Retinoids Suppress Proliferation, Induce Cell Spreading, and Up-Regulate Connexin43 Expression Only in Postconfluent 10T½ Cells: Implications for the Role of Gap Junctional Communication

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Abstract
The antiproliferative actions of retinoids in the C3H/10T½ cell system are exhibited as a decrease in proliferation rate and a decreased cell saturation density at confluence. These actions correlate with up-regulated gap junctional communication (GJC) driven by the retinoid-induced increased expression of the gap junctional protein connexin43 (Cx43). Here we examine which actions of retinoids occur only in cells making extensive intercellular contacts, and thus may be mediated through GJC, and which are exhibited in the absence of extensive intercellular contacts and thus may be independent of GJC. In confluent cultures, the synthetic retinoid tetrahydrodromethylnaphthalenylpropenylbenzoic acid (TTNPB) increased GJC, reduced the already low [3H]thymidine-labeling index of G1, growth-arrested confluent cells from 4.2 to <0.1%, and increased the area occupied by each cell by 42%. In contrast, none of these parameters was altered in logarithmic growth phase cells with very limited intercellular contacts. In order to separate cell-cell contact from cell cycle-related phenomena, non-contacting cells were arrested in early G1 by lovastatin. In this situation, Cx43 expression was low and inducible by retinoids, as in G1/G0 growth-arrested confluent cells; however, no cell spreading was induced by TTNBP. In contrast, in non-contacting cycling cells or in cells arrested by aphidicolin, Cx43 expression was higher than in confluent cells. In this situation, TTNPB did not induce Cx43 and did not induce spreading. These data demonstrate the cell cycle phase dependence of connexin43 expression and of retinoid action. Because retinoids only reduce proliferation rates and induce cell spreading in cells with extensive intercellular contacts, these data support the involvement of gap junctional communication in the antiproliferative effects of retinoids.

Introduction
The retinoids, natural and synthetic derivatives of retinoic acid, exert a variety of biological effects. Retinoic acid acts as a morphogen in developmental regulation (1), is required for normal growth and cellular differentiation (2), and is proving of clinical value as a cancer preventive agent (3), confirming results in experimental animals and cell cultures (4). In the C3H/10T½ cell in vitro model of carcinogenesis (5, 6), retinoids are potent inhibitors of neoplastic transformation, reversibly suppressing the development of neoplastically transformed foci when applied during the preinitiation phase of carcinogenesis (7, 8). The demonstration that initiated cells may also be reversibly inhibited from undergo- neoplastic transformation when in intimate contact with normal cells (9) suggested that cell-cell communication may be involved in retinoid action. This hypothesis has been supported by the recent discovery that retinoids dramatically enhance GJC, an action which correlates statistically with the ability of tested retinoids to inhibit transformation and enhance growth control (10, 11). In this hypothesis, retinoids act by stabilizing carcinogen-initiated cells, through interactions with surrounding normal cells, thus preventing their conversion to fully transformed cells (11). The retinoid-enhanced GJC is caused by the up-regulated expression of Cx43 at the mRNA and protein level (12). Cx43 is the only gap junctional gene known to be expressed in 10T½ cells (12). These data provide a molecular basis for the action of retinoids in 10T½ cells as cancer preventive agents.

Gap junctions are water-filled channels which connect adjacent cells of most cell types and serve as conduits for the transport of small molecules and ions (13, 14). Evidence is growing that junctional communication serves many physiological functions, among these, the control of growth and differentiation are highly relevant to the process of carcinogenesis (14). By enhancing junctional communication, retinoids could thus facilitate the passage of putative growth regulatory signals from normal cells to initiated cells, thereby enhancing growth control and inhibiting transformation (11).

In many cell systems, retinoids are antiproliferative, slowing the transit time of cells through the cell cycle (15). In the 10T½ cell system, however, retinoids do not reduce the proliferation rate of logarithmically growing cells but do reduce the final saturation density of both normal and carcinogen-transformed cells in a dose-dependent manner (16). Since these cells form a confluent stable monolayer at confluence, the reduction in saturation density is accompanied by an increase in area occupied by each cell. This reduction correlated statistically with the degree of induction of junctional communication (17). We equate this action on saturation density and cell spreading with enhanced

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The abbreviations used are: GJC, gap junctional communication; Cx43, connexin43; TTNPB, tetrahydrodromethylnaphthalenylpropenylbenzoic acid; C3H/10T½, 10T½.
growth control because both are opposite to the phenotypic changes that occur during the neoplastic transformation of 10T½ cells (6).

These results do not define whether the action of retinoids on growth control are cell-cell contact dependent or independent; while effects were seen only in confluent cells, these cells differ from logarithmically cycling cells in both cell cycle and in cell contact parameters. If these actions were cell contact dependent, it would implicate junctional communication as a primary mediator of retinoid action on growth control. Alternatively, enhanced cell spreading in the absence of intercellular contact would indicate that effects on junctional communication and final saturation density may only be a secondary consequence of retinoid action on cell-substrate or cell-cell interactions. Such effects of retinoids have been documented (18). Because of the increasing interest in the use of retinoids as cancer chemopreventive and chemotherapeutic agents (19) and the increasing evidence for the role of junctional communication in regulating many physiological processes (20), we have examined in more depth the association between growth control, junctional communication, and retinoid action.

Results
Effect of Retinoids on Cell Spreading
Retinoid treatment of 10T½ cells decreased the cell saturation density at confluence; as expected, this was of necessity accompanied by an increased area occupied by each cell (cell spreading) so as to maintain a confluent monolayer of cells. To determine if this increased spreading occurred prior to confluence, and was thus responsible for driving the observed decreases in saturation density, we performed the following experiments. 10T½ cells were seeded at 10³ and 4 x 10³ cells per 60-mm dish (i.e., about 2 and 80% of the normal saturation density) and were treated for 72 h with various doses of retinol, retinoic acid, and the synthetic benzoilid retinoid, TTNPB. After this time, these cultures were logarithmically proliferating or formed a confluent monolayer, respectively. Microscopic examination of proliferating control cultures showed that, in a population of 400 randomly chosen cells, 60% were without direct contact with other cells, 16% had contacts in the form of long thin cytoplasmic processes, usually with one other cell, while 22% had contacts, usually with one or two other cells, in the form of single or double pseudopodia, which occupied only a small fraction of the peripheral cell membrane. Only 2% of cells had extensive intercellular contacts (>20% membrane occupied; Fig. 1, A and B). TTNPB-treated cultures were equivalent. Confluent cultures were exactly that, with each cell completely surrounded by other such cells (Fig. 1C). Fig. 1D shows TTNPB-treated cultures, where the decrease in cell density is apparent (compare Fig. 1C with 1D). Note that in both cases (Fig. 1, C and D), cells covered the entire surface of the culture dish.

As shown in Fig. 2A, in sparsely seeded 10T½ cells, retinoids caused no increase in cell area; on the contrary, a tendency for a reduction in cell area was observed at low concentrations of retinoic acid and retinol. These differences were not, however, statistically significant. The large variations in cell area observed in both control and treated logarithmic cultures were presumably due to size differences within cells distributed throughout the cell cycle of these proliferating cells. In contrast, treatment of confluent cultures caused large increases in cell area. The highly potent retinoid TTNPB increased cell area by 41% at 10⁻⁸ M, which was the maximum response seen, while both retinol and retinoic acid caused similar increases but required 100–1000 fold higher concentrations than TTNPB (Fig. 2B). It is interesting to note that, in previous studies, in which inhibition of neoplastic transformation (8) and stimulation of junctional communication (21) were measured, the retinoids exhibited comparable structure/activity relationships.

The increase in cell area induced by retinoid treatment of confluent cells was accompanied by a large reduction in the proliferation rate of these cells measured as a decrease in the thymidine labeling index (solvent control, 4.2 ± 0.4%; TTNPB 10⁻⁸ M, <0.1%). In contrast, the same treatment of proliferating, sparsely seeded cells resulted in no reduction in proliferation rate (solvent control, 19.3 ± 2.8%; TTNPB 10⁻⁸ M, 18.0 ± 1.2%; Table 1). These data demonstrate that increased cell spreading and reduction in proliferation rate are not primary actions of retinoids but require growth-arrested confluent cells. Since these cells also exhibit a high degree of cell-cell contact and up-regulate their expression of Cx43 in response to retinoids, our results indicate that gap junctional communication may be required for the antiproliferative action of retinoids and their ability to cause cell spreading.

The Molecular Action of Retinoids on Induction of Cx43 Expression Also Requires Confluent Cells
Our previous studies of retinoid action had only used confluent 10T½ cells because it is in these cells that retinoids exert their chemopreventive activity (7). In this situation, retinoids elevate junctional communication and enhance expression of Cx43 (12, 21). To determine if the proliferation status of 10T½ cells influences this molecular response to retinoids, we analyzed Cx43 mRNA levels in TTNPB-treated cells when proliferating and when confluent. TTNPB was used because of its high potency and low toxicity. As shown in Fig. 3, both the basal level of expression and the induction of Cx43 mRNA by TTNPB are dependent on the proliferative status of the treated cells. In sparsely seeded, actively proliferating cells, Cx43 expression was higher than that in confluent cells, where Cx43 mRNA was below detection levels. Retinoid treatment, as expected, resulted in a large induction of Cx43 mRNA in confluent cells but surprisingly did not alter its expression in proliferating cells (Fig. 3). It is important to note that in TTNPB-induced confluent cells levels of Cx43 mRNA, as a proportion of total cellular RNA, were greater than those seen in proliferating cells, indicating that the inability of TTNPB to increase Cx43 mRNA in proliferating cells was not due to the Cx43 gene being transcribed at its maximum level. To determine whether Cx43 protein exhibited similar differential regulation as observed for Cx43 mRNA, sparse and dense cultures of 10T½ cells were treated with TTNPB (10⁻⁸ M) for 3 days. Cx43 levels were then quantitated by Western blotting. As expected, proliferating cells expressed higher basal levels of Cx43 protein than did confluent cells, and this basal level was unchanged by TTNPB treatment (Fig. 4, Lanes 1 and 2). In contrast, basal levels of Cx43 were very low in confluent 10T½ cells, and TTNPB treatment caused the expected dramatic increase (Fig. 4, Lanes 3 and 4) to levels of Cx43 level approximating those seen in
proliferating cells. That Cx43 mRNA levels were so different in the two situations suggests either that translation efficiency was higher in proliferating cells or that Cx43 protein is more stable in the former situation.

The location of Cx43 was probed by indirect immunofluorescence using a polyclonal antibody to the COOH-terminal region of Cx43. As shown in Fig. 5, in both acetone-treated controls (Fig. 5A) and in those treated with TTNPB as above (Fig. 5B), cells exhibited a fine punctate pattern of immunostaining over the cytoplasm and as a halo around the nucleus. Fluorescence over the nucleus was also seen; this was in a focal plane above that seen in the highly

Fig. 1. Photomicrographs of 10T1/2 cells. A and B, proliferating cells 72 h after plating sparsely at 10^4 cells/60-mm dish, acetone control; A, low magnification; B, magnification as in (C) and (D); A, a long cytoplasmic process connecting two cells; \( \Rightarrow \), a "pseudopod" connecting two cells; Panels C, D, confluent cells 72 hours after plating heavily at 4 x 10^5 cells/60-mm dish. C, acetone control; D, after treatment for 3 days with TTNPB 10^{-6} M. A, bar, 0.5 mm; B, C, and D, bar, 0.1 mm.
flattened cytoplasm and is thought to represent sites on the nuclear periphery contributing to the halo described above. In these cells, which were seeded sparsely in confluent cultures described above, cell-cell contacts were few, and junctional plaques of a size similar to those observed previously in confluent cells were not seen at sites of contact. Because these fibroblasts when at low cell density are highly motile, as exemplified by the formation of widely scattered diffuse colonies of cells (Fig. 1), it seemed probable that cell-cell contacts were only transient and not conducive to junctional assembly. To address this question, other cultures were seeded at \( \times 10^3 \) the cell number (i.e., \( 2.5 \times 10^3 \) cells/dish) and treated with acetone or TTNPB as before. In these cultures, cell-cell contacts were more extensive, and at these sites, the expected junctional plaques were observed. Fig. 5C shows two acetone-treated contacting cells with a number of junctional plaques. No difference in the formation of junctional plaques was seen in acetone-treated control cells versus TTNPB-treated cells (data not shown), which contrasts greatly with the situation described previously in confluent cultures where plaques are infrequent in confluent control cultures. Cells labeled with preimmune serum exhibited a less intense, but nevertheless similar, pattern of cytoplasmic and nuclear fluorescence (Fig. 5D). The major qualitative difference in staining pattern was that, even in densely seeded cultures, junctional plaques were never observed. This is demonstrated in Fig. 5D, which shows a region of contact between adjacent cells. Because of the need to use an antifade mounting medium for these slides, phase contrast images were of poor quality and are not shown.

The presence of a \( M \), 45,000 protein band was also observed after TTNPB treatment of confluent cells (Fig. 4, Lane 4), but not after treatment of proliferating cells. We have previously shown this band to represent a phosphorylated form of Cx43 (12), suggested to be important in the assembly of Cx43 into junctional plaques (22). Our data support this conclusion since plaque formation is extremely limited in these sparsely seeded cells with few junctions. These molecular data, furthermore, demonstrate that, similar to the enlargement of cell area (Figs. 1 and 2b), induction of Cx43 expression by TTNPB is observed only in confluent cells.

**Expression and Induction of Cx43 Is Cell Cycle Dependent**

To determine if Cx43 down-regulation is controlled by cell cycle-dependent events or, alternatively, by intercellular contact-dependent events, proliferating cells were arrested at various phases of the cell cycle, and Cx43 levels were examined. To eliminate intercellular contact as a variable, only sparsely seeded cells were used.

**Cell Cycle Arrest by Aphidicolin.** Aphidicolin, a fungal mycotoxin (23), inhibits DNA polymerase B and arrests several cell lines at the G1-S boundary of the cell cycle (24). To control for potential toxicity, we assessed the reversibility of cell cycle arrest after aphidicolin treatment of 10T½ cells. Confluent growth-arrested cells were reseeded and treated with 2 \( nM \) aphidicolin for 24 h. This reduced the \( ^{[3]}H \)dThD labeling index from 70 to 7.6%. Twenty-four h after drug withdrawal, the labeling index was 80%, demonstrating complete reversibility. Higher concentrations or 48-h treatment times caused irreversible arrest (data not shown). To avoid these toxic effects of aphidicolin, 10T½ cultures were treated with TTNPB (10\(^{-8}\) M) for 3 days, followed by 2 \( nM \) aphidicolin for the final 24 h before harvesting and Western blotting. We would expect these cells to be arrested in S phase and at the G1-S boundary. In these cells comparable Cx43 levels to those seen in actively proliferating cells were detected and, similar to proliferating cells, aphidicolin-arrested cells did not exhibit any significant increase in Cx43 after TTNPB treatment (Fig. 6). To ensure that the Cx43 bands seen in this study were synthesized after aphidicolin treatment, additional cultures were treated with 10 \( \mu M \) cycloheximide for the last 10 h of the experiment. This almost completely abolished Cx43 expression in control or TTNPB-treated cultures (Fig. 6, Lanes 5 and 6), confirming that cells were still capable of Cx43 synthesis during aphidicolin treatment and demonstrating the rapid turnover of this protein, as has been reported by others (25, 26). Thus, noncycling, aphidicolin-arrested cells resemble cycling cells in their high level of expression and lack of inducibility of Cx43 by TTNPB.

**G1 Arrest by Lovastatin.** Confluent 10T½ cells are arrested in G1-G0 of the cell cycle (27) and additionally differ from cycling cells in their extensive intercellular contacts. To separate these two variables requires the growth arrest of sparsely seeded cells in early G1. Unfortunately, culturing in 1% serum did not result in an acceptable level of cell cycle arrest; we thus used lovastatin, an inhibitor of the cholesterol biosynthetic pathway, which has been shown to cause early G1 arrest in many cell types (24). When sparsely seeded proliferating cells were treated with lovastatin for 24 h, the labeling index fell from 19.3 ± 2.8% in controls to 0% in treated cells. In lovastatin-arrested cells, the basal level of
Table 1  Effect of TTNPB on cell area in proliferating versus growth-arrested confluent cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth phase</th>
<th>[^{1}H]dThD labeling index (%)</th>
<th>Cell area ((\mu m^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>Logarithmic</td>
<td>19.3 ± 2.8</td>
<td>3725 ± 215*</td>
</tr>
<tr>
<td>TTNPB (10^{-8} M)</td>
<td>Logarithmic</td>
<td>18.0 ± 1.2</td>
<td>3433 ± 176*</td>
</tr>
<tr>
<td>Acetone</td>
<td>Confluent</td>
<td>4.2 ± 0.4</td>
<td>4787 ± 152h</td>
</tr>
<tr>
<td>TTNPB (10^{-8} M)</td>
<td>Confluent</td>
<td>&lt;0.1#{i}</td>
<td>6789 ± 113h</td>
</tr>
<tr>
<td>Acetone</td>
<td>G1-arrested by lovastatin</td>
<td>&lt;0.1#{i}</td>
<td>5019 ± 481f</td>
</tr>
<tr>
<td>TTNPB (10^{-8} M)</td>
<td>G1-arrested by lovastatin</td>
<td>&lt;0.1#{i}</td>
<td>5276 ± 286f</td>
</tr>
</tbody>
</table>

\* Sparse 10T1/2 cultures were treated as in Fig. 7. Cell areas were determined by digital image analysis. Values represent means ± SE of about 40 cells chosen to be without intercellular contacts.

\(P\) values were taken from Fig. 1.

- Significantly different (\(P < 0.05\)) from appropriate acetone controls.
- Labeled cells present, but none in the 1000 cells counted.
- No labeled cells counted or observed.

Cx43 expression was markedly decreased to a level comparable to confluent G1/G0-arrested cells (Fig. 7). Furthermore, TTNPB treatment increased Cx43 expression as in confluent cells. Although lovastatin-arrested and confluent-arrested cells were similar in respect to Cx43 regulation, they differed when cell spreading was measured. No spreading was induced by TTNPB in sparsely seeded, lovastatin-arrested cultures where cell-cell contact was denied (Table 1), whereas in contrast, as previously demonstrated, TTNPB treatment of confluent cells results in extensive cell spreading. Furthermore, only under these latter conditions did TTNPB act as an antiproliferative agent (Table 1). The major variable in these two situations is that extensive intercellular contacts and junctional communication can only occur in confluent cultures.

**Discussion**

In the present study, we demonstrate that in C3H/10T1/2 cells, certain actions of retinoids, both at the cellular and molecular level, are cell cycle dependent, while others are dependent on intercellular contact. The expression of Cx43 and its induction by TTNPB is clearly cell cycle dependent. In confluent G1-G0, growth-arrested cells and in cells arrested by lovastatin in early G1, Cx43 expression is low but is strongly inducible by TTNPB (Fig. 7). In contrast, cycling cells and those growth arrested by aphidicolin express high levels of Cx43, but this expression cannot be further increased by TTNPB (Fig. 6). Since the extent of Cx43 mRNA expression is lower than that observed in TTNPB-induced confluent cells, this lack of response does not appear due to Cx43 being transcribed at its maximum level (Fig. 3). In contrast to these molecular events, the behavioral responses to retinoids examined appear to require extensive intercellular contacts; both the antiproliferative action of retinoids and their ability to cause enhanced cell spreading were only observed in cells allowed to reach confluence and, by definition, to form extensive intercellular contacts (Table 1). Here, under conditions of retinoid stimulation, we have previously shown by indirect immunofluorescence using an anti-Cx43 antibody that all C3H10T1/2 cells are surrounded by large numbers of junctional plaques (12). We would suggest that these contact-dependent responses are causally associated with the ability of retinoids to stimulate gap junctional communication, which can only occur to its maximal extent at confluent cell densities. The immunofluorescent studies presented in Fig. 5 indicate that the mere possession of Cx43 by cells does not ensure the formation of junctional plaques. We did not detect such plaques in cells seeded at low densities, presumably because of the brief nature of contacts in these motile cells, and retinoid treatment did not cause plaques formation. Only when cells were seeded at higher densities were junctional plaques seen of a size previously recorded in confluent cultures (12). Here, too, retinoid treatment did not affect the formation of plaques, suggesting that retinoids do not function to influence connexin assembly in this...
system. Unfortunately the immunofluorescent micrographs gave little definitive information regarding the localization of Cx43 when incorporated into junctional plaques. This was due partly to nonspecific cytoplasmic fluorescence observed in cells labeled with preimmune serum, and partly, we would suggest, to difficulties in detection of small amounts of signal distributed throughout the plasma membrane as opposed to being localized into junctional plaques containing possibly thousands of connexons (13).

This proposed role of gap junctional communication in mediating contact-dependent responses of retinoids stems from our previous observations that modulation of junc-

Fig. 5. Immunofluorescent localization of connexin43. Sparsely seeded cultures of 10T1/2 cells (25,000 cells/dish) were labeled with anti-Cx43 antibody (A and B) or with preimmune rabbit serum (D), then with goat anti-rabbit fluorescein isothiocyanate-conjugated IgG. C, a culture seeded heavily at 250,000 cells/dish and labeled with anti-Cx43 antibody then fluorescein isothiocyanate-conjugated IgG. Arrows, fluorescent junctional plaques. Bar, 10 μm.
during the experiment, respectively. Cx43 levels were determined by Western blotting as in Fig. 4. Lane 1, acetone control; Lane 2, TTNPB; Lane 3, acetone + aphidicolin; Lane 4, TTNPB + aphidicolin; Lane 5, acetone + cycloheximide; Lane 6, TTNPB + cycloheximide.

Fig. 6. Effect of cell cycle arrest by aphidicolin on the expression of Cx43. Sparsely seeded 10T½ cells were treated with TTNPB (10⁻⁸ M) or acetone for 3 days. These cells were also treated with aphidicolin or with cycloheximide during the final 24 or 10 h of the experiment, respectively. Cx43 levels were determined by Western blotting as in Fig. 4. Lane 1, acetone control; Lane 2, TTNPB; Lane 3, acetone + aphidicolin; Lane 4, TTNPB + aphidicolin; Lane 5, acetone + cycloheximide; Lane 6, TTNPB + cycloheximide.

Fig. 7. Effect of early G₁ arrest on the expression of Cx43. Sparsely seeded cells were treated with acetone or TTNPB (10⁻⁸ M) for 3 days and were G₁ arrested for the final 24 h with lovastatin. Cx43 levels were determined as in Fig. 4. Lane 1, acetone control; Lane 2, TTNPB; Lane 3, acetone + lovastatin; Lane 4, TTNPB + lovastatin.

Functional communication by retinoids strongly correlates with their effects on growth control and neoplastic transformation (10, 21). These observations thus support the original hypothesis of Loewenstein (28) that gap junctions transfer growth regulatory signals. The chemical or physiological nature of these signals are as yet unknown. However, since establishment or enhancement of junctional communication strongly correlates with decreased cell proliferation (10), while conversely inhibition of communication by tumor promoters and certain oncogenes results in decreased growth control (29), we are tempted to speculate that these putative signals are growth inhibitory in nature. If so, and if these signals play a physiological role in growth control, then cells responding to these signals would be expected to be arrested in G₁-G₀, as would cells transmitting these signals. Experimental evidence in favor of this comes from the observation that transformed cells are inhibited in G₂-G₀ of the cell cycle when in junctional communication with confluent (17) but not when cocultured with proliferating 10T½ cells (30).

Recently, several groups of investigators have confirmed these correlative studies by transfecting connexin genes into neoplastic cells. Stable integration of Cx43 into transformed 10T½ cells led to enhanced growth control (31) and to suppression of tumorigenicity in nude mice. Interestingly, the two tumors that did develop in these mice had lost the transfected gene (32). In other cells, too, transfection of Cx43 has been shown to increase growth control (33, 34). Transfection of Cx32 also reduced tumorigenicity (35). Functional expression of gap junctions thus has many of the characteristics expected of tumor suppressor genes; indeed, a search for such genes in human mammary carcinoma cells identified Cx26 (36).

It is known that gap junctional communication can be modulated by transcriptional and by posttranslational modifications. The present results confirm our previous report that retinoids up-regulate expression (12). In myometrium, estrogen will also up-regulate expression of Cx43 mRNA, while progesterone antagonizes this increase (37). The molecular basis for this inhibition is not understood; however, the steroids and retinoic acid activate the same family of receptors. Sequencing of the genomic up-stream non-coding region of Cx43 has revealed putative estrogen and retinoic acid responsive elements as well as AP1 and AP2 elements (37). The former would explain estrogen and retinoid responsiveness; the latter may explain the observed cell cycle regulation of Cx43 expression. AP1, a cell cycle-dependent transcriptional complex (38), has been shown to antagonize transcriptional activation of retinoid and glucocorticoid responsive genes (39, 40). Interactions between AP1 and retinoic acid receptors can be expected to be dynamic in cycling cells and could explain much of the cell cycle dependency of Cx43 expression and retinoid responsiveness. The physiological reason for down-regulation of Cx43 expression in cells growth-arrested in G₁-G₀ is not apparent at this time. It may be speculated that cells produce an excess of Cx43 while in logarithmic growth phase in order to maximize the potential for establishing GJC upon making the initial intercellular contacts; once cells have achieved extensive communication, this need decreases and synthesis is reduced. Because this phase of growth corresponds to confluence and the need for growth control, Cx43 expression may have become regulated by growth arrest in G₁-G₀. We also must consider that the observed down-regulation is a consequence of the original criteria used to select the 10T½ cells; not only were these cells selected to be highly growth inhibited at confluence, they also were selected because of their capacity to exhibit neoplastic transformation after treatment with carcinogens (6). If, as others have shown in closely related BALB/3T3 cell lines, GJC is not down-regulated at confluence, then transformation is latent because of the suppressing effects of surrounding normal cells (41, 42). When this down-regulation is prevented in 10T½ cells by retinoid treatment or, alternatively, when carcinogen-initiated cells are placed in intimate contact with normal cells, transformation is also latent (9).

We do not know how commonly down-regulation of Cx43 expression occurs; in human dermal fibroblasts, Cx43 is expressed at high levels at confluence and is retinoid inducible (43), whereas in human mammary epithelial cells, Cx43 is expressed at low levels throughout the cell cycle. In these cells, Cx26 is differentially regulated within the cell cycle but inversely to the regulation of Cx43 reported here; expression is low in cells arrested in early G₁ by lovastatin but increases greatly in S-phase cells (44). Additionally, retinoic acid failed to induce Cx43 in these human mammary epithelial cells (44), which contrasts with its effects in human and mouse fibroblasts. Thus, the regulation of connexin genes appears highly dependent upon the cell type examined.

Posttranslational control of connexin function is believed to occur via extensive phosphorylation of Cx43. This can be observed on Western blots as multiple phosphoforms of immunoreactive protein migrating in the M, 45,000–46,000 region of the gel (Ref. 22; Fig. 2, Lane 4). In confluent 10T½
cultures, three bands can usually be seen; the most distinct are two phosphorylated forms (notated P1 and P2 by Musil and Goodenough (22)), together with an indistinct nonphosphorylated form in the M, 42,000–43,000 region (12). Phosphatase treatment of retinoid-treated 10T1/2 cells extracts prior to electrophoresis results in a single band in the predicted M, 42,000–43,000 region (12). It is of interest that only when TTNPB-treated 10T1/2 cells were allowed to form extensive contacts and functional junctions were higher molecular weight forms of CX43 observed (Fig. 2, Lane 4). Induction of CX43 by TTNPB in lovastatin-treated, G1-arrested, sparsely seeded cells did not result in the presence of the M, 45,000 CX43 (presumably phosphorylated) band (Fig. 7, Lane 4). These results imply that phosphorylation of CX43 is dependent on cell-cell contact which, in communication-competent cells, results in assembly into junctional plaques. Our studies did not address the question of the location, membrane or cytoplasmic, of CX43 in sparsely seeded cells. These observations confirm and extend the reports of Musil et al. (22, 25) that phosphorylation of CX43 is associated with, and may be functionally necessary for, the assembly of CX43 into functional gap junctions.

Materials and Methods

Chemicals. Lucifer Yellow CH, retinoic acid, retinol, and aphidicolin were purchased from Sigma Chemical Co. (St. Louis, MO). TTNPB was a gift from Hoffmann-La Roche (Nutley, NJ). Lovastatin was kindly provided by Dr. A. W. Alberts (Merck, Sharpe and Dohme, NJ).

Cell Culture and Drug Treatment. C3H/10T1/2 cells were cultured in basal Eagle’s medium with 5% fetal bovine serum and gentamicin (25 μg/ml). For studies in sparse cultures, cells were seeded at 2.5% of their final saturation density (i.e., 25,000 cells/100-mm dish) and treated with retinoids for 3 days, after which cell density was about 10% of final saturation density (100,000 cells/dish). Dense 10T1/2 cultures were obtained by seeding at 80–90% of the final cell saturation density (i.e., 8–9 × 10⁶ cells/100-mm dish). Retinoids and aphidicolin were dissolved in acetone and absolute ethanol, respectively, and added directly to the culture medium in a dose volume of 20 μl/10 ml of culture medium. Lovastatin stock solution (10 mM) was prepared as described (24). In studies to induce cell cycle arrest with aphidicolin, cells were seeded from a confluent growth-arrested culture and treated for 24 h. This induced a parasympathetic wave of DNA synthesis upon drug withdrawal. The studies with lovastatin used asynchronous cells plated from log-phase growth phases cultures.

Quantitation of Cell Area. Digitized images of sparse 10T1/2 cells were used for the determination of cell area. The boundary of each cell was identified, and cell area was quantitated by using software from Loats Associated, Inc. (Westminster, MD). For each treatment group, 25–35 cells were measured. Since in confluent 10T1/2 cells, cell boundaries were not well defined, average cell area in these cells was determined by dividing the culture dish area by the cell saturation density measured by Coulter counter. Phase-contrast microscopy revealed that, in confluent cultures, cells occupied the entire surface area (Fig. 1C).

[3H]Thymidine Incorporation Assay. Cells were incubated with [3H]thymidine (1–5 μCi/ml) for 2 h (aphidicolin) or for 1 h in studies described in Table 1 (lovastatin) and then fixed with methanol:acetic acid (3:1, v/v) for 30 min. Dishes were air dried after washing with methanol, coated with photographic emulsion (Kodak), and developed after 5–7 days of exposure. Only heavily labeled cells (above 50 grains/cell) were scored as positive.

Immunofluorescent Analysis. 10T1/2 cells were seeded at a density of 25,000 or 250,000 cells into dishes containing glass slides. They were then treated as described above. Cells were fixed overnight in −20° methanol and then immersed for 2 min in acetone. Labeling with a rabbit polyclonal anti-connexin43, prepared against the COOH-terminal residues 368–382 of the predicted sequence of rat CX43 (13) at a 1:40 dilution, followed by goat-anti-rabbit fluorescent isothiocyanate-labeled IgG (1:40; Sigma), was as described (12). All photomicrographs shown in Fig. 5 were prepared under identical conditions of exposure, development, and printing.

Analysis of CX43 mRNA. 10T1/2 cells were harvested in cold phosphate-buffered saline/10 mM EDTA, and total cellular RNA was isolated using a commercially available kit (Cinna-Biotech Laboratories, Inc., Houston, TX). Equal amounts of total RNA were added to each lane, electrophoresed on a formaldehyde-agarose gel, and transferred onto a nylon membrane (Schleicher & Schuell, Inc., Keene, NH). Connexin43 message levels were examined by hybridizing with 32P-labeled CX43 cDNA obtained from the G2A clone kindly provided by Dr. E. Beyer (Washington University, St. Louis, MO) as described previously (12).

Analysis of CX43 Protein. Separation of cellular proteins by sodium dodecyl sulfate gel electrophoresis, Western blotting, and identification of CX43 by a polyclonal anti-CX43 antibody raised against the COOH-terminal domain (residues 368–382) were performed as described previously (12). Equal amounts of protein were added to each lane.

References


