was probed after an additional 48 h and compared with corresponding controls treated only with TPA. This treatment and probing sequence was repeated at weekly intervals. This modified schedule (i.e., weekly instead of bi-weekly) was instituted to avoid the complexities of multiple drug treatments after preliminary studies had shown TTPNB to be stable in cell cultures. As seen
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Fig. 5. Effects of TPA on constitutive and TTNPB-induced communication. Cultures of IOTI/2 cells were treated on day 8 post-seeding with TTNPB 10⁻⁹ M or acetone control as in Figure 1. Twenty-four hours later they received TPA (0.1 or 0.01 µg/ml) and were probed for junctional communication after an additional 48 h. Cultures were reseeded with TTNPB on days 15, 22 and 29 immediately after medium change; they then were reseeded with TPA after 24 h and were probed for communication after an additional 48 h as above. Open bars, acetone control; hatched bars, TPA (0.01 µg/ml); solid bars, TPA (0.1 µg/ml). Symbols over bars: #, significantly different from acetone controls. P < 0.05; *, significantly different from TTNPB-treated controls. P < 0.05. Communication data were obtained as in Figure 2.

Fig. 6. Effects of TTNPB on heterologous communication between IOTI/2 and INIT/IOTII2 cells. INIT/AOTI/2 cells were labeled with fluorescent beads then overlaid onto confluent monolayers of IOTI/2 cells or unlabeled INIT/IOTII2 cells. After 4 h they received TTNPB and/or TPA and were probed after 72 h. Closed, heterologous communication between
INIT/IOTII2 and IOTI/2 cells: triangles, homologous communication between INIT/IOTII2 cells. Closed symbols, TPA 0.1 µg/ml; open symbols, acetone 0.2%. Data points represent the number of communicating cells plotted as the mean ± SE of three separate experiments. Error bars smaller than symbols if not shown. In addition, the communication frequency is shown in parentheses above each data point. The injected cell was always bead labeled. Linear regression resulted in an r² value of 0.94. Junctional communication was enhanced by TTNPB 10⁻⁵ M (P < 0.01) in the homologous situation, but did not induce a significant response in any other group. TPA significantly reduced communication (P = 0.001) in both communication systems.

in Figure 5, when probed after the first treatment sequence, TPA caused a dose-dependent decrease in both constitutive and retinoid-induced communication. However, when probed after the second and subsequent sequences, TPA failed to inhibit constitutive communication but retained its ability to inhibit TTNPB-enhanced communication. It will be noted that at no time did TPA decrease communication in TTNPB-treated cells below the level seen in the acetone-treated controls. The progressive rise in communication with culture age, both baseline and retinoid-induced, is consistent with our previous observations.

Effects on INIT/IOTII2: IOTI/2 cell communication
A question not yet addressed in these studies is the ability of retinoids and TPA to influence heterologous communication between IOTI/2 and initiated cells. In view of the effects of colony size on the transformation frequency of these cells when surround­
ed by IOTI/2 cells (17), these interactions may be of crucial im­portance. To measure these interactions, the initiated cell line was labeled with fluorescent beads, then plated onto a confluent monolayer of IOTI/2 cells. After 4 h cultures were treated with TTNPB, and bead-containing cells were probed 72 h later. Because this procedure was dissimilar in many respects to the protocols employed in other experiments reported here, we also plated these initiated cells onto confluent monolayers of other initiated cells so as to compare directly homologous with heterologous communication under identical conditions. Initiated
cells coupled less efficiently with IOTI/2 cells than with themselves (63 versus 100% communication frequency respectively) (Figure 6). Statistical analysis over this more limited dose range did not show a significant dose—response relationship in the heterologous situation. Homologous junctional communication was enhanced by 10⁻⁵ M TTNPB (P < 0.01) as previously observed. TPA caused a highly significant decrease in communication for both systems.

165
Discussion

The discovery that retinoids are potent up-regulators of intercellular communication between both 10T1/2 cells and between their carcinogen-initiated counterparts could explain many of their actions as cancer chemopreventive agents. This up-regulation correlated strongly with inhibition of transformation; junctional permeability in the initiated cells was enhanced by TTPNB and retinol at $10^{-10}$ to $10^{-9}$ M and by retinoic acid at $10^{-7}$ to $10^{-6}$ M, the same concentrations that inhibited the formation of transformed foci (Figures 1 and 3).

The correlation between junctional communication and transformation also held in the negative sense; with $10^{-6}$ M retinoic acid, a concentration at which junctional permeability was reduced, focus formation was increased.

In the 10T1/2 system, the expression of the neoplastic phenotype by transformed cells (i.e., uncontrolled growth and focus formation) has been previously shown to be inhibited by the growth regulatory effects of co-cultured normal 10T1/2 cells (17, 18). The degree of growth inhibition was enhanced by elevation of cAMP levels (18, 19), and this effect correlated with the induction of gap-junctional communication between the normal and the transformed cells; conversely, inhibition of communication released cells from growth inhibition (10). This effect is consistent with known interactions between cAMP and junctional communication (20) and conforms to a recently proposed model of growth control with discrete regulatory centers (21). We now propose that this concept of the local control of phenotypic behavior also extends to carcinogen-initiated cells. What is being controlled here is their conversion to fully neoplastic cells. Unlike neoplastic cells, which communicate poorly with non-transformed 10T1/2 cells (10), initiated cells are only marginally restricted in their abilities to form communicating junctions with non-transformed cells (Figure 6). Furthermore, also unlike fully transformed cells, which are inhibited in their communication with non-transformed cells by retinoids (10), retinoids do not inhibit heterologous communication between our line of initiated cells and 10T1/2 cells (Figure 6). As a consequence, initiated cells have the potential of being strongly influenced by the proximity of non-transformed cells. The model of growth control with discrete regulatory centers (21) is consistent with this new data. In this model, growth-controlling signals diffuse through the cell population from discrete signal sources scattered throughout the cell population; the signal distribution and the associated field of growth control are governed by the junctional permeability. The model sets no constraints on signal sign (i.e., either negative or positive growth control signals are possible) as long as the signal is $< 2000$ daltons, the size limit for passage through the junctional channel. Stabilization of the initiated state of retinoids would thus depend on the growth control field delineated by junctional communication. Originally formulated for homogeneous cell populations, it applies as well to mixed cell populations, so long as there is communication between the different cell types, as was the case between the initiated and normal 10T1/2 cells in the present experiment. Thus, in terms of this model, the inhibition of transformation by retinoids would result from an increased growth-control field delineated by the retinoid-enhanced junctional communication.

Changes in the distribution of carcinogen-initiated cells within the mass of normal cells can have interesting consequences for the induction of carcinogenesis. This distribution can fall between two extremes: when the initiated cells are widely scattered among the normal cells, and when they are clustered among those cells, forming islands. In the first case, each initiated cell is simply a part of the signal field of the normal cells in the model, and the extent of the growth-control field would be determined only by the junctional permeability of the normal cell population. In the second case, many initiated cells, particularly those in cluster centers, are far removed from normal cells, and so in larger clusters many initiated cells are effectively insulated from the normal cell field, when the junctional permeability is low. In the first situation, the model predicts maximum inhibition of transformation; in the second it predicts that this inhibition will decrease with increasing cluster size. This is precisely what is observed: when initiated cells are distributed in colonies of cells, their transformation frequency is essentially zero; with progressively larger colonies the transformation frequency increases up to $70\%$ for colonies of $- 2000$ cells (17). Analogously, in de novo transformation experiments, an increase in the initial cell seeding density will also lead to a decreased colony size of all surviving cells since the final saturation density is constant. In the model this will lead to a decreased efficiency of transformation (transformation frequency), which is also what is observed (13). Of further interest, in the original description of 10T1/2 cell transformation, in dishes seeded heavily and producing few transformed foci, most foci were found at the dish edge (13) where a real physical barrier to contact with normal cells exists.

Junctional communication thus seems to modulate malignant transformation in vitro, and may provide an important natural defense against carcinogenesis in vivo—a concept that has been central to the growth control hypothesis (6). In vivo we would expect that the development of some types of tumors may be retarded by interactions with normal host tissue (the size limit to the tumor or initiated cell mass susceptible to inhibition is given by the constant A' in the model). In these cases, tumor development could result from a proliferative stimulus that would inequally favor the outgrowth of initiated cells, thereby insulating them from growth control. Wounding or TPA treatment, both of which uncouple communication and act as tumor promoters (22, 23) could be such a stimulus. Recently a line of putative initiated keratinocytes was shown to be inhibited from forming papillomas when co-injected with a large excess of normal keratinocytes (24), providing evidence that such interactions may indeed exist in the skin. That retinoids may up-regulate communication in skin is suggested by the observation of increased gap-junctional area in retinoid treated skin tumors (25). The recent studies by Frits et al. (26) on junctional permeability in intact skin provide the opportunity for in vivo investigations. The tumor promoter, TPA, a potent activator of protein kinase C (PKC) causes a disruption of junctional communication in many cell types (27–29). The transient nature of the response (30–33) is presumably due to the down-regulation of PKC in the continuous presence of TPA (34, 35). In rat liver cells for example, communication recovers after 2 – 5 h and 22 h of treatment with TPA $10 \text{ng/ml}$ in confluent and growing cultures respectively (32). In 10T1/2 and initiated cells and in co-cultures of these cells, a single exposure to TPA inhibited communication (Figures 5 and 6). However, constitutive communication in 10T1/2 cells was found to become refractory to repeated TPA treatments, as has been previously reported in 10T1/2 cells (33) but not BALB/3T3 cells (36). The retinoid-enhanced communication behaved differently in that it was continuously suppressed; however, at no time did it decrease below the level of constitutive communication (Figure 5). Since TTPNB does not protect PKC from down-regulation (J.E.Rundhaug and J.S.Bertram, submitted), we speculate that constitutive and retinoid-enhanced
communication may be mediated by channels of different sensitivities. This antagonism between TPA and retinoids on communication is in accord with their effects on the transformation of initiated 10T1/2 cells (9).

Initiated cells are not blocked in their heterologous communication with normal cells, as are transformed cells in response to retinoids (10), but instead remain in extensive junctional communication. This allows them to exist within the communication field of normal cells and thereby be influenced by it. We suggest that the enhancement of the communication field by retinoids may explain many of their effects as chemopreventive agents.

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Retinoid-Enhanced Gap Junctional Communication Is Achieved by Increased Levels of Connexin 43 mRNA and Protein

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Natural and synthetic retinoids are potent inhibitors of experimental carcinogenesis in animals and cause reversion of premalignant lesions in humans. In the model C3H 10T1/2 cell system, retinoids enhance postconfluent growth control, reversibly inhibit carcinogen-induced transformation, and enhance gap junctional intercellular communication. These effects are highly correlated. 10T1/2 cells were found to express low levels of connexin 43, a gap junctional protein first found in the heart. After treatment of confluent 10T1/2 cells with the synthetic retinoid tetrahydroretinamethaphenylethylene/propionylbenzoic acid (TTNPB), levels of connexin 43 mRNA and protein increased within 6 h of treatment, while elevation of junctional communication was detected within 12–18 h. The maximally effective concentration of TTNPB (10−8 M) caused an approximate 10-fold elevation of connexin 43 gene transcripts after 72 h. Indirect immunofluorescence microscopy using a polyclonal antibody to the synthetic C-terminal region of connexin 43 demonstrated that TTNPB induced many fluorescent plaques in regions of cell-cell contact. These results provide a molecular basis for the retinoid-enhanced junctional communication in 10T1/2 cells. It is proposed that one action of retinoids is to modulate the intercellular transfer of signal molecules. These could mediate many of the physiological actions of retinoids on growth control and carcinogenesis.

Key words: Cell-cell communication, cancer chemoprevention, vitamin A

INTRODUCTION

Vitamin A is required for vision, for normal differentiation of many tissues, for normal growth of the organism, and for fertility in both sexes. Apart from the role of 11-cis retinal in the visual process, the mode of action of vitamin A is poorly understood at the molecular level. Vitamin A is supplied to tissues as retinol and metabolized to retinoic acid. This metabolite appears to be the effector of growth and differentiation [1]. Natural and synthetic forms of vitamin A capable of maintaining normal growth and differentiation are called retinoids. In this study, we utilize one of these, tetrahydroretinamethaphenylethylene/propionylbenzoic acid (TTNPB), a chemically stable benzoic analogue of retinoic acid [2]. The recent discovery of a family of nuclear retinoid acid receptors (RARs) with strong homology to the steroid and thyroid binding proteins suggests that retinoids function by regulating gene transcription [3,4,5].

In addition to maintaining normal differentiation of epithelial tissues, retinoids can also correct abnormal differentiation. As such they have found wide use in dermatology [6] and are being evaluated as preventive agents in human cancer [7]. In animal models, retinoids are potent inhibitors of carcinogen-induced neoplasia at several anatomic sites [8] and will inhibit chemically induced neoplastic transformation in the well-characterized C3H 10T1/2 mouse cell line [9].

A recently discovered action of retinoids, which may be relevant to inhibition of transformation in 10T1/2 cells, is their ability to upregulate homologous gap junctional communication in 10T1/2 cells, in carcinogen-initiated cultures, and in neoplastically transformed derivatives [10,11]. Enhanced communication was statistically associated with a reduced confluent saturation density, an in vitro manifestation of growth control, and the ability of retinoids to inhibit neoplastic transformation in carcinogen-exposed cultures. These results provided further evidence that gap junctions can serve as conduits for growth control signals as originally proposed by Loewenstein [12]. It had previously been demonstrated that nontransformed 10T1/2 cells normally not in communication with
neoplastic cells can nevertheless cause reversible growth arrest of neoplastic 10T1/2 cells under conditions where heterologous junctional communication is increased by cyclic adenosine monophosphate (cAMP) [13]. Retinoids, for reasons not yet apparent, block this increased heterologous communication between normal and neoplastic 10T1/2 cells, yet enhance homologous communication when tested against each cell type separately. In all cases examined, enhanced conditions of heterologous or homologous communication are associated with enhanced growth control and vice versa. It is proposed that junctional transfer of growth regulatory signals plays a major role in the control of proliferation in density-inhibited cells [10,13].

We now show that this enhanced communication is in whole or in part driven by synthesis of connexin 43. This protein, first identified as a major component of the intercalated disc of rat heart [14,15], is a member of a family of connexin molecules that differ primarily in the composition and length of their cytoplasmic domains [16]. The current work establishes retinoids as the first compounds known to modulate the expression of connexin 43.

MATERIALS AND METHODS

Materials

Oligolabeling kit was obtained from Pharmacia (Piscataway, NJ); RNA ladder and formamide were purchased from Bethesda Research Laboratories (Gaithersburg, MD); 3H molecular weight markers and 125I-labeled protein A (70-100 kDa) were obtained from New England Nuclear (Boston, MA); 32P-deoxyctydine triphosphate (dCTP, 300 Ci/mol) was acquired from ICN Biomedicals Inc. (Costa Mesa, CA); and FITC-conjugated goat antirabbit IgG, F(ab') fragment, as well as all basic chemicals, were obtained from Sigma Chemical Co. (St. Louis, MO). TTNPB was a gift from Hoffmann-La Roche (Nutley, NJ). Dr. E. Beyer, Harvard, generously supplied the G2A cDNA clone of connexin 43 [16]. cDNA probes for sea urchin ribosomal RNA [17] were obtained from Dr. T. Humphreys, University of Hawaii.

Cell Culture

C3H 10T1/2 cells were passaged weekly in Eagle's basal medium with 25 μM ganciclovir and 5% fetal calf serum (HyClone Laboratories Inc., Logan, UT) as previously described [18]. For experiments, 106 cells were seeded in 150-mm culture dishes in 20 ml medium and were grown to confluence for 1 wk. They were then refed with medium and, 3 d later, stimulated with TTNPB in 40 μl acetone or with acetone as control. The cultures were harvested at the times indicated according to the procedures stated below.

Measurement of Junctional Intercellular Communication

Immediately prior to cell harvest, two dishes of each treatment group were assessed for gap junctional communication by microinjection of 10% Lucifer yellow CH in 0.33 M LiCl as previously described [11]. The number of communicating cells (i.e., number of fluorescent cells surrounding the dye-injected cell) was quantitated by a blinded observer 10 min after dye injection.

Northern Blotting and RNA/cDNA Hybridization

Total RNA isolation, agarose gel electrophoresis, cDNA isolation, and labeling were performed as described in Maniatis et al. [19], except where noted. The hybridization procedure was carried out according to Fregien et al. [20].

At the times indicated, total RNA was isolated from about 2 x 106 cells/time point. Cultures were washed once in cold phosphate-buffered saline (PBS)/10 mM EDTA and scraped from the dish into the same solution; the cell pellet was then dissolved in guanidine isothiocyanate buffer. This cell lysate was overlaid onto cesium chloride solution and centrifuged at 174,000 g for 21 h at 20°C. The RNA-containing pellet was then brought into solution and extracted with phenol/chloroform, followed by a cold ethanol precipitation. The resulting pellet was washed twice in 70% ethanol, vacuum dried, and dissolved in 20 μl sterile water. Quantitation and purity of the total RNA was determined by UV spectroscopy at 260/280 nm.

Ten micrograms of the isolated total RNA was run on 1% agarose/16.5% formaldehyde gels using RNA ladder for molecular weight determination. After electrophoresis, the RNA was partially hydrolyzed in 50 mM NaOH/10 mM NaCl for 45 min, neutralized in 0.2 M Tris, pH 7.4, for 45 min, and then presoaked in 20 x SSC (1 x SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) for 60 min. The gel was then inverted and blotted overnight onto a 0.22-μm nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) by capillary action using 20 x SSC buffer for transfer. After transfer, the blot was washed gently in 20 x SSC and vacuum dried at 80°C for 2 h.

The northern blot was hybridized to the 1.5-kb G2A cDNA clone of connexin 43. The blot was prewashed three times, using 4 x SET (1 x SET, O.015 M NaCl, 0.002 M EDTA, 0.03 M Tris, pH 7.4) and 50% formamide, with 10% Denhardt's solution, 0% sodium dodecyl sulfate (SDS), 1% sodium pyrophosphate/10 μg/ml polyethylene glycol 250 μg/ml salmon sperm DNA as blocking agents. The blot was hybridized using similar solutions as above except for the addition of 100 μg/ml salmon sperm DNA, 5% Denhardt's solution, 10% dextran sulfate, and 10 x cpm/ml, of a 0.5 x 106 cpm/ml salmon sperm DNA as blocking agents. The blot was hybridized using similar solutions as above except for the addition of 100 μg/ml salmon sperm DNA, 5% Denhardt's solution, 10% dextran sulfate, and 10 x cpm/ml, of a 0.5 x 106 cpm/ml salmon sperm DNA as blocking agents. The blot was hybridized using similar solutions as above except for the addition of 100 μg/ml salmon sperm DNA, 5% Denhardt's solution, 10% dextran sulfate, and 10 x cpm/ml, of a 0.5 x 106 cpm/ml salmon sperm DNA as blocking agents. The blot was hybridized using similar solutions as above except for the addition of 100 μg/ml salmon sperm DNA, 5% Denhardt's solution, 10% dextran sulfate, and 10 x cpm/ml, of a 0.5 x 106 cpm/ml salmon sperm DNA as blocking agents.
Antibody preparation and characterization

Indirect immunofluorescence microscopy

10T1/2 cells were grown on plastic slides (Pipemex; Nunc Inc., Naperville, IL) and treated postconfluence for 4 d with TTPNB. Slides were first fixed by briefly washing in warm PBS, then immersed in dry –20°C methanol for 2 min, transferred to 4°C PBS, then blocked for 1 h in a solution of PBS, 5% bovine serum albumin (BSA), and 0.2% sodium azide, pH 7.5. The primary rabbit anti-connexin 43 antibody was applied at 1:30 dilution in blocking solution at 4°C for 30 min. Slides were rinsed in cold PBS and washed for 20 min at 4°C with high-salt PBS (PBS + 0.5 M NaCl), rinsed in PBS, then exposed to the FITC-conjugated secondary antibody (goat anti-rabbit IgG F(ab′)2 fragment) in blocking buffer at 4°C for 30 min. Slides were washed as above and mounted in 50% glycerol in PBS, pH 8.0, containing 100 μg/mL p-phenylenediamine hydrochloride [24] to retard photobleaching. The coverslips were sealed with clear nail polish and were stable for 4–6 d at 4°C. Epifluorescent microscopy was performed using a Zeiss Axioplan microscope and photographed on Kodak TMAX 3200 film.

RESULTS

Expression of Connexin 43 Transcripts

Northern blots of total RNA from control and TTPNB-stimulated 10T1/2 cells were probed with cDNA for connexins 21, 32, and 43. Under high stringency conditions, a 3.1-kb band hybridized with the connexin 43 cDNA; other cDNAs failed to hybridize (data not shown). Connexin 43 thus appears to be the only member of the gap junction family of genes so far characterized that is expressed in 10T1/2 cells.

We have previously reported that junctional communication is enhanced by natural and synthetic retinoids and that this increase is detectable within 12–18 h of treatment [10, 11]. To determine if this was associated with increased levels of connexin 43 gene products or, alternatively, with increased permeability of existing junctional channels, we performed northern and western blotting on 10T1/2 cells at various times after stimulation with graded doses of TTPNB. TTPNB was used because it was the most potent of a series of retinoids in its ability to inhibit transformation and enhance communication [11] and because retinoic acid itself is rapidly metabolized by 10T1/2 cells [25].

Time Course of Connexin 43 mRNA Induction

Northern blots of total RNA from 10T1/2 cells demonstrated that connexin 43 mRNA was elevated within 6 h of treatment with 10−8 M TTPNB and that levels continued to rise over the 72-h duration of the experiment (figure 1). In three independent experiments, an 8- to 15-fold increase of the 3.1-kb transcript over basal unstimulated levels was measured by digital image analysis. Rehybridization of blots to cDNA of ribosomal RNA revealed a variation between key lanes of only about 20% in hybridization to this probe. Since it was shown that most of the effect of retinoids on cell-cell communication is reversible within 6 h of removal of TTPNB [10], the reversibility of the mes-
sage induction was investigated. Within 6 h of the removal of TINPB, the elevated level of gap junction mRNA had decreased to below detectable levels (Figure 2). The northern blot shown in Figure 1 was quantitated by digital densitometry and arbitrary transmission values plotted together with the functional communication data obtained from the same cultures (Figure 3). Increased levels of connexin 43 mRNA clearly preceded increases in communication prior to 12 h posttreatment with TINPB [10, 11].

Western Blotting

Western blotting of lysates of confluent 10T1/2 cells was performed under reducing and nonreducing conditions. This was found necessary because the immunoreactivity

![Figure 2](image2.png)

Figure 2. Connexin 43 mRNA levels decrease rapidly after removal of TINPB. Confluent 10T1/2 cultures were treated with TINPB 10^{-4} M for 96 h or with acetone as control. In half of the cultures, ribosome-containing medium was removed and replaced with fresh medium with or without TINPB 10^{-4} M; the other cultures were untouched. After 6 h, cultures were harvested; total RNA isolated, and equal amounts of RNA electrophoresed, blotted, and hybridized against labeled connexin 43 cDNA. Lane 1, control, acetone treated; lane 2, TINPB 10^{-4} M; lane 3, TINPB 10^{-4} M then retreated with TINPB 10^{-4} M for 6 h; lane 4, TINPB 10^{-4} M then treated with acetone for 6 h. The reduction in hybridization in lane 3 versus lane 2 is consistent with previous observations that fresh culture medium causes transient reduction in dye transfer.

![Figure 3](image3.png)

Figure 3. Comparative time course for induction of connexin 43 mRNA and functional gap junctions by TINPB in confluent 10T1/2 cells. The autoradiograph of the northern blot shown in Figure 1 was quantitated by digital image analysis and plotted in arbitrary units of transmission. Communication data was obtained from dye injection into 10 cell groups immediately prior to harvesting for RNA isolation. Because of problems of protein aggregation, western blots were not analyzed. Comparable results were observed in three separate experiments. Closed and open triangles, 3.1- and 10.4-kDa band intensities on northern blots, treated and control, respectively; closed and open squares, mean number of fluorescent cells surrounding a single dye-injected cell, treated and control, respectively.

![Figure 4](image4.png)

Figure 4. Time course of TINPB-induced increase in connexin 43. Confluent cultures were treated with TINPB 10^{-4} M for the times indicated. Cell lysates were reduced prior to electrophoresis, equal protein amounts applied to each lane, and western blots performed. Immunoblotting with polyclonal serum gave no signal. Lane 1, 0 h control; lane 2, 6 h TINPB; lane 3, 24 h TINPB; lane 4, 48 h TINPB; lane 5, 96 h TINPB; lane 6, 96 h control. The bands in the 70-kDa region (lanes 3 and 4) are believed to represent dimers of connexin 43 (see Figure 3). Positions of molecular weight markers (× 10^{-2} D) are shown.

![Figure 1](image1.png)

Figure 1. Time course of TINPB-induced increase in connexin 43 mRNA. Confluent 10T1/2 cells were stimulated with 10^{-4} M TINPB for the times indicated. 10 μg total RNA was loaded per lane, electrophoresed, and transferred to nitrocellulose by blotting. RNA/DNA hybridization using the 32P-labeled cDNA clone connexin 43 [16] was done at 42°C; posthybridization washes included two stringent washes of 0.5× SSPE at 65°C. Lane 1, control, 0 h; lane 2, TINPB, 6 h; lane 3, TINPB, 12 h; lane 4, TINPB, 24 h; lane 5, acetone, 48 h; lane 6, TINPB, 48 h; lane 7, acetone, 72 h; lane 8, TINPB, 72 h. This experiment is a representative sample of three experiments. Rehybridization of the blot to the cDNA of ribosomal RNA followed by digital image analysis demonstrated approximately equal hybridization in key lanes; for example, lanes 3, 4, and 6 gave a mean transmission value of 54.1 ± 6.1, while lane 5 (TINPB control) had a value of 63.2 arbitrary transmission units.
RETI N OIDS AND CONNEXIN 43 SYNTHESIS

10T1/2 cells exhibit two clearly distinct immunoreactive bands at apparent molecular masses of 43 and 45 kDa, when electrophoresed under reducing conditions. As described above, similar doublets were seen under nonreducing conditions at higher molecular masses. To determine if this heterogeneity is due to protein phosphorylation, lysates of TTNPB-treated 10T1/2 cells were immunoprecipitated; a portion of the immunoprecipitated cells was then subjected first to phosphatase digestion and then to electrophoresis under reducing conditions. As seen in Figure 5, TTNPB-treated cultures demonstrated three immunoreactive bands: a weak band at 43 kDa and two stronger bands at approximately 44 and 45 kDa. Phosphatase digestion resulted in a single strong band at 43 kDa, parallel to the weak band seen in nondigested controls. Inhibition of phosphatase action by vanadate inhibited this band shift. These data indicate that a major portion of retinoid-induced connexin 43 becomes phosphorylated in 10T1/2 cells.

Immunofluorescent Localization of Connexin 43

10T1/2 cells were seeded onto Permanox plastic slides for fluorescent microscopy since conventional plastic dishes

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Figure 5. Dose response for induction of connexin 43 by TTNPB. Confluent cultures of 10T1/2 cells were treated with TTNPB or acetone vehicle for 96 h. Equal amounts of total cell protein were electrophoresed without reduction (Figure 5A, upper panel), or after reduction with 2-mercaptoethanol for 5 min at 25°C (Figure 5B, lower panel), and western blotting was performed. Lane 1, acetone control; lane 2, TTNPB 10^{-8} M; lane 3, TTNPB 10^{-9} M; lane 4, TTNPB 10^{-10} M. Immunoblotting with preimmune serum did not give a signal (data not shown). Note the aggregation of connexin 43 into probable dimers and tetramers under nonreducing conditions (Figure 5A) and the incomplete conversion to monomers under reducing conditions. More vigorous denaturation at 50°C reduced the immunoreactivity of this antigen. Positions of molecular weight markers (x 10^{-3} Da) are shown.

Figure 6. Effects of phosphatase digestion on connexin 43. Lysates from cells treated with TTNPB 10^{-8} M for 96 h were immunoprecipitated with N-terminal connexin 43 antibody and proteins incubated with or without alkaline phosphatase. In some samples, phosphatase action was inhibited with 1 mM vanadate. Resulting digests were electrophoresed and immunoblotted. Lane 1, TTNPB treated; lane 2, TTNPB treated + alkaline phosphatase; lane 3, TTNPB treated + alkaline phosphatase + 1 mM vanadate. Molecular weight markers of 19, 24, 36, 43, 55, and 55 kDa were used to calibrate this gel. Calculated molecular masses of immunoreactive bands based on scanning plots of these markers were about 43-45 kDa for the lower and upper bands. Based on these calibrations, the 43-kDa marker shown ran anomalously slowly on this gel.
RETINOIDS AND CONNEXIN 43 SYNTHESIS

on which cells were grown for all the biological and molecular studies autofluoresce and obscure the FITC signal and T011/2 cells, when grown on glass, have a morphology quite distinct from that on plastic. When fixed and stained with antibodies to the C-terminal domain of connexin 43, control untreated cells showed occasional small fluorescent plaques localized in regions of cell-cell contact (Figure 7A). After exposure to 10^-8 M TNPB for 96 h, these plaques had dramatically increased in number and size, such that the region of intercellular contact with neighboring cells was clearly defined (Figure 7C). This region is poorly visualized by phase optics because of the highly flattened cytoplasm of T011/2 cells, particularly after treatment with retinoids. Surveys of many thousands of cells exposed to 10^-8 M TNPB demonstrated that all cells were surrounded by fluorescent plaques and would be expected to be part of a junctional network as predicted by the microinjection studies [11]. Cells treated with 10^-9 M TNPB (Figure 7E) appeared to possess more numerous junctional plaques, but these plaques were smaller than those observed in cells receiving 10^-8 M TNPB (Figure 7C). Fluorescent plaques were limited to regions of cell-cell contact, not just peripheral regions, as was demonstrated in late logarithmic growth phase cells which had not yet formed a confluent monolayer (Figure 7G,H).

DISCUSSION

Retinoids have previously been demonstrated to upregulate homologous junctional communication in normal, chemically initiated, and transformed T011/2 cells [10,11]. We now show that this enhanced communication is associated with major increases in connexin 43, which in turn is driven by increases in message for this protein. The response of T011/2 cells to retinoids at the molecular level reflects their behavioral responses to retinoids (i.e., alterations in saturation density, gap junctional transfer, and the suppression of neoplastic transformation) since all of these effects are reversible in T011/2 cells and follow a similar dose-response relationship [9,10,11]. The causal linkage between increased cellular levels of connexin 43 and increased junctional communication is suggested by the following evidence: (1) connexin 43 is a major protein associated with the region of the intercalated disc of rat heart concerned with junctional signal transfer [22]; (2) microinjection of connexin 43 mRNA into frog oocytes confers gap junctional competence [27]; (3) connexin 43 shares homology with a family of known gap junctional proteins [16]; (4) increases in connexin 43 detected by western blotting immediately preceded observed retinoid-induced increases in dye transfer (Figure 3); (5) retinoid-induced increases in connexin 43 became associated with plaques in regions of cell-cell contact (Figure 7).

The increase in connexin 43 mRNA could be due to message stabilization or transcriptional activation. Our data cannot distinguish between these alternatives. Nor do we know if this represents a direct action of retinoids or requires prior activation of other genes. While there are several reports of retinoids altering mRNA levels [28,29], these most probably reflect secondary changes induced during retinoid-stimulated differentiation or are a consequence of correcting a state of retinoid deficiency [30]. There is clear evidence for direct transcriptional activation by retinoids of two genes: tissue transglutaminase in macrophages [31] and laminin in F9 teratocarcinoma cells [5]. In macrophages, increases in mRNA occurred within 15 min of stimulation, a far more rapid time course than for the induction of connexin 43 mRNA reported here. These differences could reflect differences in the regulatory elements of these two genes. In the case of laminin gene activation in F9 cells [5] and granulocytic differentiation of HL60 cells [32], there is evidence that retinoic acid acts by binding to a nuclear RAR, which in turn interacts with genomic retinoic acid responsive elements (RAREs). The receptor itself, and the DNA responsive element to which it binds, are closely related to the thyroid, steroid, and vitamin D receptors and their respective responsive elements [3]. At least four RARs have been described with distinct tissue-specific expression [33,34], each potentially interacting with unique RAREs. This, coupled to interactions with thyroid responsive element [35], provides a likely mechanistic basis for the multiple effects of retinoids in diverse tissues.

Immunoreactive connexin 43 was found to migrate on SDS polyacrylamide gels as a doublet in both reduced and nonreduced conditions (Figure 5). While both bands increased in intensity after retinoid treatment, the upper band, which we show to be a phosphorylated form of connexin 43 (Figure 6), generally exhibited the most dramatic increase. Connexin 43 doublets have also been seen in intact tissues and in cultured vole cells where the upper band has also been shown to be a phosphorylated form of this protein [21]. The functional significance of the phosphorylation of connexin 43 is at present unclear. In the heart, the upper band is predominant; in this organ, gap junctions are clearly required to be in an open state. In the brain, the lower band predominates (R. Kadle and B.J. Nicholson, submitted for publication); unfortunately, however, the physiological state of gap junctions in the brain is not known. In T011/2 cells, the extensive junctional communication seen in response to retinoids and the extensive phosphorylation of connexin 43 in retinoid-treated cells treated with 10^-8 M TNPB, fluorescent and respective phase images; panels I and J. Higher magnification view of regions of cell-cell contact in cultures treated with 10^-8 and 10^-9 M TNPB, respectively. In all treated cultures, the brightest fluorescence was observed in regions of cell-cell contact, regions which are poorly imaged by phase contrast microscopy. The lack of such fluorescent plaques in peripheral regions not contacting other cells (arrows) can be seen in logarithmically growing cells (panels G and H). Magnification of panels A-H x 750; I-J x 2000.
cells both suggest that open gap junctions consist of phosphorylated proteins. The potential modulatory effects of phosphorylation of connexins are not understood; however, both A-kinase [36] and elevation of cAMP levels [13] have been shown to stimulate junctional communication. Conversely, the ability of pp60^src to inhibit junctional communication in vivo has been associated with the phosphorylation of tyrosine residues on connexin 43 of the cells [21].

The observation that the upregulation of gap junctional communication induced by retinoids in 10T1/2 cells is strongly correlated with enhanced growth control [10] and suppression of the neoplastic transformation of initiated cells [11] suggests a functional relationship between these phenomena. In an extension of our observation that normal cells can reversibly suppress the growth of neoplastic cells when these cell types are induced to communicate across junctions in response to cAMP [13], we have hypothesized that similar junctional transfer of signal molecules results in the suppression of neoplastic transformation of carcinogen-initiated cells. Initiated cells, which occur as a position intermediate between normal and malignant in the carcinogenic process, can communicate extensively with nontransformed 10T1/2 cells. Furthermore, retinoids upregulate junctional communication in both cell types. Thus, in mixtures of normal and initiated cells which are produced after exposure of normal cells to a carcinogen, retinoid treatment would place these initiated cells within an expanded network of communicating cells. Consistent with this model, just as transformed cells become growth arrested when placed in junctional communication with growth-arrested normal cells [13], initiated cells are inhibited from progressing to neoplasia under two circumstances: (1) if distributed in small colonies in close contact with surrounding normal cells [27] or (2) when distributed in large colonies where close contact is denied, if the network of communicating cells is expanded by retinoids [11]. Conversely, the tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA), which blocks communication, inhibits the retinoid-induced suppression of transformation [11].

Both the identity of the postulated signal(s) and the question of whether proliferation and transformation are influenced by the same signal(s) are completely unresolved. The constraints of gap junctional pore size and the expectation that signals should not be membrane permeable predict a water-soluble, charged ion or molecule of less than about 1000 Da. For example, cAMP [38], inositol triphosphatase, and Ca^2+ [39] have been shown to traverse gap junctions and to possess these physicochemical properties.

Regulation of gap junctional communication by retinoids in mesenchymal cells (and in other cell types expressing connexin 43) in the intact animal could help explain certain physiologic and pharmacologic effects of retinoids in addition to their role in cancer chemoprevention. For example, it is known that gap junctional communication is developmentally regulated during embryogenesis [40], that disruption of communication in hydra leads to abnormalities [41], that a retinoid acid gradient determines pattern formation in the chick limb bud [42], and that retinoids are teratogenic [43]. Our results suggest that in addition to direct effects of retinoids on genes controlling growth, differentiation, and development, retinoids could affect these functions indirectly by modulating the intracellular transfer of regulatory signals.

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177


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182


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