INHIBITED INTERCELLULAR COMMUNICATION AS A MECHANISTIC LINK BETWEEN TERATOGENESIS AND CARCINOGENESIS

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> Cancer cells should be seen not as exclusively a problem in cell proliferation, but rather as a problem combining the processes of proliferation and differentiation."
>
> Van. R. Potter, 1978

I. INTRODUCTION*

The quotations used to begin this analysis are designed to focus on one level at which teratogenesis and carcinogenesis might be biologically linked, namely by interfering with the process of normal differentiation. Many cancer researchers have described cancer as a "disease of differentiation",[1,2] and Potter has coined the description, "oncogeny is blocked or partially blocked ontogeny".[3] It is suggested that an increased understanding of the complex mechanisms of cellular differentiation might reveal clues to teratogenesis and carcinogenesis, since the normal process of differentiation is disrupted in both cases. This analysis will explore the potential role of inhibited intercellular communication as a mechanism of disruption of differentiation common to both teratogenesis and carcinogenesis. A potential association between teratogenesis and carcinogenesis has been noted many times.[4-10] It remains to be resolved whether the association is fortuitous or real, and if real, what the shared mechanistic components are. Several major observations have suggested a mechanistic link between the two phenomena: (1) many genetic syndromes that predispose to cancer also increase the risk to congenital anomalies;[6,11-17] (2) there seems to be a high risk of cancer in certain cases of experimental animals and human beings exposed to teratogens;[6,18-23] and (3) many carcinogens are teratogenic,[6] although not all teratogens are carcinogenic.[8] Alone, each of these observations is tempting; together, they make it difficult to ignore a possible mechanistic link between these two processes.

Many genetic syndromes which predispose individuals to specific cancers have been associated with a variety of congenital malformations. This suggests that the gene or chromosomal mutations which enhance the chance of producing specific cancers in the individual

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also influence normal development during embryogenesis. Examples of associations are found in Down's syndrome,[11] Bloom's syndrome,[13] Fanconi's syndrome,[17] xeroderma pigmentosum,[17] ataxia telangiectasia,[17] and retinoblastoma.[15]

Cells from patients with xeroderma pigmentosum or Bloom's syndrome have an increased chance of incurring mutations compared to normal cells,[24-28] due to either faulty DNA repair or replication processes, respectively.[29-31] It has been suggested that the hypermutability of the cells of individuals with these syndromes may be responsible for the increased cancer risk.[32] The relationship of hypermutability to congenital malformations has generally not been considered. Down's syndrome cells do not seem to be either hypersensitive or hypermutable compared to normal cells when exposed to certain mutagens,[33] suggesting that the chromosomal mutation inherited in Down's syndrome affects teratogenesis and carcinogenesis by some mechanism other than increased sensitivity to cytotoxicity or mutagenesis.[9]

There are also several reports of children having congenital malformations associated with cancers in the absence of clearly definable genetic syndromes.[6,21,34-41] It is suspected that these situations result from exposure of the embryo to environmental conditions or chemicals which alter normal differentiation and lead directly to congenital malformations and the formation of tumors later in life. For example, children diagnosed as having the fetal hydantoin syndrome or the diethylstilbestrol syndrome face increased risk to a variety of cancers later in life.[6] There may also be a higher association of tumors in children born with congenital malformations due to the unique postnatal exposure to "carcinogenic" treatments for the sequelae related to the malformations (i.e., long-term exposure to phenobarbital given to a child born with convulsions, repeated surgery, or multiple X-ray treatments for bone deformities, etc.).

Several reports have noted that many carcinogens are also teratogens.[4,6] These analyses are sometimes complicated, however, by difficulties inherent in the assessment of teratogenesis and carcinogenesis. One significant concept ignored in most previous analyses is that carcinogenesis is a multistep process for many, if not all, cancers, involving initiation, promotion, and progression phases.[42,43] This complex process entails the evolution of phenotypes over a relatively long period of time and includes several different mechanistic processes.[44] Without suggesting that teratogenesis is the result of a single or simple errant mechanism, there is little evidence that teratogenesis involves the same multistep mechanistic processes that are found in carcinogenesis. Nonetheless, the initiation and promotion phases of carcinogenesis may share molecular bases with teratogenesis.

The initiation phase of carcinogenesis is widely believed to involve a mutagenic event.[45] Likewise, gene and chromosomal mutations inherited via the germ cells have been associated with teratogenesis and other manifestations of embryotoxicity.[46] While theoretically possible, it is less obvious if mutations in the somatic cells of an embryo can result in malformations.[47-49]

Correlations between mutagens and teratogens have been found.[50] However, most correlation studies have been rendered weak by complications due to: (1) inadequate in vivo and in vitro test models; (2) faulty interpretations of both negative and positive data due to artifacts of mutation test models; and (3) problems of extrapolation from in vitro test conditions to real life.[8]

The molecular basis of the promotion phase of carcinogenesis is more controversial. Most tumor promoters show little or no mutagenic activity as discussed by Trosko et al.[44] It is more likely that tumor promoters exert their effects by either direct interactions with membranes[51] or by altering gene expression.[52] Membrane interactions are likely very important components of normal embryogenesis also.[53,54]

One means of membrane interaction involves direct cell-to-cell communication via junctional structures.[55-59] Most tumorigenic cells have been found to have modified junctional communication abilities, and it has been suggested that interference with intercellular com-

munication could be part of the tumorigenic process.[44,53,55] Likewise, it has been suggested that junctional communication may have a vital role in embryogenesis.[53,60-62]

Inhibition of intercellular communication may provide a mechanistic link between carcinogenesis and teratogenesis.[9,10,53] Alteration of this membrane function may explain how some carcinogens and teratogens redirect cells and tissues into a path of abnormal differentiation. In the following analysis, arguments will be presented to support this viewpoint.

II. THE GAP JUNCTION AS A COMMUNICATION CHANNEL

Direct intercellular communication is known to occur between adjacent cells in a variety of conditions. A large body of evidence convincingly implicates the gap junction as the communicating channel.[55-57] Gap junctions have been found in all multicellular species of the animal kingdom investigated.[57] In mammals they are found in most tissues, both excitable and nonexcitable. The structures are conspicuously absent from circulating blood cells, adult skeletal muscle cells, and many adult nerve cells.[57]

A. Gap Junction Structure

Communicating junctions can form between cells from many different tissues,[56,58] leading to the speculation that there might be a single type of gap junction structure common to all cells. The prominent exceptions are the arthropod junction and the mammalian lens fiber junction. Both of these cell types apparently fail to form communicating junctions with other mammalian cells,[62a,62b] and the junctions appear structurally different from other vertebrate junctions.[56,58,62a,66] Even among various nonlens mammalian tissues there is considerable heterogeneity in the size and shape of the gap junctions.[56,58,59]

The gap junction appears in cross-section electron micrographs as a septalaminar membrane structure where opposing membranes of adjacent cells are separated by a 2- to 4-nm gap.[57] Using freeze-fracture techniques, *en face* views of the structure reveal plaques of 6- to 10-nm sized particles that apparently transverse the gap between the cell membranes. The size of the patches can vary widely.[56,58,59] In most mammalian tissues, each particle, known as a connexon, consists of six subunits surrounding a water-filled core.[63-65] The number of subunits appears to differ for some tissues, however, such as mammalian lens.[66] The connexons may assume a variety of packing arrangements in the membrane. The hypothesis that the less-ordered arrangements reflect the gap junction in its permeable state[56] has been recently contradicted by findings of variable packing arrangements in rapidly frozen tissues.[66a,66b] The connexons are firmly bonded to each membrane, since mechanical and chemical cell separation techniques result in the entire structure being removed to only one of the cells.[67] Gap junctions can be "unzipped" with hypertonic sugar solutions.[68]

The diameter of the core of the communicating junctions has been explored using fluorescent dyes as tracers (see Section II.B). It has been reported that insect junctions passage larger molecules, up to 1800 daltons, while mammalian junctions restrict passage to molecules of approximately less than 1000 daltons.[69] Mammalian junctions also appear to discriminate against passage of negatively charged molecules, suggesting that the core has a net negative charge.[69] It is becoming increasingly apparent that junctions may be able to modulate communication, restricting passage to smaller molecules under specific conditions.[70-72]

Gap junctions consist mainly of protein, with some lipid components. While there is disagreement as to the exact protein composition, a major gap junction protein of 26,000 to 28,000 daltons has been reported with most consistency.[56-59] Partial amino acid analyses of gap junction proteins isolated from rat liver and lens revealed no homology in the protein structures.[73] Two-dimensional peptide maps showed considerable homology among gap junction liver proteins from four mammalian species,[74] however, suggesting that these struc-

tures are not species specific. Preliminary sequence data of the amino terminal region of a gap junction protein isolated from rat heart shows about 40% homology with a rat liver gap junction protein.[74a] In addition, antibodies raised to rat[74b] and mouse[74c] liver gap junction proteins cross-reacted with a variety of tissues in several species, but not with mammalian lens fiber tissue. With the current, incomplete, information now available, it appears that the gap junction structures of many, but not all, mammalian tissues may indeed be similar.

Gap junctions are apparently dynamic structures.[59] In some tissues, gap junctions form at distinct times, as in the myometrium where gap junctions increase with parturition[75] or the pancreatic B-cells where gap junctions increase during secretion.[76] Additionally, gap junction proteins appear to turn over relatively rapidly, having an approximate half-life of 5 to 10 hr in rat liver[74a,77] and 5 hr in mouse liver.[78]

Gap junctions appear to form and grow in many tissues through coalescence of small aggregates of intramembrane particles which later fuse to enlarge the junction.[59] This was first described by Johnson et al.[79] with reaggregating Novikoff hepatoma cells in vitro. Junction formation is often very rapid, beginning in the first few minutes of contact in reaggregating cell systems.[79] This suggests that the components necessary for junction formation already exist in these cells and only require the appropriate stimulus to trigger rapid assembly. In support of this argument, most attempts to block formation of gap junctions using inhibitors of protein and RNA synthesis have failed.[80,81] Protein synthesis may be required in some cases where cAMP changes are involved, however.[82,83]

Two basic mechanisms have been proposed for the disassembly of gap junction structures.[58,59] Dispersion of the particles of the junctional plaques has been suggested, operating as the converse of the assembly process discussed above. Alternatively, it is suggested that gap junctions are endocytosed and broken down by lysozymes. The latter argument is supported by findings of invaginated gap junction membranes and acid phosphatase-positive gap junction vesicles inside of the cell.

B. Measurement of Junctional Communication

A variety of means have been devised to monitor direct intercellular communication.[55,84] We will briefly describe those techniques which will contribute to an understanding of later discussions.

1. Electrocoupling

The spread of passive electrical potential between contiguous cells is known as electrocoupling. Microelectrodes are inserted inside two neighboring cells and current pulses are passed into one of the cells. The electrical potentials of the two cells are measured simultaneously with respect to the external medium. The ratio of the voltage change in the second cell to that of the injected cell is the coupling coefficient or coupling ratio. When the cells are joined by communicating channels the coupling ratio is relatively high. For greater detail concerning this technique, the reader is referred to the review by Socolar and Loewenstein.[84]

This technique has proved to be a very sensitive test for the presence of communicating junctions. However, it only discerns the passage of the smallest types of molecules, those inorganic ions that carry electrical current. Furthermore, care must be taken in interpreting changes in the coupling ratio, since the measure is dependent on the electrical resistance of both the junctional and nonjunctional membranes.

2. Junctional Conductance

Measurement of junctional permeability can be obtained more directly and quantitatively by measuring junctional electrical conductance. This method is technically more difficult and restrictive than electrocoupling, requiring multiple microelectrodes with simultaneous measurement of several parameters. It is difficult to apply to complex systems such as cell

sheets and has therefore been used mostly with cell pairs or short cell chains. A major advantage of the technique is that it controls for changes in nonjunctional membrane resistance. Again, the reader is referred to the review by Socolar and Loewenstein[84] for greater detail.

3. Dye Transfer

Junctional communication can be observed using fluorescent dyes as tracer molecules. In most cases these dyes are injected into cells using microelectrodes. The dyes are generally hydrophilic and do not readily cross the nonjunctional cell membrane. Quantitative information concerning the permeability of the junctions can be obtained using dyes of different molecular weights and charges.[69-71] Fluorescent dyes can also be introduced into cells by transmembrane diffusion as nonpolar esters of diacetate, dipropionate, or dibutyrate.[85,86] Inside the cell the compounds are rapidly hydrolyzed by esterases to the fluorescent, hydrophilic compound.

4. Metabolic Cooperation

The intercellular exchange of metabolites through direct cell contact is known as metabolic cooperation. Subak-Sharpe et al.[87] first demonstrated the phenomenon using metabolically normal cells cultured with mutant cells that were metabolically deficient. When co-cultured in the presence of a radiolabeled precursor that required metabolism by the affected pathway, the mutant cells were able to incorporate the metabolite into their cellular products as visualized by autoradiography. Pitts and Simms[88] introduced a variation of this technique in which donor cells were prelabeled with a radioactive precursor such as ^{3}H-uridine and then co-cultured with unlabeled recipient cells. This technique has the advantage of not requiring mutant cells.

Fujimoto et al.[89] introduced a different method for the detection of metabolic cooperation that measured survival of mutant cells in the presence of a toxic precursor. Wild-type cells with the ability to metabolize the compound to its toxic metabolite died when cultured in the presence of the compound, but mutant cells that lack the ability to metabolize the compound normally survived the exposure. When wild-type and mutant cells were co-cultured, the mutant cells received the toxic metabolite from the wild-type cells if the cells were junctionally coupled. Junctional communication was thus measured as decreased mutant cell survival in co-cultures compared to mutant cells cultured without wild-type cells.

By monitoring the transfer of radiolabeled nucleotides between cells in culture, Gilula et al.[90] demonstrated that metabolic cooperation was associated with electrical coupling and the presence of gap junctions.

5. Electronmicrograph Visualization

Since gap junctions are the assumed structures responsible for direct intercellular communication, morphological detection of gap junctions has been used as an indicator of junctional communication. This requires electron microscopy of thin sections of the tissue and/or freeze-fracture of the membranes to reveal the internal membrane structure. Large areas of the membrane must be scanned to obtain accurate estimates, rendering the technique time-consuming. Additionally, negative results must be interpreted with caution. Recently, Meyer et al.[91] demonstrated that the gap junctional membrane decreased 100-fold in regenerating liver while electrocoupling was reduced only 10-fold. Indeed, there are several reports now of electrical and dye coupling existing in tissues in which no gap junctions could be observed.[58] It appears that electrical coupling is possible with very small numbers of communicating channels which may be difficult to locate in electron micrographs. Regardless, the gap junction is the membrane structure most consistently associated with direct intercellular communication.

C. Control of the Gap Junction

The communicating junction is apparently modulated in a variety of ways. Communication can be blocked completely or it can become restricted so that only small molecules pass. Some modifying influences which may be important for endogenous cellular control of the junction will be discussed here.

1. Calcium

Calcium can dramatically alter the permeability of intercellular communication in a variety of cells. Intracellular injection of calcium solutions into cells of the salivary gland of the insect *Chironomous* decreased both electrical and dye coupling.[70] This condition was reversed by lowering calcium concentrations. Furthermore, calcium reduced communication in these cells in a stepwise fashion, allowing selective restriction based on size of the transferred molecule.[70] Using the calcium-sensitive compound, aequorin, Rose and Loewenstein[92] showed that calcium levels need to become elevated only in the region of the junction of the *Chironomous* cells to decrease transfer.

Similarly, intracellular injection of calcium uncoupled normal cardiac cells, as did experimental procedures which elevated intracellular calcium levels.[93] Mammalian cells in culture were also uncoupled by treatments expected to elevate intracellular calcium levels. In experiments using rat epitheliod liver and rat fibroblast cell lines, Flagg-Newton and Loewenstein[71] found that metabolic inhibitors and a calcium ionophore partially uncoupled the cells. A combined treatment of metabolic inhibitors blocked transfer of all fluorescent dyes tested, but electrical coupling remained in many cases. In addition, treatment with the ionophore blocked transfer of the larger fluorescent dyes only, allowing dyes with molecular weights of up to 559 daltons to sometimes pass between cells. The cultured mammalian cells thus proved more resistant to uncoupling compared to invertebrate cells and mammalian cells in general.[71]

2. Acidity

Decreased intracellular pH has been associated with reversible uncoupling in a variety of cells, including rat epitheliod liver and fibroblast cell lines,[71] *Chironomous* salivary gland cells,[94] *Xenopus* embryonic cells,[95,96] *Ambystoma* and *Fundulus* embryonic cells,[97,98] and mouse pancreatic acinar cells.[99]

Because intracellular calcium and hydrogen ion levels are interdependent, it has been argued that changes in calcium may be secondary to changes in pH, and that the converse may also be true. The data of Rose and Rick[94] suggested that in *Chironomous* salivary gland cells, pH changes either resulted from or caused increased levels of calcium, and that calcium was primarily responsible for uncoupling. They used coupling coefficients for their measurements of electrocoupling. Rink et al.[96] and Spray et al.,[97,98] reported that the intracellular calcium concentrations did not change during acidification-induced uncoupling of *Xenopus*, *Fundulus*, or *Ambystoma* cells. Spray et al.[97] found that cellular acidification altered nonjunctional conductance in a variable fashion, while junctional conductance declined in a simple, directly related manner. They concluded that pH is a more likely physiological mediator of junctional conductance than calcium, since calcium depressed junctional conductance in their experiments only at very high concentrations. The discrepancy between the findings of the laboratories could be due to intrinsic differences in vertebrate vs. invertebrate junctions or the inconsistent effects on the nonjunctional membrane resistance, since coupling coefficients are dependent on both the junctional and nonjunctional conductances.

3. cAMP

Increased intracellular levels of cAMP increase the permeability of the gap junction under certain circumstances. Treatment of mammalian cell cultures with cAMP, dibutyryl cAMP,

or phosphodiesterase inhibitors, all of which increase intracellular cAMP, restored dye coupling to cells previously unable to communicate.[100,101] Electrical coupling was also improved in the salivary gland of *Drosophila* with similar treatments.[102] The ability of low serum concentration and low cell density to stimulate junctional communication has been attributed to increased cAMP levels.[100,101]

4. Hormones

Gap junctions are known to be hormonally responsive in several types of tissues.[59,222] Some excellent examples are found in the female reproductive system. The granulosa cells of theovarian follicle have gap junctions that increase with estrogen and decrease with ovulatory doses of human gonadotropin.[103,104] In uterine myometrial cells, gap junction formation can be inhibited by progesterone and stimulated by estrogen and certain prostaglandins in many animal species.[105-107] Another interesting example is the increase in gap junctions observed in pancreatic B-cells when stimulated to secrete by insulin.[76] It is possible that some of these hormonal effects are mediated by protein kinase activation by calcium or cAMP, since many hormones are known to increase intracellular levels of these substances.

5. Phosphorylation

Recent evidence suggests that phosphorylation may be involved in the regulation of junctional communication. It has been reported that a lens fiber junction protein (MP26) is phosphorylated in vitro by cAMP-dependent protein kinase in isolated membranes of bovine lens.[108] In addition, Wiener and Loewenstein[109] have been able to restore junctional competency to a noncommunicating Chinese hamster ovary cell line by inserting the active subunit of cAMP-dependent protein kinase. One interpretation of these reports is that cAMP may stimulate junctional communication by phosphorylating a protein on the gap junction through activation of cAMP-dependent protein kinase. Some caution may be warranted in evaluating the findings with the lens junction protein, however, since there are many apparent differences between the lens and other vertebrate junctions, and recent studies have questioned the origin of this protein.[109a,109b]

Potent inhibitors of junctional communication, phorbol esters, activate a different kinase known as protein kinase C or calcium- and phospholipid-dependent protein kinase.[110] The phorbol ester substitutes for diacylglycerol in the activation of the enzyme, thereby lowering the requirement for calcium substantially. Diacylglycerol is formed at the same time as inositol trisphosphate with the cleavage of phosphatidylinositol 4,5-bisphosphate.[110a] Various membrane receptor actions are now believed to function by activation of inositol metabolism,[110b] and inositol trisphosphate has been implicated as a second messenger for calcium mobilization from intracellular stores.[110a] Since these phorbol esters activate a phosphorylation reaction whose consequences are largely unknown, and they can profoundly depress junctional communication, it is possible that the junction effects may be related to the phosphorylation reactions.

III. THE INITIATION AND PROMOTION MODEL OF CARCINOGENESIS

The nature history of cancer formation seems to indicate, both in experimental animals and in naturally occurring human tumors, that the tumor is the end result of a complex evolution of celluar phenotypes derived from a single normal cell which had "gone wrong".[111,112] Despite the obvious heterogeneity of phenotypes and genotypes within a tumor,[113] there seems to be strong evidence of the clonal origin of tumors.[114-116]

From a variety of animal and organ system cancer models,[117] this complex carcinogenic process appears to be dissectible into initiation, promotion, and progression phases.[42,43,111,112,118] Operationally, the initiation phase involves the biological conversion of a single normal cell

to a "premalignant" cell by means of a rather irreversible mechanism. Promotion refers to the clonal amplification of this single premalignant or initiated cell, such that a conversion to the malignant state occurs. Progression refers to the pleiotropic diversion of phenotypes occurring in the malignant cells.

Although the molecular mechanisms of these distinct biological phases are not yet known, it is clear that the initiation phase is distinctly different from that of promotion. Initiators must beagents or conditions which can induce rather stable and irreversible genomic changes. Mutations (gene or chromosomal), produce stable and "irreversible" changes in the genome. In addition, most mutagens are either carcinogenic initiators or "complete" carcinogens.[119] Clearly, one can also conceive of stable, nonmutagenic changes in the genome, which could then be considered as an initiation event.[119]

Promoters, by causing the release of a premalignant, initiated cell from its latent state and by clonally amplifying the initiated cell, are acting as selective mitogens.[44] Among the many pleiotropic effects of promoters and promoting conditions on cells,[52,120] sustained hyperplasia seems to be obligatory.[121] The fact that some noncytotoxic promoters can induce divergent responses in many cell types, such as differentiation of one type and proliferation of another,[122] suggests they can interfere with normal regulatory mechanisms controlling proliferation and differentiation.

Although operationally, initiation and promotion appear to involve distinct mechanisms, the chemicals which influence carcinogenesis do not seem to fit into neat categories of "pure" initiators or promoters.[119] The reason seems fairly straightforward in that chemicals rarely, if ever, do just one thing in living cells. For example, agents which can initiate cells by inducing mutations can also kill cells, which, in turn, could promote tumors by inducing the surviving, initiated stem cells to proliferate. Other agents working at physiological levels might modulate gene activity without killing or mutating cells. On the other hand, these same agents at high concentrations might cause membrane and microtubule disruption leading to aberrations in chromosomal distribution (aneuploidy) and/or to cell death (Figure 1).

Finally, the various theories of carcinogenesis (i.e., mutation vs. epigenetic theories, oncogene theory, etc.) must accomodate the initiation/promotion model of carcinogenesis. The fact that an oncogene of human bladder tumor cells differs from the cellular homologue at the oncogene by a single base change directly implicates mutations with some aspects of the carcinogenic process.[123,124] Mutagens at non-necrogenic doses appear to be good initiators and at higher cytotoxic levels, "complete" carcinogens (i.e., they can perform both the initiating and promoting functions). Nonmutagenic chemicals, given after an animal has been exposed to a mutagen/initiator, seem to act as promoters (mitogens or growth factors) by allowing selective proliferation and accumulation of initiated cells. Examples of promoters include phorbol esters,[42,125] phenobarbital,[126,127] DDT,[126,128,129] polybrominated biphenyls,[130,131] bile acids,[132,133] steroid hormones,[134,135] di(2-ethylhexl)phthalate,[136,137] chloroform,[138,139] and carbon tetrachloride.[127,140] Most recently, several reports discount the potential genotoxic damage of promoters.[141-143]

The demonstration that multiple oncogenes are needed for transformation[144,145] suggests that one set of oncogenes puts cells in the initiated state, whereas other oncogenes, functioning as growth factors (i.e., c-sis as homologue to platelet-derived growth factor[146]) would act as promoters to increase the target size of the initiated clone of cells. Further exposure of these initiated cells to mutagens and increased cell division have been predicted to increase the probability of their conversion to the malignant state.[147-149] Observations confirming these predictions have been made.[142,143,150,151]

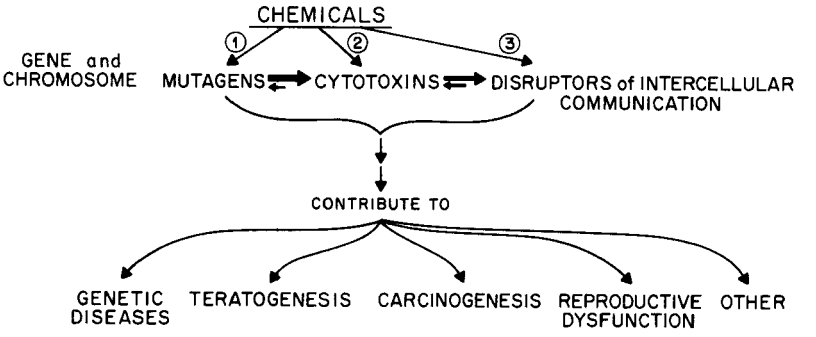

FIGURE 1. A heuristic scheme to classify chemicals on the basis of three biological end points: mutagenicity, cytotoxicity, and inhibition of intercellular communication. The arrows between the biological end points are designated to mean that chemicals can have multiple biological consequences in the direction indicated (i.e., mutagens can kill cells and the death of cells can cause the modulation of gene expression in some surviving cells). In addition, some chemicals that can inhibit intercellular communication at noncytotoxic levels could at much higher concentrations kill cells, possibly also inducing some chromosomal mutations at these high doses. It further depicts the speculated disease processes in which mutagenesis, cell death, and disrupted intercellular communication can play a role. (From Trosko, J. E. et al., *Ann. N.Y. Acad. Sci.*, 407, 316, 1983. With permission.)

IV. POTENTIAL ROLE OF JUNCTIONAL INTERCELLULAR COMMUNICATION IN TUMOR PROMOTION

Since tumor promotion involves the selective expansion of initiated stem cells by their proliferation and blocked differentiation, one must surmise, in the absence of tumor promotion, that there are mechanisms which suppress the initiated cell. In other words, there are factors regulating proliferation and differentiation in these single initiated cells from the surrounding normal cells.

Furth[152] and Iversen[153] suggested that cancer might be the result of dysfunctional homeostatic control. This concept was supported by the observations that malignant cells lacked contact inhibition[154,155] and that many cells from tumors did not perform gap junction-mediated intercellular communication.[55,156,157] Furthermore, those theories suggesting that cancer is a "disease of differentiation"[1,2] or that "oncogeny is blocked or partially blocked ontogeny"[3] are also consistent with the idea that abnormal cell communication exists between the cancer cell and the surrounding normal cell.

Many studies seem to suggest that gap junctional communication plays a very important role in the adaptation of multicellular organisms (see later discussion). The compromised gap junctional communication in most tumor cells, although not a universal observation,[55,57,58] suggests that, among the many things which occur during the neoplastic transformation of a normal cell, the loss of gap junctional communication is important and not just a random phenotype found in malignant cells.

The potential link between gap junctional communication and the tumor promotion phase of carcinogenesis was made when Yotti et al.[147] and Murray and Fitzgerald[158] observed that noncytotoxic and nongenotoxic tumor-promoting chemicals, such as the phorbol esters, inhibited junctionally mediated metabolic cooperation. Enomoto et al.[159] have strengthened this association with their observation that the tumor-promoting phorbol esters reversibly inhibited electrocoupling between cells. Later, Yancey et al.,[160] using freeze-fracture analysis, showed that tumor promoter-treated Chinese hamster V79 cells had far fewer gap junctions on their plasma membranes than did control cells. More recently, Kalimi and Sirsat[161,161a] observed in transmission electron micrographs that phorbol esters also caused

the disappearance of gap junctions from the interfollicular epidermis of treated mouse skin. This effect was observed only at tumor-promoting doses. Finally, using a variety of in vitro cell strains and lines, several investigators have shown that many known tumor promoters (natural products, food additives, pollutants, drugs, pesticides, herbicides, components of cigarette tar, biological toxins, etc.[128,162-176]) could inhibit gap junctional intercellular communication.

Evidence from somatic cell genetics experiments also suggests a link between gap junctional communication and tumorigenicity.[177] In brief, using somatic cell hybrids between junctionally competent and incompetent cells, there seems to be a good correlation between depressed junctional communication and cancerous growth. These observations might provide the basis for linking epigenetic and genetic mechanisms of transformation. Nongenotoxic chemicals, such as TPA, might reversibly block junctional communication, thereby bringing about a transformation phenotype by epigenetic means, while mutations affecting the regulation of gap junctional function or structure could permanently bring about the tumorigenic phenotype.

Finally, a hypothesis has been proposed that some oncogenes could act as tumor promoters by inhibiting gap junctional communication.[178] Atkinson et al.[179] reported that temperature-sensitive avian sarcoma virally transformed rat kidney cells cannot perform gap junctional communication at permissive temperatures, suggesting that the oncogene product somehow renders the gap junctions nonfunctional. If this observation can be generalized to other viral and cellular oncogene functions, a unification of viral and chemical carcinogenesis might be possible.

V. MOLECULAR BASIS OF ABNORMAL EMBRYOGENESIS

A. Definition of Teratogenesis

Teratogenesis is the process of abnormal embryonic development. The definition of teratogenesis is often restricted to structural abnormalities, but this distinction may artificially limit discussion of mechanisms since other nonstructural deficits can also be manifestations of abnormal embryonic development. Examples of the latter include behavioral deficits, mental and physical growth retardation, immunological compromise, and increased susceptibility to carcinogenesis. Stillbirth, premature delivery, and spontaneous abortion are additional manifestations of embryotoxicity which may share common mechanisms with teratogenesis. We mention these embryological responses to toxic agents simply as a reminder that structural deficits are not the sole abnormal embryological effects.

B. General Principles of Teratogenesis

From experimental studies with laboratory animals, several general principles of teratogenesis have been derived and discussed extensively by Wilson.[54] These principles define parameters that influence the teratogenic outcome when embryos are exposed to toxic substances. These parameters include dose, route of delivery, metabolism, embryonic stage of development, and genetic predisposition. A complete discussion of these principles can be found in Wilson's review, so we will include only those points that are most important to our analysis.

1. Dose

One aspect of dose-response relationships observed in teratological studies is the apparent existence of a threshold, below which the substance fails to elicit a teratogenic response. If only structural deformities are considered, it appears that teratogenesis may be similar to the promotion phase of carcinogenesis in the likely existence of thresholds.[54,180-182] Because of insufficient information, it would be premature to generalize the concept of teratogenic

thresholds to other manifestations of embryotoxicity, such as mental dysfunction, carcinogenesis susceptibility, sterility, and growth retardation.

2. Developmental Stage

One of the most prominent determinants of a teratogenic response is the stage of development at which the embryo is exposed to the toxic agent. It is generally accepted that "critical periods" exist in the development of an embryonic tissue at which it is particularly susceptible to teratogenic insult. During the critical period, a tissue may be adversely affected by a relatively low exposure to a teratogenic agent. With increased dosage, similar teratogenic responses can sometimes be elicited at times surrounding the critical period. These periods typically occur at the organogenesis stage of development, when the embryo is forming the primordial structures of the organs. The time of maximum sensitivity differs among the tissues, probably reflecting the different schedules of development.

It is suggested that the tissues are on development schedules which have narrow limits of tolerance, and that the organ primordia must achieve the basic structures necessary within a narrow window of time; otherwise, the tissues will be unable to interact in the proper manner to form the correct final structures. Anything which alters the development sequelae is expected to have a significant impact on the structural outcome.

3. Genetic Predisposition

The genetic background of an animal is known to influence the teratogenic response to exogenous agents. Thalidomide is a well-known example of this characteristic, being potently teratogenic in humans but only weakly so in most nonprimate species. Strain differences within species of experimental animals are also well documented. For example, the A/J mouse strain is particularly susceptible to cleft palate induction by various agents compared to the C57BL strain.[183] Dagg[184] and Cole and Trasler[185] have provided clear examples of synergism between genes and chemical teratogens, reporting that mutant genes and chemical teratogens interacted to produce specific malformations at dosages below their respective individual thresholds. It is believed that similar interactions may account for certain malformations being associated with genetic syndromes in humans, as discussed in the Introduction.

C. Molecular Mechanisms of Teratogenesis

Wilson[54] has delineated those early events which could contribute to abnormal embryonic development and hence teratogenesis. The list is quite extensive, reflecting the complexity of the morphogenetic process and the vast opportunity for interference with the system. The mechanisms most relevant to our discussion include cell death, abnormal membrane characteristics, and altered amounts of metabolic components. These initial events might subsequently alter morphogenetic fields, genetic programs, or developmental schedules.

1. Cell Death

Of the mechanisms proposed for teratogenesis, the cell death hypothesis has received perhaps the most attention.[186] It is suggested that excessive cell death which exceeds the proliferative repair capabilities of a tissue may leave the tissue with too few cells to carry out a necessary developmental event within a critical time period. The relationship is not always consistent, however, with some tissues exhibiting increased cell death with no structural consequences and other tissues exhibiting little cell death with deleterious outcomes in the development of the tissue.[186] In general, however, there is more agreement with the hypothesis than against the hypothesis.

2. Altered Membrane Characteristics

Altered membrane characteristics have been studied to a lesser extent in teratogenesis, perhaps due to the difficulties involved. Nonetheless, the importance of the membrane in

embryonic development has long been appreciated. Several necessary processes of morphogenesis are believed to be dependent on the membrane, including tissue induction, cell migration, cell recognition, cell shape changes, responsiveness to hormonal stimuli, and junction formation and integrity. While these membrane-mediated phenomena are believed to be necessary for morphogenesis, the developmental consequences of alteration of membrane properties is not well understood.

VI. POTENTIAL ROLE OF JUNCTIONAL COMMUNICATION IN TERATOGENESIS

Intercellular channels have the potential for playing a pivotal role in developmental processes because they offer a convenient mechanism for restricting diffusion of regulating substances. Several models have been proposed which suggest schemes of how junctional communication may be involved in embryogenesis.[60-62]

A. Junctional Communication and Normal Morphogenesis

1. The Formation and Disappearance of Junctional Communication

The formation and disappearance of gap junctions and/or junctional communication have been associated with major morphogenetic events in several embryonic species and tissues. We will describe a few salient examples here, and the reader is referred to the extensive review by Bennett et al.[187] for more details and examples.

In an important study, Lo and Gilula[188] monitored developmental changes in the junctional competency of early mouse embryos. In the very early embryos, ionic coupling and the transfer of horseradish peroxidase (40,000 daltons) were observed only between sister blastomeres. This transfer was attributed to cytoplasmic bridges because of the pattern of transfer and the large size of the communicating channels. Electrocoupling and transfer of the smaller dye, fluorescein (330 daltons), was first observed throughout the entire embryo in the late eight-cell stage, coincident with compaction and the appearance of gap junctions. These findings were later verified and expanded by McLachlin et al.[81] and Goodall and Johnson.[86,189]

In a compansion study, Lo and Gilula[190] continued to monitor junctional coupling in the post-implantation embryos. They reported that dye transfer became restricted to certain areas of the embryo although all of the cells remained electrically coupled. Dyes that originally crossed freely between the embryo proper and the extra-embryonic tissues were unable to do so later in the post-implantation stages. Additional areas of compartmentalization were also recognized within the embryo proper as the embryo developed. It was suggested that this type of compartmentalization may represent early tissue determination.

In older embryos undergoing organogenesis, changes in electrocoupling have been observed between presumptive tissues prior to important differentiation steps. Warner[191] found that the cells of the neural epithelium are initially electrocoupled to surrounding ectoderm, but as the neural folds fuse, the neural cells become uncoupled from the overlying ectoderm while still maintaining coupling among themselves. In a similar study, Blackshaw and Warner[192] reported that electrocoupling between presumptive somites was interrupted at the intersomite border prior to somite formation. The important findings of these studies are that groups of cells were able to selectively stop junctional communication with a particular type of cell while maintaining communication among themselves, and that this occurred at an important transition of development.

Ginzberg and Gilula[193] examined the pattern of gap junctions in the embryonic chick otocyst. Alterations in the distributions of gap junctions were associated with differentiation of the epithelium. Gap junctions initially joined the homogenous population of epithelial cells during formation of the otic pit, but as the epithelium differentiated into sensory and support cells, the junctions reorganized so that only the support cells remained linked to

each other by gap junctions. This coincided with innervation of the sensory cells by the underlying ganglion cells.

2. Tissue Induction

The ability of one tissue to induce morphological changes in a neighboring tissue is a well-documented finding. Initially, experiments with membrane filters separating the tissues were interpreted to mean that cell contact was not necessary for induction to occur, and that the inducing agent must be diffusible in the extracellular space. It is now known that cell processes can in fact transverse the filters used in most of these studies, and it is generally agreed that cell contact cannot be discounted in studies using filters to separate the tissues.[53]

Cell contact is not believed to occur in the inductive effects between certain epithelial and mesodermal tissues. In one such tissue, the limb, gap junctions have been found to exist within the distinct tissues but not between the tissues involved in the inductive process.[194,195] The gap junctions in the inductive apical ectodermal ridge increase during the period of induction.[194] These findings suggest that junctional communication is possibly involved in the tissue's production of or response to inductive signals, but not in the actual transmission of the inductive signals.

A morphometric study of the embryonic chick limb shows that the gap junctions have different organization patterns in different parts of the limb.[196] Based on the findings of Peracchia[56] and De Mello,[93] the authors suggest that the different junction patterns in the limb reflect differences in junction competency. While interpretation of these data was admittingly speculative, the authors suggested that the junctional network within the apical ectodermal ridge could be used to control the inductive signal from the ridge, and that coupling among the subridge mesoderm might be important for maintaining its proliferative, "embryonic" nature.

3. Morphogenetic Fields

Morphogenetic fields are those areas in a developing organism over which a specific morphogenetic event is restricted. Often "morphogens", diffusible substances which elicit the morphogenetic event, are evoked to explain morphogenetic fields. In such cases the boundaries of the morphogenetic field are defined by the extent of influence of the morphogen. Wolpert[62] has discussed several schemes by which morphogenetic fields could be established. Most significantly, he has concluded that direct intercellular communication via junctions would be a powerful and likely method to control the distribution of small morphogenetic substances.

An important recent finding supports a role for junctional communication in morphogenetic field determination. The development of segmentation in insect larvae cuticles strongly suggests the presence of discrete morphogenetic fields within each segment. Warner and Lawrence found that cells across segmental borders were electrocoupled[197] but were unable to transfer the dye Lucifer Yellow® (450 daltons).[72] A smaller tracer molecule, lead-EDTA (374 daltons), was freely transferred irrespective of the segmental boundary. Recent studies with *Drosophila* have generalized these findings and showed that the border cells separating the compartments of the wing imaginal disk were distinct from other disk cells in their inability to transfer fluorescent dyes with molecular weights greater than 376.[198-200]

Less direct evidence from studies of head regeneration in hydra was used by Wolpert and colleagues to devise some models of morphogenetic fields.[62,201,202] A study by Wilby and Webster[203] suggested that an inhibitory substance was emitted from the head region which prohibited formation of another head over a distance of 0.5 mm. This inhibition is apparently dependent upon cell contact.[204] Since gap junctions are formed within a few hours after grafting of a head, Wolpert[201] has suggested that the inhibitory signal may be transmitted through the gap junction. Recent isolation and purification of the head inhibitor has revealed

that it is a hydrophilic protein with a molecular weight of less than 500 daltons,[205,206] which could easily pass through junctional structures.

B. Inhibition of Junctional Communication and Teratogenesis

Recently, Warner and colleagues have made some very interesting and important findings on the possible role of gap junctions in embryonic development.[207] They injected antibodies raised against purified rat liver gap junction proteins into single cells of *Xenopus* embryos at the eight-cell stage. The antibodies produced abnormal morphogenesis without increasing cytotoxicity or inhibiting cell division. Indeed, Kageura and Yamana[207a] have shown that removal of a single cell in the eight-cell *Xenopus* embryo does not usually cause substantial alteration of development. When the antibodies were injected into the same cell, characteristic, reproducible malformations occurred in the mature tadpoles. The data thus suggest that gap junction communication is involved in morphogenetic pattern formation. This study is the first direct demonstration that inhibition of gap junction-mediated intercellular communication is associated with abnormal embryonic development.

Inhibition of metabolic cooperation in a V79 Chinese hamster cell system has recently been used to test several teratogens for their ability to block junctional communication. Several teratogens not known to be particularly genotoxic are able to block metabolic cooperation in this system, including mirex,[208,209] phenobarbital,[209,210] diphenylhydantoin,[209,210] various alkyl glycol ethers,[211] ethanol,[212] warfarin,[209] and chlorpromazine.[213] While this in vitro system is rather different from the in vivo situation in mammals, these data nonetheless suggest an association between inhibited junctional communication and teratogenesis. Furthermore, if inhibition of junctional communication can be substantiated as a mechanism of teratogenesis, in vitro assays such as this may provide useful, rapid screens for teratogens.

VII. INHIBITION OF INTERCELLULAR COMMUNICATION AS A COMMON MECHANISM OF TERATOGENESIS AND CARCINOGENESIS

Associations between teratogenesis and carcinogenesis have been recognized on several levels, as noted in the Introduction. Because of the complexities of each phenomenon, it is likely that no single mechanism will be found common to both that includes all situations. The possibility that junctional communication is involved in the control of growth and differentiation,[55] however, suggests that inhibition or alteration of this process may be one common link between teratogenesis and carcinogenesis (Figure 2).

A. Indirect Effects of Cytotoxicity

As already noted, many carcinogens are also teratogenic.[4-10] One large class of such agents are those that are genotoxic.[5,8,50] Genotoxic agents administered by themselves are generally not carcinogens at low doses, but require doses large enough to elicit cytotoxicity in the target tissue in order to become complete carcinogens.[119] This cytotoxicity is accompanied by subsequent hyperplasia.[214,215] Many teratogens behave in an analogous manner, causing marked necrosis followed by "compensatory hyperplasia".[186] This finding is so widespread that the "cell death hypothesis" is a widely held theory of teratogenesis.[54] The hypothesis predicts that the primary cause of abnormal development is the lack of a critical number of cells needed to carry out a necessary developmental event.[186] The cytotoxicity of these compounds, however, has the additional effect of isolating cells from each other and in that fashion interrupting intercellular communication.[10] While the mechanism of communication disruption is indirect in this situation, it is nonetheless a manner in which these types of carcinogens and teratogens could be included in the theory proposed in this article.

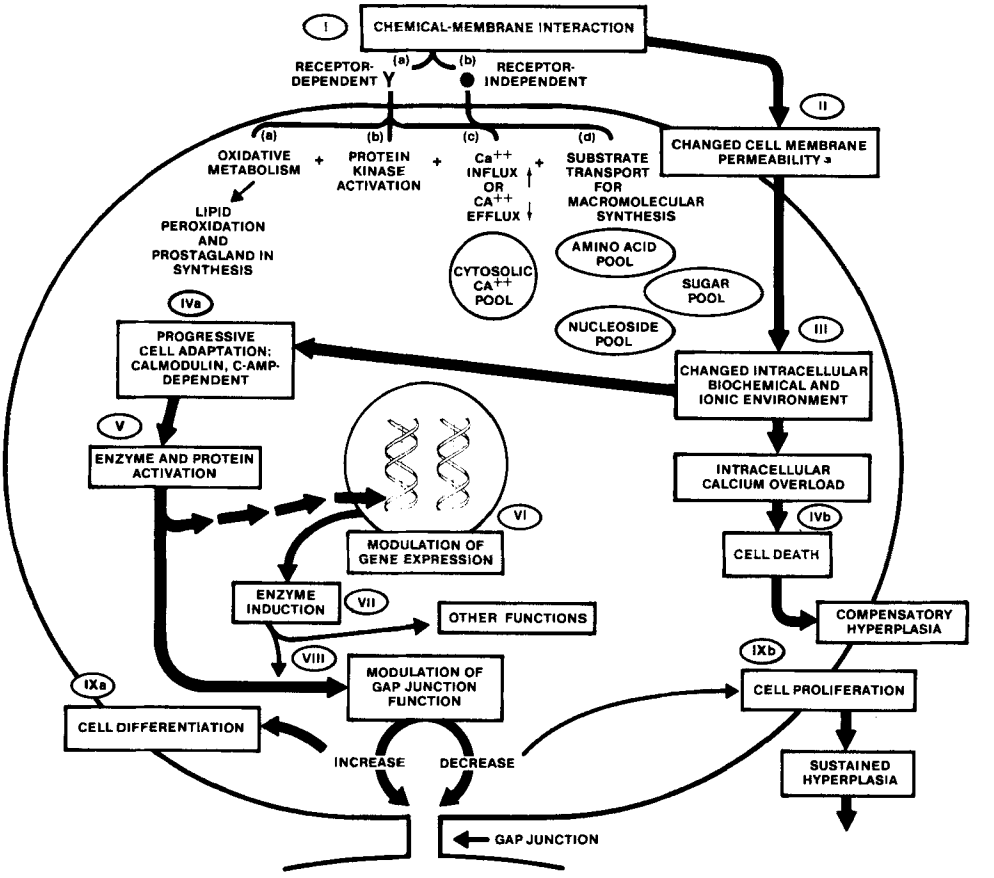

FIGURE 2. A schematic representation of pleiotropic membrane-triggered reactions affecting mitogenesis, differentiation, and cytotoxicity. The hypothesis represented is based on membrane receptor-independent or dependent chemicals causing an immediate physical/chemical alteration in membrane permeability to ions and molecular substrates (i.e., Ca^{++}, amino acids). In addition, activation of pre-existing enzymes and protein structures takes place, which in turn sets off a cascading series of reactions. With altered substrate pools, cytosolic free ion concentrations, free radical generation, and activated enzymes, modulation of gap junction function and new gene expression and enzyme induction occur. This, in turn, alters the physiology of the cell. Depending on the initial cell physiology, the nature and amount of the membrane signal, and the nature of feedback homeostatic systems, the premitotic cell either divides or differentiates and the post-mitotic cell adaptively responds with altered gene expression. The hypothesis is based on the assumption that all of these pleiotropic membrane-triggered responses are tightly coordinated in normal cells. (From Trosko, J. E. and Chang, C.-C., *Pharmacol. Rev.*, 36, 137s, 1984. With permission.)

B. Membrane Effects

More recently, it has been recognized that many tumor promoters are also teratogenic. Examples include phenobarbital,[126,216,217] synthetic estrogens (including DES),[38,134,218,219] and TPA.[42,220,221] It is generally believed that tumor promoters are not genotoxic and that they exert their effects primarily at the cell membrane.[51] The cell membrane is a potential target site for teratogens also, since many morphogenetic processes are believed to be membrane-dependent (see Section V.C.2). Direct interruption of junctional communication may be a common mechanism of action for these types of compounds, since many have the ability to interrupt metabolic cooperation in V79 Chinese hamster cells in vitro.[147,209,210,219]

Substances which act primarily at the cell membrane may elicit their responses by activating

receptors and/or their reactions. A large number of substances activate cAMP reactions upon binding to their receptors. Since increased intracellular cAMP levels[100-102] and cAMP-dependent protein kinase activity[109] have been associated with recovery of junctional competency, it is possible that substances which activate the cAMP cascade may also influence junctional communication. Indeed, gap junctional changes have been associated with cAMP-dependent hormones in several systems.[59,222]

Another class of substances now appears to activate a different second messenger system upon binding to their receptors.[110a] These compounds elicit the breakdown of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol trisphosphate.[110b] Diacylglycerol activates protein kinase C,[223] and inositol trisphosphate mobilizes calcium from intracellular stores.[110a] The breakdown of phosphatidylinositol 4,5-bisphosphate can be inhibited by cAMP.[226] For a series of phorbol compounds, the ability to activate protein kinase C has been correlated with the ability to inhibit junctional communication and promote tumor formation in mouse skin.[110,147,158] Furthermore, increased intracellular calcium has been associated with depressed junctional communication in a variety of cells.[70,92-93a] It is possible, then, that activation of this system, or part of this system, may have consequences for junctional competency.

When considered together, the speculation arises that substances which activate protein kinases may alter normal growth and differentiation by phosphorylation reactions which ultimately affect junctional communication (Figure 3). Further investigation in this area is needed to substantiate the possible interrelationships of these events.

C. Proposed Model

1. Carcinogenesis

The hypothesis has been presented that cancer may be a problem of aberrant differentiation sustained by failed intercellular communication. The multistage model of carcinogenesis predicts that the irreversibly initiated cell is at least partially regulated by the surrounding cells of a tissue, possibly by the secretion of extracellular factors (chalones)[224] or by junctional communication of regulating substances. The initiated cell remains inactive until stimulated to proliferate by a tumor promoter. It is suggested that tumor promoters may release the initiated cell from control of the surrounding tissue by interrupting intercellular communication.

Several tumor promoters have now been shown to interfere with junctionally mediated metabolic cooperation in mammalian cell cultures.[128,147,158-176] Additionally, many tumorigenic cells have decreased abilities to junctionally communicate.[55,156,157] These findings support the hypothesis that tumor promoters act by inhibiting junctional communication between the initiated cell and the surrounding normal cells.

Several tumor promoters, including phorbol esters,[110] teleocidin,[225] and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD),[230] activate protein kinase C. The mechanism of activation is most clearly understood for the phorbol esters, which substitute for the endogenous kinase substrate, diacylglycerol, and lower the calcium requirement for activation.[110] Diacylglycerol is rapidly metabolized by the cell, however, whereas TPA and other phorbol esters are only poorly metabolized.[226] It has been suggested that the unusually prolonged activation of protein kinase C could be responsible for some of the effects of TPA in the cell, including its potent tumor-promoting ability.[226] Alternatively, it may be that an imbalance of second messengers, caused by activation of protein kinase C without concomitant mobilization of calcium by inositol trisphosphate, is responsible for the tumor-promoting effects.[110a]

In certain cells the tumor promotion process can be interrupted by chemicals which increase intracellular levels of cAMP.[227] Since increased cAMP levels have been associated with improved junctional communication,[100-102] it is possible that junctional communication effects mediate the antitumor promoter activity. Lending support to this hypothesis are Kanno et al.,[226a] who reported that dibutyryl cAMP and aminophylline prevented uncoupling of cells by a tumor-promoting phorbol ester.

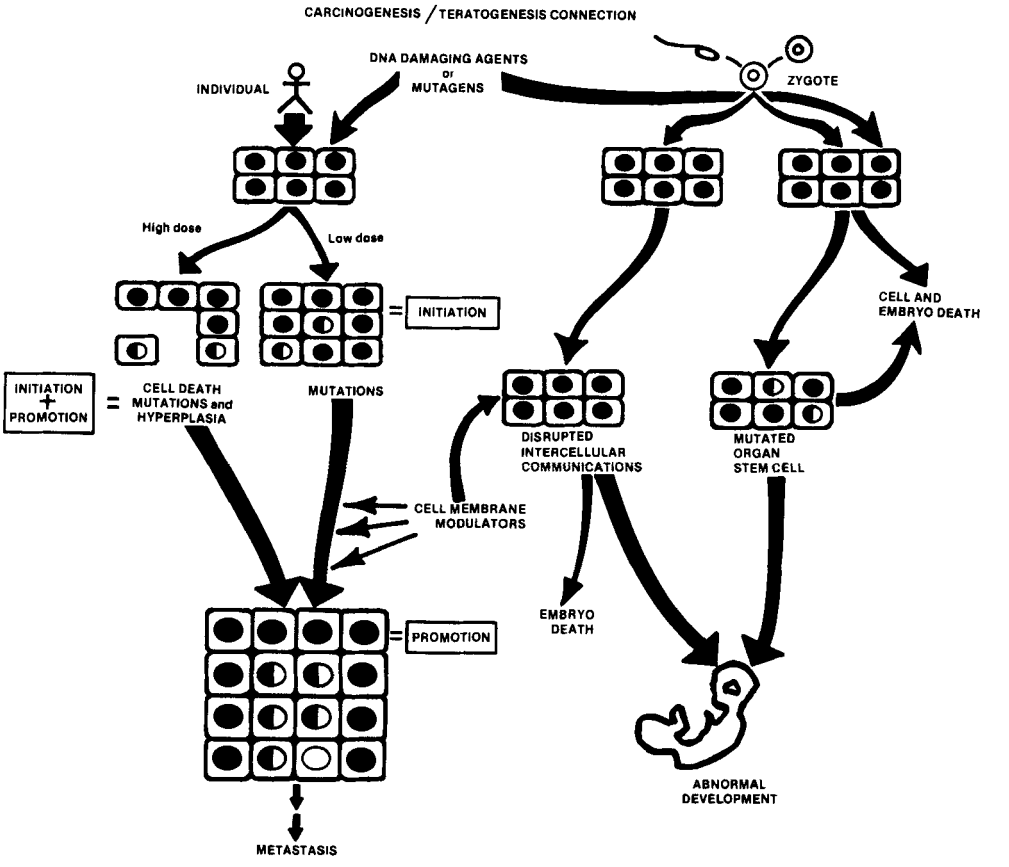

FIGURE 3. This diagram illustrates a hypothesis linking mutagenesis, cell death, and disrupted intercellular communication with the initiation/promotion phases of carcinogenesis and teratogenesis. An early conceptus or fetus exposed to a mutagen at a low dose could conceivably have a critical gene mutated in a stem cell leading to a congenital defect. At high cytotoxic doses of a mutagen, fetal toxicity would be expected. Exposure to non-genotoxic, but cytotoxic chemicals could also lead to congenital defects or fetal toxicity. Chemicals which inhibit intercellular communication could disrupt regulation of proliferation and differentiation of tissues if given at a critical period of development. The consequences would be either congenital defects or embryo- or fetotoxicity. The same chemicals and actions of chemicals can influence carcinogenesis as either complete or "incomplete" carcinogens. (From Trosko, J. E. and Chang, C.-C., *Methods for Estimating Risk of Chemical Injury: Human and Non-Human Biota and Ecosystems*, Vouk, V. B. et al., Eds., John Wiley & Sons, Sussex, England, 1985, 181. With permission.)

2. *Embryogenesis*

Intercellular communication has been an integral consideration in most theories of embryogenesis. It has been reasoned that the cells of an embryo must be able to communicate with each other to define tissue specificity and pattern formation, and to coordinate morphogenetic events. Additionally, neighboring tissues have long been known to exert inductive influences on each other,[53] requiring some form of signal transmission.

It has been hypothesized that direct intercellular communication of developmental signals via junctions would offer a convenient and efficient means of controlled diffusion of morphogenetic signals.[60-62] Many studies have chronicled alterations in gap junctional communication that occur coincident with major developmental events (see Section VI.A.1). Recent studies demonstrate that junctional communication may only be modified, not completely abolished, at boundaries of morphogenetic fields.[72,198-200] The role of gap junctions

in the process of tissue induction is less clear, and at the present time appears to involve the induction of or response to inductive signals, but not the actual transmission of the signals. These studies support a central role for junctional transmission of development-regulating substances. The logical extension of this conclusion is that inhibition of junctional communication in the embryo would result in abnormal development.

A recent study has provided direct evidence that inhibition of gap junction-mediated communication may interfere with embryonic development.[207] Studies with mammalian cell cultures have also shown that several teratogens interfere with gap junction-mediated metabolic cooperation.[208-213] The challenge now is to determine whether this is a phenomenon that can be extended and generalized as a mechanism of teratogenesis common to many substances and conditions.

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