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CELL-CELL COMMUNICATION IN CARCINOGENESIS

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1. ABSTRACT

To explain the complex carcinogenic process by which a single normal cell in human beings can be converted to an invasive and metastatic cancer cell, a number of experimental findings, epidemiological observations and their associated hypothesis/theories have been integrated in this review. All cancers have been generally viewed as the result of a disruption of the homeostatic regulation of a cell's ability to respond appropriately to extra-cellular signals of the body which trigger intra-cellular signal transducting mechanisms which modulate gap junctional intercellular communication between the cells within a tissue. Normal homeostatic control of these three forms of cell communication determines whether the cell: (a) remains quiescent (Go); (b) enters into the cell proliferation phase; (c) is induced to differentiate; (d) is committed to apoptose; or (e) if it is already differentiated, it can adaptively respond.

During the evolution from single cell organisms to multicellular organisms, new cellular/biological functions appeared, namely, the control of cell proliferation ("contact inhibition"), the appearance of the process of

differentiation from committed stem cells of the various tissues and the need for programmed cell death or apoptosis. Interestingly, cancer cells have been characterized as cells: (a) having been derived from a stem-like cell; (b) without their ability to control cell growth or without the ability to contact inhibit; (c) which can not terminally differentiate under normal conditions; and (d) having altered ability to apoptosis under normal conditions. During that evolutionary transition from the single cell organism to the multicellular organism, many new genes appeared to accompany these new cellular functions. One of these new genes was the gene coding for a membrane associated protein channel (the gap junction), which between coupled cells, allowed the passive transfer on ions and small molecular weight molecules. A family of over a dozen of these highly evolutionarily–conserved genes (the connexin genes) coded for the connexin proteins. A hexameric unit of these connexins in one cell (a connexon) couples with a corresponding connexon in a contiguous cell to join the cytoplasms. This serves to synchronize either the metabolic or electrotonic functions of cells within a tissue. Most normal cells within solid tissues have functional gap junctional intercellular communication (GJIC) (exceptions are free-standing cells such as red blood cells, neutrophils, and several, if not all, the stem cells). On the other hand, the cancer cells of solid tissues appear to have either dysfunctional homologous or heterologous GJIC. Therefore, among the many differences between a cancer cell and its normal parental cell, the carcinogenic process involves the transition from a normal, GJIC-competent cell to one that is defective in GJIC.

The review examines how GJIC can be either transiently or stably modulated by endogenous or exogenesis chemicals or by oncogenes and tumor suppressor genes at the transcriptional, translational, or posttranslational levels. It also uses the gap junction as the biological structure to facilitate cellular/tissue homeostasis to be the integrator for the "stem cell" theory, "disease of differentiation theory", "initiation/promotion/progression" concepts, nature and nurture concept of carcinogenesis, the mutation/ epigenetic theories of carcinogenesis, and the oncogene/ tumor suppressor gene theories of carcinogenesis. From this background, implications to cancer prevention and cancer therapy are generated.

2. INTRODUCTION: CANCER AS A 'DISEASE OF HOMEOSTASIS'

In order to understand the disease of cancer, one must recognize that, while there are multiple cancer types found in most organs, there are some "universal features" associated with all cancers. Cells that are cancerous appear not to respond to "contact inhibition"(1,2), fail to terminally differentiate (3-5), appear to be clonally-derived from a stem like cell (6-12), and continue to genotypically and phenotypically change as the tumor grows (13,14). More recently, the biological processes of "signal transduction" (15,16) and programmed cell death or apoptosis (17-19) appear also to be altered in cancer cells compared to their normal parental cells.

During the course of evolution from single-celled organisms to multi-cellular organisms, new genes and cellular functions had to accompany that transition. Single-cell organisms survived changes in the environment by adaptively responding to physical (temperature, radiations) and chemical (nutrients, toxins, toxicants) agents by intracellular signals which led to cell proliferation modifications. In the multi-cellular organism, a delicate orchestration of the regulation of cell proliferation for growth and tissue repair/wound healing and of the differentiation of cells had to occur after the fertilization of the egg cell, during embryonic/fetal development, sexual maturation and adulthood/aging of the individual organism. That orchestration of specific cell/tissue/organ and organ system functions is referred to as "homeostasis".

In the multi-cellular organism, homeostasis is mechanistically governed by three major communication processes: **extracellular-communication** via hormones, growth factors, neurotransmitters and cytokines which trigger **intracellular-communication** via alterations in second messages (e.g., Ca^{++}, diacylgycerol, pH, ceramides, NO, c-AMP, reactive oxygen species) and activated signal transduction systems to modulate **intercellular-communication** mediated by gap junction channels (20) (figure 1). Cell adhesion and cell-matrix interactions are considered a subclass of intercellular communication molecules.

All of these communication processes in a multi-cellular organism are intimately interconnected to maintain its normal development and health. In effect, this communication processes must control a cell's ability (a) to proliferate; (b) to differentiate; (c); to apoptose; and (d) if differentiated, to respond, adaptively. Disruption of any one of these three forms of communication could lead to increased or decreased proliferation; to abnormal differentiation; to increased or decreased apoptosis and to abnormal adaptive responses of differentiated cells.

3. EVOLUTION AND CANCER

Single cell organisms survive changes in their environment by having in their populations a few individual cells with mutations in some gene that might give the individual cell a selective advantage. In turn, this individual would survive to leave offspring and carry on the species. Limitations in nutrients, as extracellular-communication signals, can control cell proliferation of single cellular organisms.

When, during the course of evolution, the first multi-cellular organism appeared, new genes and biological functions had to parallel the appearance of the control of cell proliferation within the multi-cellular individual, as well as the induction of differentiation of cells at critical

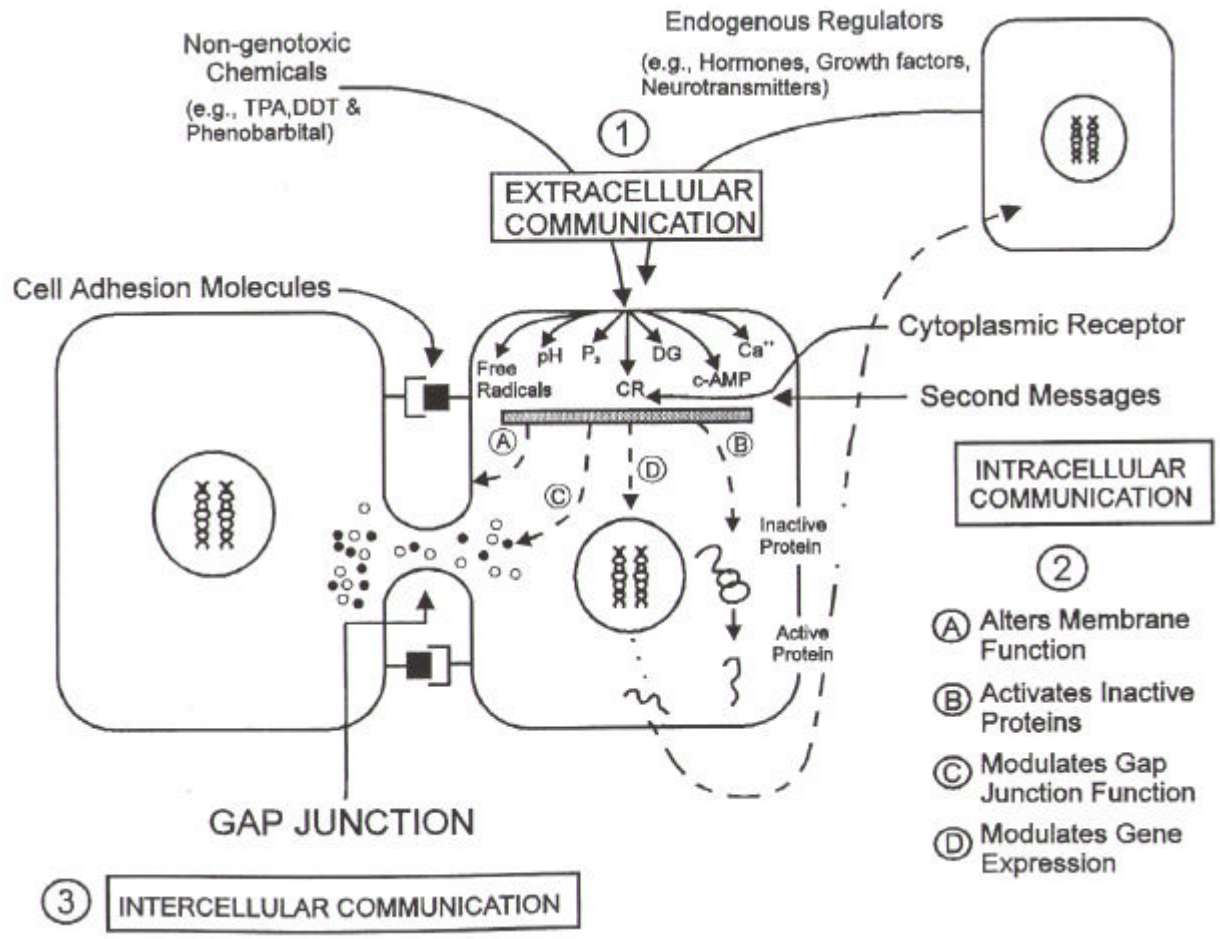

Figure 1. Scheme of the postulated link between extracellular communication and gap junctional intercellular communication via various intracellular signal transducing mechanisms (second message) mechanisms. Diagram illustrates how exogenous non-genotoxic agents can either interfere with, or mimic, endogenous extracellular signals (Reprinted from J.E. Trosko and T. Inoue, Stem Cells 15 (Suppl. 2), 59-67, 1997, AlphaMed Press; used with permission).

times, the control of programmed cell death and the adaptive responses of the differentiated cells. One family of highly evolutionary-conserved genes, the genes that code for the gap junction proteins or connexins, appeared at the time multi-cellularity appeared (21).

Philosophically, the appearance of cancer, a disease of multi-cellular organisms, seems as though the process of carcinogenesis is a "throw-back" in the evolutionary process. The cancer cell, unlike the normal multi-cellular counter-part, no longer has any growth control (except by nutrient depletion) and can not terminally differentiate. In effect, a cancer cell resembles a bacterial cell that survives by uncontrolled cell proliferation and can not differentiate. Normal cells of a multi-cellular organism have connexin genes while single-cellular organisms do not. Cancer cells, which do not contact inhibit, do not have growth control, do not terminally differentiate and usually have abnormal apoptosis responses, do not appear to have functional gap junctional intercellular communication (22-27). Is it just coincidence that the control of cell growth, terminal differentiation and the appearance of apoptosis appeared when the gap genes appeared during the evolution of a multi-cellular organism or is it causal?

4. THEORIES OF CARCINOGENESIS: OVERVIEW

In order to examine the major thesis of this exercise, namely that reversible disruption of gap junctional intercellular communication plays a role during the tumor promotion phase of carcinogenesis and that stable down-regulation of GJIC leads to the conversion of a premalignant cell to an invasive and metastatic cancer cell, a brief review of the major theories of carcinogenesis will be undertaken. One of the first concepts that must be understood when trying to unravel the complicated carcinogenic process is the "**hierarchical**" nature of multi-cellular organisms (28). The idea that the "whole is greater than the sum of its parts" comes from this idea. From

atoms and molecules which are organized into organelles within cells, from cells which are organized within tissues to form organs, and the integration of organs into organ systems, the emergence of features not found in any of the individual components of any one level can be found. The negative and positive feedback of molecular, biochemical, cellular and physiological information via the three forms of communication processes (extra-, intra-, and inter-) within and between the hierarchical levels is the basis for the **cybernetic** concept (29). When these cybernetic feedback systems help maintain the hierarchical nature of multi-cellular organisms, then it can be said that homeostasis is achieved and the organism is in a state of health.

In the case of a human being, starting from a single fertilized egg (the "toti-potent" cell), 100 trillion cells, consisting of pluri-potent stem cells, progenitor cells and terminally differentiated cells, are organized and orchestrated via the communication processes to produce an adult by cell proliferation, differentiation, apoptosis and adaptive responses of the differentiated cells. The pluripotent and progenitor cells are capable of further growth, differentiation, wound healing and adaptive responses before the break down of the hierarchy which leads to the death of the higher level individuals (recall that the death of the individual does not necessarily coincide with the dead of cells). Early concepts, derived over the decades by Claude Bernard, W.B. Cannon, P. Weiss, J.L. Kavanaugh, O.H. Iverson, E.E. Osgood and V.R. Potter (see reviews, 30,31), postulated the existence of positive and negative regulatory factors that existed between stem progenitor cells and their differentiated daughters to control growth and differentiation.

The mechanistic basis for the cybernetic feedback system consists of positive factors (growth factors, hormones, cytokines, and neurotransmitters) [extra-cellular signals] that are secreted by one cell type and that trigger receptors and transmembrane signal transducting elements in distal cells [intra-cellular signals]. These signals are, in turn, either transmitted to, or blocked from, the neighboring cells when the gap junction channels are up- or down-regulated. After receiving these signals, the targeted cells alter their physiology and produce negative extracellular signals that feedback to the original positive-signalling cells. If this basic view of homeostasis in a multi-cellular organism is accepted, then, by logic alone, the breakdown of any one of these three steps (extra-, intra- or inter-cellular communication) should lead to the dysregulation of cell proliferation, differentiation, apoptosis and adaptive responses of differentiated cells. While this seems to describe what has happened in cancer cells, it remains to be experimentally verified that this is, indeed what happens during carcinogenesis.

Any scientific hypothesis or theory must, by definition, explain observations in order to produce testable predictions which can falsify the hypothesis or theory. Some of the major observations that would have to be explained by any theory of carcinogenesis include: (a) normal cells are contact inhibitable, while cancer cells are not (1); (b) normal cells derived from stem and progenitor cells are capable of terminal differentiation; cancer cells under normal situations are not [teratomas represent a special case] (32); most, if not all, tumors appear to be derived from a single cell (6-12); and (d) during the long carcinogenic process, the tumor cell acquires multiple genotypic and phenotypic changes(13).

Over the decades, while many theories have elements that explain some of the observations, none of them provide the framework for a complete explanation. From Boveri's idea that cancers are formed because of chromosomal abnormalities (33) to current ideas that altered activation and de-activation of tumor suppressor genes are the "cause" of carcinogenesis (34). The following two quotations set the stage for understanding the "reductionalistic " versus a "holistic" view of the problem:

> "The understanding of the cellular basis of cancer means being able to describe the biochemical of the regulated pathways between the cell surface and the nucleus that control cell growth." (35).

> "The cancer problem is not merely a cell problem, it is a problem of cell interaction, not only within tissues, but with distant cells in other tissues." (36)

Several the major theories have stimulated research: (a) the idea that cancer is a "**disease of differentiation**" (3-5); the **"stem cell"** theory of cancer (6-12) has been pitted against the **"de-differentiation"** theory of cancer (37); the idea that combines these former two theories is found in **"oncogeny as partially blocked ontogeny**" (5); the **"initiation/promotion/progression"** concept of carcinogenesis was conceived as an operational description to explain distinct steps during the multi-step process (38); the **"nature versus nurture**" theory (39) has been argued to explain whether genetics or the environment was the determinant in the cause of cancer; classic disagreements have appeared as to whether **mutagenic versus epigenetic** mechanisms are responsible for carcinogenesis (40); more recently, the "**oncogene and tumor suppressor gene"** theory has been a driving force in cancer research. The hypothesis that " **cancer was the result of dysfunctional gap junctional intercellular communication",** which was postulated by Loewenstein (22), has been modified to integrate some of the aforementioned ideas (40,41).

The working hypothesis of this review will that the "dysfunctional gap junctional intercellular communication" theory can integrate all of the other theories because each of them can related to GJIC. The hypothesis to be developed here is that, starting with a pluripotent stem cell or early progenitor cell, a stable alteration (a mutation or, in some cases, a epigenetic

repression at the transcriptional level) of a gene (a proto-oncogene or tumor suppressor gene) that controls terminal differentiation of this cell but does not alter the control of the proliferative ability of the cell (i.e., contact inhibition or some form of GJIC would still remain in cells of solid tissues and some form of growth inhibition would exist for soft-tissue cells). This would explain the " initiation " phase of carcinogenesis . As long as this initiated stem-like cell is communicating with other normal cells (heterologous GJIC) or other initiated cells (homologous GJIC), there will be no cell proliferation. When GJIC in these initiated cells is inhibited in a sustained fashion by endogenous chemicals (e.g., growth factors, hormones, cytokines) or by exogenous non-genotoxic chemicals, then clonal expansion of these initiated cells can occur. This would constitute the tumor promotion phase. The process of the down regulation of GJIC by both classes of chemicals is reversible. Therefore, the process of tumor promotion is either interruptible or even reversible (42). Because of their inability to terminally differentiate (43) these initiated cells will slowly accumulate as a focus of benign mono-clonally derived focus of non-terminally differentiated cells. The observation that tumor-promoting chemicals seem to block apoptosis of cells, the accumulation of initiated cells would also contribute to the clonal expansion of these non-terminally differentiated cells (19,44). Cell death or surgery could act as an "indirect tumor promoter" by releasing a surviving initiated cell from mitotic suppression (40).

When during this clonal expansion process of the initiated, partially differentiated, but gap junctionally-coupled cells accrues other stable events in the genome (either mutational or epigenetic transcriptional repression of genes) that brings about a genomic inhibition of GJIC (e.g., activation of an oncogene; de-activation of a tumor suppressor gene; mutation or transcriptional repression of a gap junction gene or a cell adhesion molecule), then the progression phase of carcinogenesis could occur.

Clearly, an individual can inherit mutated or possibly altered imprinted genes that directly affect the initiation or promotion phases of carcinogenesis (e.g., xeroderma pigmentosum-DNA repair deficient and hypermutable syndrome; Bloom's syndrome) or that affects an oncogene or tumor suppressor gene (i.e., Li-Fraumeni syndrome). On the other hand, agents in the environment (e.g., ultraviolet light and asbestos) can influence one or more stages of carcinogenesis. These illustrate that the "nature and nurture" theory of carcinogenesis is the appropriate way to conceptualize the role of the interaction of genes and environmental factors (39).

5. STEM CELL VERSUS THE DE-DIFFERENTIATION THEORIES.

One of the major debates in carcinogenesis concerns the question whether all cells of the multicellular organism are potential targets for carcinogenesis or whether only few special cells can given rise to cancer. One of the universal characteristics of a cancer cell is that it appears to be "immortalized" and partially, but not terminally-differentiated. Normal cells appear to be "mortal" and to have the capability to become terminally differentiated. It is therefore important to define some terms. Stem cells ought to be characterized: a "toti-potent cell is one that can give rise to all cell types within the organism. A pluripotent stem cell is derived from the toti-potent stem cell and has been restricted (committed) to give rise by what appears to be a finite number of cell divisions to only a specific lineage of cell types within the organ in which it gives rise. The daughter cells (progenitor cells) of these pluripotent stem cells which are limited to give rise to one cell type would give rise to the terminally-differentiated cells of that lineage. By definition these terminally-differentiated cells can never proliferate.

One of the prevailing paradigms in the cancer field is that the first major step in carcinogenesis is for a "mortal", normal cell to be "immortalized" and then, subsequently neoplastically transformed. While it would seem obvious that some terminally differentiated cells could not support the De-Differentiation theory (e.g., red blood cells, polyploid hepatocytes, lense cells, neurons, keratinocytes), proponents of this hypothesis argue that some differentiated cells (progenitor cells) could be plastic enough to revert back to a early progenitor or pluripotent cell.

While cells of a tumor appear to be derived from a single cell ("mono-clonal" theory), this does not argue in favor of the stem cell over the de-differentiation theory. While the evidence does not rigorous support one theory over the other, several lines of evidence seems mere consistent with the stem cell theory. **First**, if one defines a stem cell as a cell that has the capacity to divide asymmetrically (i.e., one daughter is committed to terminally differentiate and the other daughter must remain "stem-like", then the stem-like cell is, by definition "immortal", while the other is committed to become "mortal". If that is the case, then the first step in carcinogenesis is not to "immortalize" a normal, "mortal" cell, but to prevent the " mortalization" of a normal, immortal stem cell (see "initiation/ promotion/progression" theory below).

The **second** line of evidence in favor of the stem cell theory is the observation made by Nakano *et al*, (45) that there are only a few target cells in a population of Syrian baby hamster cells which are susceptible to neoplastic transformation. These cells were operationally characterized as being "contact-insensitive".

The **third** line of evidence comes from experiments which isolated and characterized presumptive pluripotent stem cells from human kidney and human breast tissue (32,46). These studies indicate that pluripotent stem cell have no expressed connexin genes or functional GJIC. Cancer cells are also characterized

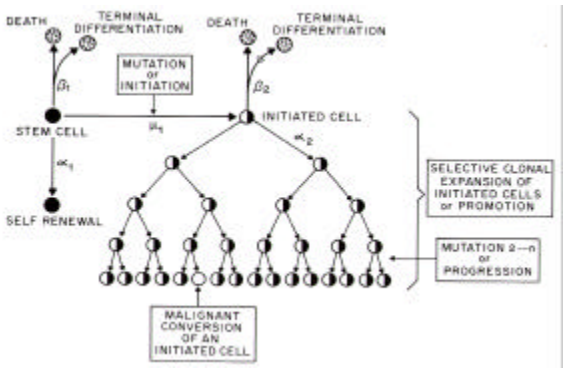

Figure 2. The initiation/promotion/progression model of carcinogenesis. β_1 = rate of terminal differentiation and death of stem cell; β_2 = rate of death, but not of terminal differentiation of the initiated cell ($\rightarrow$); α_1 = rate of cell division of stem cells; α_2 = rate of cell division of initiated cells; μ_1 = rate of the molecular event leading to initiation (i.e., possibly mutation); μ_2 = rate at which second event occurs within an initiated cell. (From Trsoko *et al*., In: Modern Cell Biology; Vol. 7, Gap Junctions, E.L. Hertzberg and R.G. Johnson, eds., pp. 435-448, 1998; with permission from Alan R. Liss, Inc., New York).

with the same phenotype (41). The major difference is that the normal human stem cell can be induced to differentiate very easily, whereas, while some cancer cells can be induced to differentiate, it is much harder to do. The fact that some cancer cells can be induced to terminally differentiate is, itself, evidence they behave as pluripotent stem cells (47).

The recent hypothesis that telomerase activity is necessary for immortality was derived from observations that in normal cells the telomerase activity decreases as the cell senescences while in immortal and neoplastic cells the telomerase activity is "restored"(48). This hypothesis does not seem to hold with recent demonstration that human breast epithelial pluripotent stem cells have high telomerase activity which remains high when they are prevented from terminally differentiating by SV40 transformation. The activity remains high even when these cells are neoplastically transformed (49). Only when the normal pluripotent stem cell is induced to terminally differentiate does the telomerase activity decrease.

6. INITIATION/PROMOTION/PROGRESSION THEORY OF CARCINOGENESIS

The multi-stage nature of carcinogenesis, originally conceptualized during mouse skin chemical carcinogenesis studies (42), consisted of the operational concepts of "initiation", "promotion", and "progression" (figure 2). No mechanism for each step can be directly implied from these experiments. However, because the initiation phase appears to be irreversible, and relatively easily induced by DNA damaging agents, mutagenesis has been the implied underlying mechanism responsible for this step (50). It can not be rigorous ruled out, however, that stable "epigenetic" events, which might transcriptionally alter some proto-oncogene or tumor suppressor gene, could also be an initiating agent.

Biologically, the initiating event, if it takes place in a pluripotent stem cell, must prevent the "mortalization" or terminal differentiation of a normal, immortal" pluripotent stem cell. Some evidence from mouse skin "initiation"/ promotion studies does seem to indicate that initiated mouse skin cells do not terminally differentiate under conditions were normal skin cells do and that these non-differentiated cells, when place back into the mouse, give rise to papillomas, indicating they still retain their proliferative potential. This initiation event seems to tie the stem cell theory to the multi-step theory of carcinogenesis.

The promotion phase of carcinogenesis, operationally, is an interruptible process (and reversible up to a certain point) (42). This implies that the initiated cell can be stimulated to proliferate but not terminally differentiate. The promotion process can be implied to be an epigenetic process. Mitogenesis, rather than mutagenesis, best describe the promotion process (40). Also, since tumor promoters appear to block apoptosis, these initiated cells can accumulate as dysfunctional, non-differentiated cells within a tissue (e.g., enzyme-altered foci in the liver; nodules in the mammary gland; polyps in the colon or papillomas in the skin).

One of the first hypothesis concerning the mechanism of tumor promotion was derived from observations that the skin tumor promoters, phorbol esters, could block gap junctional intercellular communication, at non-cytotoxic levels, in a reversible fashion (51,52). More recently, it was also postulated that tumor promoters could inhibit apoptosis at the same time they blocked GJIC (19). This infers that there might be a direct connection between GJIC and the death signal being transmitted through gap junctions.

Less is known about the mechanism(s) of carcinogenic progression. It does appear to be the step conferring autonomous growth of the initiated cell, rendering it independent of exogenous tumor promoters. It does seem to be an irreversible process, implying either a mutagenic event or a stable epigenetic event.

7. NATURE AND NURTURE THEORY OF CARCINOGENESIS

The idea that genetics might play a role in carcinogenesis was not widely accepted until relatively recently, in spite of the fact it was well known for decades that there were hereditary syndromes that predisposed individuals to cancer and that cancer cells could have chromosomal aberrations. "Is cancer caused by the individual's genes or by the environment?" was the question often heard in both scientific and lay circles. When the paradigm, " Carcinogen as mutagen" appeared (52), an interesting new insight was formed that linked "nature" and "nurture" together (40). If agents which were

mutagens and they were found in the wide environment (nurture), they must interact with DNA of the germ and/or the somatic tissue (nature) to induce mutations in various genes that affect the cancer process directly (e.g., oncogenes or tumor suppressor genes) or indirectly (e.g., DNA repair or initiator-prone genes; growth factor or promoter prone genes). It was only when molecular determinations of specific mutations were found in oncogenes or tumor suppressor genes found in the tumor cells that the question was resolved. The "nature" versus "nurture" debate seemed irrelevant in favor of the "nature and nurture" A classic example that would the xeroderma pigmentosum syndrome which inherits a defective DNA repair gene. The cells of these individuals are incapable of removing sunlight induced pyrimidine dimers formed in the DNA of skin cells, unlike the non-xeroderma pigmentosum individual. As a result, these unrepaired DNA lesion can act as substrates for both cell death or mutations. If these mutations occurred in a skin stem cell and in an oncogene or tumor suppressor gene in these cells, initiation would take place. Other cells, unable to repair these lesions could die. As a result, if an initiated cells is stimulated to proliferate as a result of other cells dying (i.e., compensatory hyperplasia), then promotion of these surviving initiated cells could occur (41,53).

This theory of carcinogenesis readily integrates the stem cell and initiation/promotion/progression theories of carcinogenesis.

8. MUTATION VERSUS THE EPIGENETIC THEORIES OF CARCINOGENESIS

While the mutation theory of carcinogenesis has had a long history [i.e., Boveri (33)], the idea that non-mutagenic events might play a role during either the whole of, or some phase of, carcinogenesis. As mentioned above, the "Carcinogen as mutagen" paradigm, together with the recent direct measurements of mutations in oncogenes and tumor suppressor genes, has almost become a "state religion". By that we mean it has paralyzed some investigators to think that mutagenesis, alone, can explain all of carcinogenesis.

First, the definition of these two terms must be understood. **Mutagenesis is that process that brings about a qualitative or quantitative alteration of the genetic information. An epigenetic process is that which alters the expression of the genetic information at the transcriptional, translational or posttranslational levels. In principle and in reality, there can be chromosomal mutations (i.e., a translocation or a non-disjunction of a chromosome) that can induce an epigenetic event (i.e., The extra chromosome 21 in Downs Syndrome can alter gene expression without a point or gene mutation).**

While the role of mutations in the carcinogenic process appears well documented, the role of epigenetic events is not as well documented or as widely accepted. Yet, it is very well documented that in the initiation/promotion/progression model of carcinogenesis, tumor promoting chemicals, such as phorbol esters, DDT, phenobarbital, saccharin, polybrominated biphenols**,** peroxisome proliferators, etc., are non-mutagenic (55). Promotion is the mitogenic process that brings about the clonal expansion of initiated cells. Cell proliferation is an epigenetic process by which a mitogenic stimulus triggers a signal transduction pathway which can block gap junction function, posttranslationally, and turn on cell cycle genes, transcriptionally (56). The fact that tumor promoting chemicals, such as phorbol esters, can activate signal transducing protein kinases, alter the phosphorylation of connexin proteins, at non-cytotoxic doses and effect 100% of the exposed cells rules against it acting as a " mutagen"(57,58). Also, the blockage of GJIC by the tumor promoters is reversible. These are not characteristics of a mutagen which acts in a random fashion on genes and is, for practical purposes, an irreversible event.

If there must be multiple "hits" to give a normal cell those phenotypic features, such as "immortality" or inability to terminally differentiate, to lose contact inhibition, to invade tissues and to metastasize, and if these "hits" are in large part the result of mutations, then with the average mutation rates of genes being relatively low, the probability of accruing all these mutations in the one cell that was originally initiated would be the product of the individual low probabilities of each independent event. One would never get a cancer if that were all that was needed. However, if in the first cell, a mutation occurred in a gene that prevented terminal differentiation but not proliferation, then one needs only to stimulate this initiated cell to proliferate a few times to produce a few million cells. At that time, the target size of cells with one hit increases the probability of a spontaneous mutation to occur in one of these cells. This cell could then proliferate a few times to create a target size of cell with the original "hit" and a second " hit", increasing the likelihood that a third "hit" could take please.

It seems clear that both mutagenic and epigenetic mechanisms need to take place to bring about the complicated, multi-step carcinogenic process. This mutation and epigenetic theory of carcinogenesis can be integrated into the stem cell, initiation/promotion/ progression and nature and nurture theories of carcinogenesis.

9. ONCOGENE AND TUMOR SUPPRESSOR GENE THEORIES

In recent years the concept of oncogenes was derived from the fact that specific DNA sequences in tumor cells, when injected into a non-tumorigenic recipient cell, could transform it into a neoplastic cell. Over the years, using this protocol, a number of "oncogenes" have been identified and they have been characterized as falling into four functional classes of genes. The counterpart of these "activated" or mutated oncogenes in normal, non-tumorigenic cells are called " protooncogenes" and they

code for (a) growth factors; (b) growth factor receptors; (c) cellular signal transducing proteins; or (d) nuclear transcription factors. These genes and their normal coded proteins appear to be involved in the regulation of cell proliferation, differentiation or apoptosis. If they are somehow overexpressed or mutated to an unregulated form, these cells contribute to the phenotype of cancer cells, namely they do not contact inhibit or proliferate in an uncontrolled fashion nor do they terminally differentiate or apoptosis normally.

For these reasons, an oncogene-activated cell resembles a stem cell which is initiated by an mutagenic event (i.e., it can not terminally differentiate but can proliferate) or which, in a limited way, it resembles a normal progenitor cell treated with a tumor promoting chemical such as phorbol ester (i.e., it transiently blocks contact inhibition, enhances proliferation, modifies differentiation) (59). It has been shown that phorbol ester-treated myc- transformed cells [which do not form tumors by itself] behave very much like the v-Ha-ras oncogene (60-62). However, although by itself, the v-Ha-ras oncogene product can trigger a mitogenic signal transduction mechanism in cells, in the non-tumorigenic cells used in the oncogene protocol system, it is not sufficient to bring about full neoplastic transformation. Together with an activated myc oncogene or phorbol ester, the ras oncogene can induce the stable neoplastic phenotype with myc or a transient phenotypic neoplastic phenotype with phorbol ester. Both v-Ha-ras and phorbol esters can also inhibit GJIC, stabily or transiently, respectively.

Together with the observations that several other oncogenes [e.g., src, neu, mos, raf] (41), can also down-regulate GJIC concurrent with the neoplastic transforming properties of their coded signal transducting potential, the oncogene theory, not only is linked to the stem cell theory, but the initiation/promotion/progression theory.

Tumor suppressor genes, conceptually, must do the opposite of the biological effects on oncogenes; namely, they must suppress cell growth and assist in the differentiation and apoptosis of cells. Operationally, these DNA sequences are identified by transfecting neoplastic cells and showing that they stop unregulated cell growth, induce differentiation or apoptosis. Recently, several studies have identified the existence of tumor suppressor gene on the normal chromosome 11 (63-72). Interestingly, in these neoplastic cells which do not have function GJIC, the non-neoplastic cells containing a genetically-engineered normal chromosome 11 have normal growth control, loss of their neoplastic potential and enhanced GJIC (73).

These latter observation with a tumor suppressor gene conceptual support the link between growth control regulation, and its oncogene counterpart of the inhibition of growth control, with GJIC and both the stem cell and initiation, promotion, progression theories. More will be said about the similarities of tumor suppressor genes, anti-tumor promoters and GJIC.

10. GAP JUNCTIONS-ANCIENT AND UBIQUITOUS MEDIATORS OF CELLULAR HOMEOSTASIS

As discussed above, the major premise of this review is that dysfunctional GJIC plays a crucial role in the tumor promotion phase of carcinogenesis and that all of the major theories of carcinogenesis can be integrated if examined from this perspective.

Cancer is a disease of “abnormal homeostasis” mediated by defects in intra-, extra-, and intercellular forms of communication that disrupt the delicate balances between cellular proliferation, differentiation, apoptosis, and adaptation. One of the most ubiquitous and ancient forms of intercellular communication that is disrupted in neoplastic cells is that mediated by gap junctions. In the remainder of this review, we will discuss the structural and functional aspects of gap junctions, the proteins that form gap junction channels (connexins), the evidence that gap junctions are involved in cellular growth regulation and how this might work, and lastly how the enhancement of GJIC and connexin expression might be beneficial in cancer therapy.

11. STRUCTURE OF GAP JUNCTIONS

Gap junctions are one type of junctional complex formed between adjacent cells and consist of aggregated channels that directly link the interiors of neighboring cells. Gap junctions have been detected in such primitive invertebrates as jellyfish and hydra and similar structures known as plasmodesmata are found in plants (74-76). In the adult mammal, gap junctions are found in most cell types with the known exceptions being skeletal muscle fibers, certain neurons, and circulating blood cells (74,75), although some blood cells may express gap junction proteins and gap junction-like structures (77).

Each gap junction channel is comprised of two hemichannels or connexons and each connexon is formed by the aggregation of six protein subunits known as connexins (figure 3) (78). Connexins are folded in the plasma membrane in the approximate shape of an “M”. The amino and carboxyl termini project into the cytoplasm while the remainder of the molecule traverses the plasma membrane four times. These membrane-spanning regions lie in parallel. The third one contains a high proportion of hydrophilic amino acids and is thought to line the interior of the channel. The four membrane-spanning domains and the extracellular loops are highly conserved between the many different connexins that have been cloned (described below). More varying are the cytoplasmic regions. As will

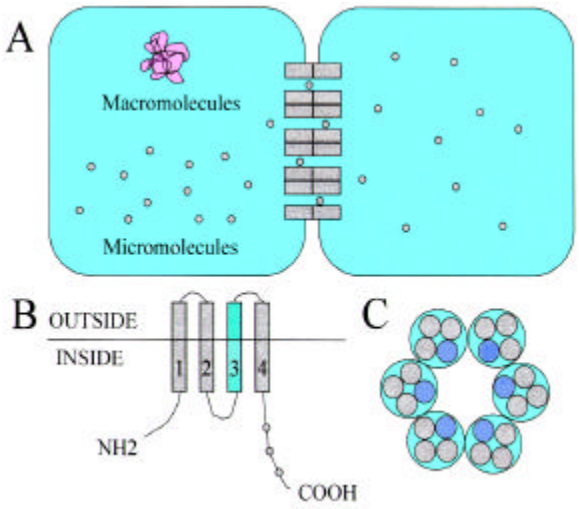

Figure 3. Diagrammatic representations of gap junctions and connexins. (A) Gap junction between two cells. Gap junction channels are too small to enable the cell-to-cell passage of macromolecules such as proteins, but will allow free cell-to-cell diffusion of micromolecules (<2,000 Da) such as calcium ion, water, and cyclic nucleotides. (B) Structure of a connexin. All known connexins are transmembrane proteins with four transmmbrane domains and cytoplasmic amino and carboxyl termini. The third transmembrane domain contains many hydrophilic amino acids and is thought to line the gap junction channel. Some connexins are phosphorylated on the carboxyl tail. (C) "End-on" view of a gap junction hemichannel (connexon). Two connexons align end-to-end to form a complete channel between neighboring cells. Each connexon is formed from six connexins; the third transmembrane domain of each connexin lines the channel.

Table 1. Mammalian connexins and organs or tissues in which they are found.

CONNEXIN	TISSUE
Cx26	Parenchymal liver (hepatocytes), pancreas, endometrium
Cx30	Brain, skin
Cx30.3	Skin
Cx31	Skin, placenta
Cx31.1	Skin
Cx32	Parenchymal liver (hepatocytes), kidney, pancreas
Cx33	Testes
Cx37	Endothelium, lung, ovary
Cx40	Endothelium, smooth muscle, myocardium, lung
Cx43	Most epithelia, heart, uterus, connective tissue, brain
Cx45	Kidney, skin
Cx46	Lens
Cx50	Lens

be discussed, these different regions may be involved in the cellular regulation of gap junction formation and channel permeability. Connexin folding as well as connexin-connexin and connexon-connexon interactions are mediated through disulfide bonds, hydrophobic protein interactions, and other more poorly understood forces.

Gap junction channels have a diameter of approximately 1.5-2 nm depending upon the type of junction-forming protein and are large enough to permit the direct diffusion of small (<2,000 Da) molecules and ions between cells (74). Many substances such as ions, water, sugars, nucleotides, amino acids, fatty acids, small peptides, drugs, and carcinogens are small enough to move between cells through gap junction channels. However, proteins, complex lipids, polysaccharides, RNA, and other large molecules are not. Channel passage does not require ATP and appears to result from passive diffusion. This flux of materials between cells via gap junction channels is known as gap junctional intercellular communication (GJIC).

One of the most significant physiological implications for GJIC is that gap junction "coupled" cells within a tissue are not individual, discrete entities, but are highly integrated with their neighbors. This property facilitates homeostasis and also permits the rapid, direct transfer of second messengers between cells to coordinate cellular responses within the tissue. On the other hand, the channel permeability size limit enables cells to maintain enzymatic and other macromolecular functions through the retention of specific enzymes, receptors, and other large molecules. Thus, GJIC can be viewed as an ancient, common, and important mechanism of cellular homeostasis and integrator of cellular functions within complex tissues.

12. THE CONNEXIN MULTIGENE FAMILY

The gap junction channel-forming connexins comprise a multi-gene family with at least thirteen mammalian connexins discovered thus far (78). Several homologous DNAs have been identified in other vertebrate species. Several connexins and tissues in which they are highly expressed are listed in table 1. The number associated with each connexin indicates its molecular mass. Connexins are expressed in a cell-, tissue-, and development-specific manner. For instance, connexin43 is the predominant connexin expressed in cardiac muscle and was first cloned from this tissue (79), although other connexins (connexin40, connexin45, and connexin46) have also been detected in cardiac tissue. In adult liver, the predominant connexins are connexin32 and connexin26 (80-82) which are expressed by adult parenchymal liver cells (hepatocytes). However, nonparenchymal liver epithelial cells, hepatic fat storing (Ito) cells, and hepatic connective tissue cells express connexin43 (83-85). Connexin 37 and connexin40 are highly expressed in lung endothelial cells (86,87), whereas connexin43 is the predominant connexin expressed by human and murine lung epithelial cells (88). Lee *et al.* (89), however, reported that connexin32 was the most abundant connexin

in freshly isolated rat type II alveolar epithelial cells and that connexin43 expression arose after culture. Connexin expression is also related to cellular differentiation. For example, the types of connexins expressed by epidermal cells changes as the cells differentiate and move away from the basal layer (90,91).

Despite the presence of conserved sequences within connexins, the diversity of these proteins is not due to alternative splicing of RNAs. Instead, there appears to be one gene per connexin. Many connexin genes have been mapped and are located on several chromosomes (92) suggesting their distribution is random throughout the genome. Why there are so many connexins is not clear, but may reflect differences in their function(s) and/or the regulation of their expression, formation, and permeability.

In those cells where multiple connexins are expressed, gap junction hemichannels may be comprised of more than one connexin (heteromeric connexons) or may be homomeric (93-96). Heteromeric channels may have different permeability and regulatory properties than homomeric ones and may provide numerous additional options for regulating the type of signals that pass through its gap junction channels. In addition, heterotypic channels consisting of two different connexons have been described (97,98). Interestingly, only certain heterotypic pairings form functional channels. For example, hemichannels of connexin43 and connexin26 and of connexin32 and connexin26 form permeable channels, but those of connexin43 and connexin32 do not (99).

13. REGULATION OF CONNEXIN GENE EXPRESSION

The structures of nearly all connexin genes identified thus far are similar and consist of two exons separated by a long intron (78). The first exons are quite short (about 100 base pairs), contain no protein coding information, and are separated from the second exons by long introns (6-8 kilobasepairs). All of the coding information for the proteins resides within the second exons. The promoters that have been identified for these genes are located upstream of exon 1.

The rat connexin32 gene is unusual, however. Initial work indicated that it consisted of a small first exon separated from a second exon by an approximately 6 kb intron and that the promoter was located upstream of exon 1 (100); this is the typical connexin gene structure just described. Recently, however, two additional promoters have been identified which are active in neural tissue, but not in the liver. These promoters are located more proximal to exon 2 and give rise to mRNAs containing the noncoding, alternate exons 1A or 1B and exon 2 (101,102). Thus, in contrast to the other connexin genes, three promoters control the expression of connexin32 and function in a tissue-specific manner.

The regulation of connexin gene expression is poorly understood. Besides the connexin32 gene, many other connexin gene promoters have been sequenced and several putative regulatory sites have been identified (103-111). Few of these sites have been examined for function, however.

In the rat and mouse connexin32 promoters located upstream of exon 1 and active in liver tissue, there does not appear to be a canonical TATA-box sequence (100,103). Instead, several GC-rich sequences near the transcription start sites appear to bind the transcription factor Sp1 and positively regulate connexin32 transcription (107,108). In addition, there is a novel 25 bp element within this region that also appears to be necessary for connexin32 transcription (108). Hepatic nuclear factor-1 and nuclear factor-1 consensus sequences are also present in this basal transcriptional region. DNAse I hypersensitive sites reside further upstream in this promoter and may silence connexin32 expression in nonexpressing cells (108). The alternate connexin32 promoters active in neural tissues contain canonical TATA boxes (101,102), but their functional activity has not been reported.

The promoters of human and mouse connexin26 have been sequenced and several interesting elements have been noted (103,106). Like the connexin32 hepatic promoter, there does not appear to be a canonical TATA box, but a TTAAAA motif is present in the human promoter approximately 20 bp upstream of the transcription start sites (106). Additionally, several GC-rich sequences, a Yin-Yang (YY)-1-like element, and consensus mammary gland factor binding sites are present further upstream (103,106). The functional activity of these elements has not been reported, however.

The promoters of the human, rat, and mouse connexin43 genes have been cloned (104,105,109). All three promoters contain a TATA box located near the transcriptional start sites and several activator protein (AP)-1 sites further upstream. The AP-1 site(s) in the human connexin43 promoter function positively since phorbol ester treatment of human uterine myometrial cells increased connexin43 expression within 6-8 h and mutation of the most proximal AP-1 site reduced the response (110). The increase in connexin43 expression was preceded by transient increases of c-Fos and c-Jun which also supports the functional role of the these sites; in response to phorbol esters, c-Fos and c-Jun heterodimerize then bind to AP-1 sites. The enhancement of connexin43 expression by phorbol ester may seem paradoxical since 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the classical phorbol ester tumor promoter, blocks GJIC in most connexin43-expressing cells. However, this block occurs rapidly (within minutes) and is due to activation of calcium-phospholipid-dependent protein kinase (protein kinase C; PKC) phosphorylation of connexin43 rather than to decreased connexin43 gene transcription (112,113).

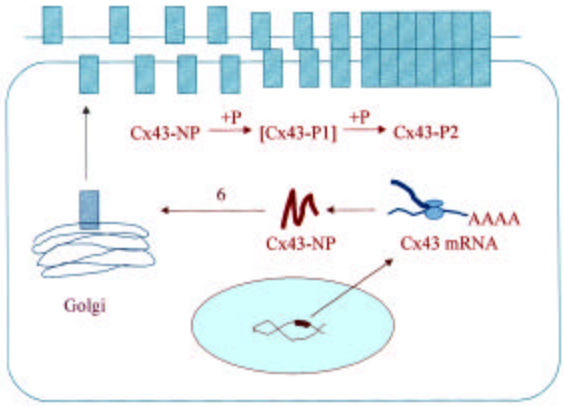

Figure 4. Formation of gap junctions containing connexin43. The connexin43 gene is expressed and the messenger is translated into protein. Six connexin43 subunits oligomerize in the Golgi apparatus into a connexon (hemichannel) and which is transported to the cell surface. There the connexons align with connexons from the neighboring cell to form complete channels. These aggregate into large gap junction plaques that may contain hundreds to thousands of channels. During the pairing of connexons and aggregation into plaques, connexin43 is phosphorylated at least twice. This results in three species of connexin43 that can be detected by Western blot, namely connexin43-NP which is not phoshorylated and connexin43-P1 and connexin43-P2 which are phosphorylated.

The rodent and human connexin43 promoters also contain several half-palindromic estrogen-responsive elements (EREs) that enhance connexin43 transcription in the presence of estrogen (111). In addition, negative and positive elements were identified within approximately the first 100 nucleotides of the mouse promoter (109).

Several physiological, pharmacological, and dietary factors alter the expression of connexins. These factors often affect connexin expression in a cell-specific manner. For example, the expression of connexin32 and connexin26 in hepatocytes, but not in liver epithelial cells, is increased by glucocorticoids (114-116). The mechanism(s) have not been identified. As noted above, estrogen increases connexin43 transcription presumably through putative estrogen-responsive elements (111). Cyclic AMP, forskolin, and glucagon increase connexin32 and connexin43 transcription in certain cells (117-121) and this may occur through activation of cAMP-responsive enhancer elements located in the connexin32 and connexin43 promoters (90,103).

Connexin messenger RNA and protein stability also appear to be important in the expression of connexins. Sequences located in the 3'-untranslated region of the connexin43 mRNA appear to stabilize this messenger (104). Besides increasing connexin gene transcription, cyclic AMP may also increase the permeability of preexisting gap junction channels, enhance the formation of gap junctions from preexisting connexin, and decrease connexin degradation (117,122,123). These effects may involve connexin phosphorylation by cAMP-dependent protein kinase (protein kinase A; PKA) (117).

In summary, several transcriptional and posttranscriptional mechanisms are involved in the regulation of connexin expression. A better understanding of these mechanisms will lead to the development of therapies designed to alter connexin expression for the treatment of diseases such as cancer (see below).

14. GAP JUNCTION FORMATION, CONTROL OF CHANNEL PERMEABILITY, AND MECHANISMS OF DISRUPTED GJIC

The mechanisms of gap junction formation and regulation of channel permeability are poorly understood. The most complete knowledge regarding gap junction formation is for connexin43 (Cx43) and is based largely upon he work of Musil and Goodenough (57,124) (figure 4). Their data suggest that six Cx43 subunits oligomerize into connexons in the Golgi apparatus and are then transported to the plasma membrane. At this point, the connexons are closed to prevent leakage of cellular contents and entry of extracellular materials. At the plasma membrane, the connexons are attracted to those on the adjacent cell by poorly understood forces and two connexons join in an end-to-end manner to form a complete channel. Subsequently, the channels aggregate into large gap junction plaques and the channels open to connect the two cells, although the order of these last two steps is controversial. Coincident with the formation of open gap junctional channels and aggregation of particles into junctional plaques, connexin43 is phosphorylated in at least two positions. The kinase(s) that perform these phosphorylations are not known, but a likely candidate is PKA. This phosphorylation may increase particle aggregation, channel permeability, and/or connexin43 stability as discussed above.

Gap junction channel formation also requires appropriate cell-cell adhesion. The cadherins appear to be especially important in this regard since gap junction formation was induced in connexin-expressing, cadherin-deficient, noncommunicating cells after transfection with cadherin expression vectors and cadherin antibodies blocked gap junction formation (57,125). In addition, membrane protein glycosylation can also impair gap junction formation. Treatment of noncommunicating cells with an inhibitor of glycosylation induced gap junction formation (127,128).

Rates of connexin synthesis and degradation and the disassembly and removal of gap junctions from the cell surface are also important in the regulation of GJIC. Biochemical studies have demonstrated that connexin43 and connexin32 have half-lives (hours) that are much shorter than most plasma membrane proteins (days) (129-133).

Gap junction degradation proceeds by several pathways. Gap junctions containing connexin43 are internalized and degraded in lysosomes or are ubiquinated and degraded in proteosomes in response to various physiological or toxicological cues. For example, the loss of gap junctions from rabbit granulosa cells during maturation of the ovarian follicle occurs by gap junctional internalization and lysosomal degradation (134). We have reported that certain pesticides induce connexin43 gap junction internalization and lysosomal degradation in rat liver epithelial cells (135). In addition, connexin43 has been reported to be ubiquinated and degraded via the proteosome pathway and to be degraded in lysosomes (136,137). The relative contribution of each pathway may be cell specific. Degradation of connexin32-containing gap junctions can be mediated by the calcium-activated proteases, μ-calpain and m-calpain (138,139) and connexin50 is processed in the ocular lens by calpain (140). Thus, there appear to be several pathways of connexin degradation and the contribution of each are cell- and connexin-specific.

After aggregation into plaques, gap junction particles are dispersed in the plasma membrane in response to certain physiological and toxicological cues. For instance, partial hepatectomy causes dispersal of hepatocyte gap junction particles (141) as well as altering connexin expression (discussed above). We have shown that a compound from licorice root, 18β-glycyrrhetinic acid, causes the disaggregation of connexin43-containing gap junction particles and their dispersal in the plasma membrane (142). Interestingly, this was associated with the dephosphorylation of connexin43 and supports the role of phosphorylation in gap junction particle aggregation (57,124). Another group has observed gap junction particle dispersal induced by the alpha isomer of this compound, but did not see changes in connexin43 phosphorylation (143).

Once formed, gap junction channels open and close and this "gating" is controlled by several mechanisms including connexin phosphorylation. Several protein kinases phosphorylate connexins and this may alter connexin tertiary structure to either open or close the channels (144). Many protein kinases have been identified that phosphorylate various connexins or that may indirectly stimulate connexin phosphorylation by other kinases. Activation of PKA induces connexin43 and connexin32 phosphorylation on serine residues located in the cytoplasmic carboxyl tail and this is associated with increased gap junction number and channel permeability (145). The phorbol ester-activated protein kinase, PKC, also leads to increased phosphorylation of connexin43 on serine residues, but this is associated with decreased channel permeability (146). In hepatocytes, PKC has also been reported to induce connexin32 phosphorylation and an enhancement of channel permeability (147), but in connexin32-transduced rat liver epithelial cells, PKC activation results in loss of connexin32-mediated GJIC (113). The src oncogene product, $pp60^{src}$, which has tyrosine kinase activity, blocks GJIC when expressed in many types of cells and this is due to the phosphorylation of connexin43 on tyrosine residues in the carboxyl tail (148). Some connexins such as connexin26 are not phosphorylated so that gating must be regulated in other ways.

Other mechanisms regulating channel gating include intracellular levels of hydrogen and calcium ions, transjunctional voltage, and free radicals (145). Decreased pH or pCa induce channel closure in a cell- and connexin-specific manner. Residues in the intracellular regions of the protein have been reported to be important in the pH gating effect (150-151). It is less clear how calcium regulates gap junction channel permeability (152-154). The calcium-binding protein, calmodulin, has been reported to be associated with connexins and this may be important in calcium-gating of gap junction channels (155-159). Free radicals are highly reactive molecules and ions generated during normal cellular metabolism or following exposure to certain toxic agents. Excessive intracellular levels of free radicals decrease gap junction channel permeability (160,161), but the mechanisms are complex. The radicals may directly attack connexins or other plasma membrane components (e.g., lipids), or may induce changes in cellular calcium levels or redox status. It is also unclear whether radicals are important physiologically in the regulation of gap junction channel permeability and turnover. From a pathological standpoint, however, free radicals may be important in the dysfunctional GJIC in cardiac myocytes that occurs during ischemic injury (161,163) and in hepatocytes during certain types of toxic injury (164).

From the above discussion, it is apparent that the ability of cells to establish and maintain GJIC is dependent not only on connexin gene expression, but also on many other factors including cell-cell adhesion, gap junction assembly/disassembly, connexin stability, and channel gating. Not surprisingly, the disruption of GJIC by various agents often involves these regulatory mechanisms.

15. MULTIPLE FUNCTIONS OF GJIC

Many physiological roles besides growth control have been proposed for GJIC and several are briefly reviewed:

1. Homeostasis. GJIC permits the rapid equilibration of nutrients, ions, and fluids between cells. This might be the most ancient, widespread, and important function for these channels (74).
2. Electrical coupling. Gap junctions serve as electrical synapses in electrically excitable cells such as cardiac myocytes, smooth muscle cells, and neurons (74,75). In these tissues, electrical coupling permits more rapid cell-to-cell transmission of action potentials than chemical synapses. In myocytes, this enables their synchronous contraction.
3. Tissue response to hormones. GJIC may enhance the responsiveness of tissues to external stimuli (165,166).

Second messengers such as cyclic nucleotides, calcium, and inositol phosphates are small enough to pass from hormonally activated cells to quiescent cells through junctional channels and activate the latter. Such an effect may increase the tissue response to an agonist.

4. Regulation of embryonic development. Gap junctions may serve as intercellular pathways for chemical and/or electrical developmental signals in embryos and for defining the boundaries of developmental compartments (167). GJIC occurs in specific patterns in embryonic cells and the impairment of GJIC has been related to developmental anomalies and the teratogenic effects of many chemicals (168).

16. ROLE OF GJIC IN REGULATING CELLULAR PROLIFERATION AND NEOPLASIA

More important to this review is that GJIC is also involved in the regulation of cellular growth and expression of the neoplastic phenotype. For some time, gap junctions have been proposed to serve as passageways for the cell-to-cell exchange of low molecular weight growth regulatory molecules (169). GJIC is frequently reduced in neoplastic and carcinogen-treated cells. It was hypothesized that this contributed to dysregulated cellular growth by isolating cells from their neighbors (170,171). As will be described below, there is now compelling evidence that this is true.

16.1 Neoplastic Cells Have Fewer Gap Junctions

Gap junction size and number, connexin expression, and cell-cell coupling (GJIC) have been studied in many neoplastic cells using ultrastructural, biochemical, and immunological means and by introduction of fluorescent or radioactive tracers and determination of tracer passage into adjacent cells. The vast majority of neoplastic cells have fewer and smaller gap junctions, express less connexins, and have reduced GJIC compared to their nonneoplastic counterparts (74,88,172,173).

There are exceptions, however, in that some neoplastic cells have normal or greater gap junction expression and cell-cell coupling (88,170,173-178). This does not indicate, however, that such cells communicate normally. Loewenstein (170) has pointed out that cells can have defective GJIC at several levels. First, they may lack functional (permeable) gap junctions. Secondly, they may have functional gap junctions amongst themselves (homologous GJIC), but are incapable of gap junction formation with nontransformed cells (heterologous GJIC). Thirdly, they may form gap junctions, but are insensitive to the gap junction signals that control growth and phenotype. Finally, they may have other defects that are sufficient to neoplastically transform the cell despite functional GJIC. In support of the second possibility, our group (88,173) and others (175-178) have identified several neoplastic cell lines that have extensive gap junction formation, connexin expression, and homologous GJIC, but little heterologous GJIC with their nontransformed counterparts. If this deficiency occurs *in vivo*, the tumor cells would be isolated from the junctional regulatory influences of their surrounding normal cells. This inability of tumor cells to communicate with normal cells is not due to the expression of different connexins, but possibly to differences in the cell surfaces (e.g., glycosylation or cell-cell adhesion molecules) that prevent adequate cell-cell contact needed for gap junction formation.

16.2 Growth Stimuli Inhibit GJIC

16.2.1. Carcinogens

A variety of chemical carcinogens have been identified that enhance or "promote" neoplastic transformation through mechanisms that do not involve direct damage of DNA. Many of these "nongenotoxic tumor promoters" selectively induce the proliferation and/or inhibit the programmed death (apoptosis) of preneoplastic cells (179). This leads to clonal expansion of the preneoplastic cell population and increased risk of additional genetic changes occurring in these cells that lead to full neoplastic transformation. Importantly, GJIC may play a role in this proliferative response. Most of the tumor promoters that have been examined (over 100) inhibit GJIC in cultured cells and cells within target tissues (180-182). The ability of tumor promoters to inhibit GJIC is one of their most common properties. These compounds are chemically diverse and include pesticides such as DDT, dieldrin, and lindane; pharmaceuticals such as phenobarbital and diazepam; dietary additives such as saccharin and butylated hydroxytoluene; polyhalogenated hydrocarbons such as dioxin; and peroxisome proliferators such as clofibrate. Not surprisingly, these agents inhibit GJIC through several diverse mechanisms (discussed below).

Preneoplastic cells are more sensitive than normal cells to the inhibitory effects of tumor promoters on GJIC (183-186). This differential response agrees with the selective proliferation and clonal expansion of preneoplastic cells versus normal cells as discussed above.

In contrast to nongenotoxic tumor promoters, most studies indicate that mutagenic ("genotoxic") carcinogens do not inhibit GJIC or induce cell proliferation (182,187) although another group has reported that several classical, mutagenic carcinogens can reduce GJIC (188). Genotoxic carcinogens instead probably induce neoplastic transformation by mutationally activating proto-oncogenes and inactivating tumor suppressor genes (189). Thus, chemical carcinogens appear to have different effects on DNA, GJIC, and cell proliferation that correlate with their genotoxic/nongenotoxic carcinogenic mechanisms.

16.2.2. Oncogenes

Oncogenes are genes derived from normal cellular genes (proto-oncogenes) that have been mutationally activated and/or are overexpressed and that function in the transformation of a normal cell into a neoplastic one. The protein products of these genes function in signal transduction, gene regulation, growth control, and many other facets of tissue and cellular homeostasis, and not surprisingly, many block GJIC.

Oncogene products that block GJIC include Ras, Neu, and Src (173,189). Oncogenes also cooperate in their ability to reduce GJIC and transform cells. Rat liver epithelial cells that expressed only raf or myc oncogenes did not have reduced GJIC and were not transformed, but GJIC was strongly inhibited and the cells were highly tumorigenic when both oncogenes were expressed (176). Similarly, overexpression of the c-Ha-ras oncogene in rat liver epithelial cells partially reduced GJIC, but v-myc overexpression did not and coexpression of the two resulted in nearly complete inhibition of GJIC and highly malignant cells (62).

16.2.3. Growth Factors

Many growth factors such as epidermal growth factor, platelet derived growth factor, basic fibroblast growth factor, hepatic growth factor, and transforming growth factor-alpha inhibit GJIC when applied to cultured cells (25). This effect may occur rapidly (minutes to hours) or may be delayed (days). The mechanism(s) of inhibition of GJIC by epidermal growth factor may be related to the stimulation of connexin phosphorylation by mitogen-activated protein kinase (MAPK) and closure of gap junctional channels (190). Platelet-derived growth factor induced the rapid inhibition of GJIC coincident with the phosphorylation of connexin43 (191). Basic fibroblast growth factor, however, inhibited GJIC after long exposures (>8 h) coincident with decreased connexin43 expression (192). Some growth factors such as transforming growth factor beta enhance GJIC in some types of cells, but decrease it in others (193-196).

Several oncogenes code for growth factors, growth factor receptors, or mitogenic signal transducing elements and several tumor promoters act as growth factors since they induce cell proliferation. As noted above, oncogenes and tumor promoters have also been associated with dysfunctional GJIC. Thus, it is evident that growth factors, oncogenes, and tumor promoters share the common properties of increasing cell proliferation and inhibiting GJIC.

16.3. Growth Inhibitors Stimulate GJIC

In contrast to the effects of growth factors, oncogenes, and tumor promoters on GJIC, many growth inhibitors and anticancer agents increase GJIC and connexin expression in target cells (25). Retinoids, carotenoids, green tea extract, certain flavonoids, dexamethasone, and cyclic AMP analogues and agonists inhibit neoplastic transformation and/or tumor cell growth and can block neoplastic transformation in some tissues. These agents also increase connexin expression and gap junction formation in target tissues (115,197-199) or block the inhibitory effects of tumor promoters on GJIC (200-202).

Certain tumor suppressor gene products also increase GJIC in neoplastic cells. As discussed above, the human chromosome 11 carries one or more tumor suppressor genes (63-72). Introduction of this chromosome into neoplastic cells restored normal growth control, reduced tumorigenicity, and enhanced GJIC mediated by connexin43 (73) despite the fact that the connexin43 gene is located on human chromosome 6 (92). This result also suggests that tumor suppressor gene products inhibit neoplastic transformation by enhancing GJIC in addition to their known actions on cell cycle genes, signal trandsuction pathways, and gene expression (203).

16.4. Cell Cycle-Related Changes in GJIC

GJIC may also have a role in the progression of dividing cells through the cell cycle. In several model systems, cell cycle-related changes in GJIC have been noted (25). For example, this has been demonstrated clearly in regenerating rat liver. The adult rat liver parenchymal cells (hepatocytes) are highly coupled by gap junctions and only a small proportion (<1%) are undergoing cellular replication. The majority of the cells are in stationary (G0) phase, but will enter the cell cycle and begin dividing in a synchronous manner when two-thirds of the liver is removed surgically (two-thirds partial hepatectomy). When followed throughout the cell cycle, gap junctions and connexin expression decrease dramatically in S-phase then reappear and persist throughout the rest of the cycle (204,205).

Cell culture studies have also demonstrated changes in GJIC at specific stages in the cell cycle (206-209). Cultured cells normally replicate in an asynchronous manner. They can be synchronized, however, by first blocking cell cycle progression at a particular point using chemical agents or nutrient or growth factor depletion then releasing the cells from the block. When such cells were analyzed for GJIC, reductions were noted in late G1 and in mitosis. In one study, the reduction of GJIC in G1 was correlated with a change in the phosphorylation state of connexin43 which could be prevented by inhibitors of protein kinase C (209). As discussed above, this kinase is one of many that phosphorylate connexins and alter gap junction permeability.

Thus, both in vivo and in vitro studies have documented cell cycle-related changes in GJIC. The specific cell cycle stage(s) at which GJIC are reduced are not the same in all studies, however. Connexin expression and phosphorylation may be involved in this reduced GJIC, but this is not fully established for all systems nor is it understood how changes in GJIC contribute to cell cycle regulation.

17. INVOLVEMENT OF GJIC IN THE GROWTH INHIBITION OF NEOPLASTIC CELLS BY NONTRANSFORMED CELLS

Cell culture model systems have illustrated that the growth of neoplastic cells can be inhibited by coculture with nonneoplastic cells. This effect has been attributed to noncontact-dependent and contact-dependent phenomena. For instance, normal cells may secrete growth inhibitors (e.g., TGF-β) into the culture

medium that inhibit neoplastic cell growth (210). Contact with extracellular matrix or plasma membrane components such as integrins may also trigger growth-inhibiting processes in neoplastic cells (211-214).

The inhibition of neoplastic cell growth by normal cells also appears to involve GJIC, however (215-217). We have studied this using cultured rat liver epithelial cells (WB-F344 cells) as a model system (217). In normal WB-F344 cells, high levels of connexin43 are expressed, numerous gap junctions are formed, and the percentage of communicating cells is high (95-100%). Neoplastic transformants of these cells which were generated by ras and neu oncogene transfection formed few gap junctions and had low incidences of communication (20-25%). Coculture of the two types of cells resulted in the growth suppression of the neoplastic cells. Inhibition occurred only when the two types of cells were permitted to make contact with each other and not when they were physically separated in the culture dish. In addition, medium from normal cells was incapable of reducing the growth of neoplastic cells. These data suggested that cell-cell contact, not secretion of extracellular factors was required for growth inhibition. To investigate whether GJIC between normal and neoplastic cells was required, GJIC-incompetent mutants of the normal cells were utilized. These cells did not inhibit the growth of neoplastic cells despite the presence of cell-cell contact. When GJIC was restored in the mutant cells by their transfection with a functional connexin43 gene, however, neoplastic cell growth was again inhibited. Thus, the inhibition of neoplastic WB cell growth by normal cells required GJIC.

18. SPECIFIC DISRUPTION AND ENHANCEMENT OF GJIC

The above discussion has reviewed indirect evidence that GJIC controls cell growth and the expression of the neoplastic phenotype. The problem with those studies is that many of the growth inhibitors, growth enhancers, carcinogens, oncogene products, etc. have many effects on cells besides altering GJIC so that the evidence is correlative. Cause and effect cannot be shown unequivocally. To demonstrate a role for GJIC in growth control and neoplasia directly, methods to specifically alter GJIC must be utilized.

Recently, many techniques have been developed to specifically inhibit or enhance the expression of a target gene in cultured cells and animals. Several approaches have been used to specifically disrupt the function or expression of connexins and GJIC in nonneoplastic cells. These include antisense blockage of connexin gene expression, targeted disruption of connexin genes (connexin "gene knockout"), and transfection of defective connexin genes whose protein products block the function of normal connexin proteins ("dominant-negative" connexin expression). Additionally, the specific enhancement of GJIC in neoplastic cells has been achieved by transfection of functional connexin genes. As discussed below, these types of approaches have provided strong, direct evidence that GJIC is involved in growth regulation and neoplastic transformation.

18.1. Connexin Antisense Studies

Antisense approaches include treating cells or animals with short (usually 15-25 nucleotides), single-stranded DNA or RNA molecules that are complementary to targeted heteronuclear or messenger RNA, or transfecting cells with vectors that continuously generate complementary RNA. Antisense molecules are thought to inhibit gene expression by binding to heteronuclear RNA or messenger RNA and inducing their degradation or by inhibiting translation.

Using antisense approaches, two groups have inhibited connexin expression in nonneoplastic cells and shown that this affected their growth. In one study, connexin antisense-transfected, nontransformed cells lost their ability to inhibit the growth of cocultured neoplastic cells (218). In another study, cells treated with connexin antisense oligonucleotides grew to a much higher saturation density (i.e., the maximal number of cells per dish) (219).

18.2. Connexin Gene Knockout

"Gene knockout" involves the disruption of a target gene by the insertion of a noncoding sequence through homologous recombination. A connexin43 knockout mouse has been developed (220). This mutation, which was lethal at birth, resulted in offspring that had enlarged, abnormally developed hearts. No neoplasms were evident in the embryos, possibly because of the young age at death or because of compensatory communication by the expression of other connexins. However, cell lines developed from the embryos exhibited abnormal patterns of growth (221,222). A connexin32 knockout mouse has also been developed (223). Interestingly, these mice exhibited elevated rates of endogenous hepatocyte proliferation and were more susceptible to spontaneous and carcinogen-induced hepatic tumor formation (223).

18.3. Dominant-Negative Inhibition of Connexin Function

Dominant-negative disruption of normal gene product function involves introduction of a mutated gene whose protein product interferes with the function of the normal gene product. Because of the oligomerization of connexins into hemichannels and the precise architecture of the gap junction channel, this approach may be especially fruitful to block GJIC. Recently, several mutated connexins that posses dominant-negative activity have been identified (224-227). One of these, a dominant-negative mutant of connexin26, inhibited connexin26-mediated GJIC, enhanced cell growth, and

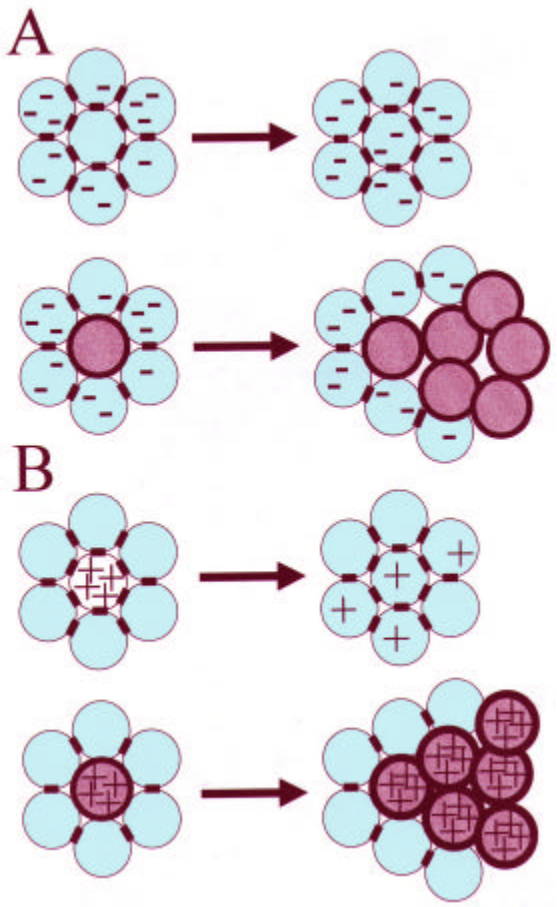

Figure 5. Illustration of the concept that growth regulatory signals may pass through gap junction channels. (A) Negative signals would enter from neighboring cells and suppress the growth of the receiving cell. (B) Positive, growth stimulatory signals would diffuse into neighboring cells resulting in a substimulatory level in the signal-generating cell. If a cell lacks functional gap junctions, its growth would not be suppressed by neighboring negative signals or would be stimulated by its accumulation of positive signals. This could lead to dysregulated growth.

increased the tumorigenicity of cells transfected with the mutant gene (227).

18.4. Connexin Transfection Studies

Connexin gene expression has been enhanced in several poorly expressing malignant cell lines by transfection of connexin cDNAs (228-236). In these "recommunicating" neoplastic cells, growth rates *in vitro* and/or tumor formation were highly reduced; these effects often correlated with the extent of reestablished GJIC. In some cases, the connexin-transfected cells also expressed altered levels of cell cycle regulatory proteins (233) or more differentiated functions (235). In another study (231), the connexin-transfected cells secreted a soluble, peptide growth inhibitor. This latter finding suggests that GJIC is integrated with other mechanisms of growth control.

19. GJIC AND OTHER GROWTH CONTROL MECHANISMS

Thus, four different approaches have been used successfully to inhibit or enhance GJIC in a specific way. These studies provide strong evidence that GJIC is involved in growth regulation and neoplasia, but do not exclude the importance of other mechanisms. With most important biological control processes, multiple, redundant mechanisms are present to prevent cellular dysfunction should a single mechanism become defective. Similarly, we believe GJIC is only one mechanism of growth control that functions coordinately with others (e.g., growth factor responses and signal transduction pathways, cell cycle controls, responses of cells to the extracellular matrix and cell-cell adhesion molecules, etc.) as previous studies suggest (231,233). The next decade should provide much insight into the interplay between these various regulatory processes.

20. GROWTH REGULATION MEDIATED BY A GAP JUNCTION SIGNAL

Two basic schemes by which growth may be regulated by a gap junction signal molecule are shown in figure 5 and are adapted from Loewenstein (170). Both inhibitory and stimulatory low-molecular weight signals might be produced in cells and diffuse to adjacent cells through gap junction channels. The negative signals would inhibit cell division and maintain differentiation whereas positive signals would stimulate growth and prevent differentiation. The loss of gap junctions or reductions in channel permeability would isolate cells from the inhibitory signals of neighboring cells and/or enable the accumulation of positive signals in signal-generating cells. These effects would result in the loss of growth inhibition or the stimulation of proliferation, respectively. If the reduction of GJIC were sustained, a noncommunicating cell could expand by clonal growth into a tumor.

While this model is clearly an oversimplification of growth and tumor formation, conceptually it provides the tissue with the ability to fine-tune cell number and function in a closed system. By regulating the amount and type of positive and negative signals generated, and the number and permeability of gap junction channels, a steady-state level of signals and cell number could be maintained. Changes in cell number following cell loss (due to toxicity, wounding, etc.) or cell gain (due to cell proliferation, impaired cell death, etc.) would change the size of the communicating network. This would result in an altered steady-state level of signals and would trigger cell growth or death until the system (tissue) reached its previous homeostatic, steady-state level.

This gap junction communicating cell network is a closed system that incorporates all the cells in the tissue and is, therefore, different than growth control mediated by extracellular factors and other types of cell-cell or cell-extracellular matrix interactions. Theoretically, growth control mediated by GJIC would enable more strict control

of cell number than would extracellular growth control mediated by growth factors, hormones, and chalones or cell-cell and cell-extracellular matrix regulation through integrins and other membrane components. The concentrations of the former could not be controlled precisely amongst all the cells and the latter would be local and would not integrate all cells in the tissue.

What is the gap junction growth regulatory signal and how does it work? This is obviously a difficult question to answer experimentally because there are so many molecules and ions capable of passing through gap junction channels. Multiple stimulatory and inhibitory signals might be involved. The answer, however, represents the "Holy Grail" of the gap junction/growth control field. Several criteria for a gap junction growth regulatory molecule can be proposed to simplify the problem:

1. The signal must be capable of passing between cells through gap junction channels.
2. The signal should affect cell growth in some manner.
3. The levels of the signal should oscillate within the cell that generates the signal and these oscillations should be important in growth control.
4. The amplitude of the oscillations should be dampened by GJIC.
5. The oscillatory signal should regulate cell growth through a known mechanism since it is unlikely that the passage of a signal through gap junctions per se would affect growth.

As first suggested by Loewenstein (170), one possible growth regulatory, gap junction signal molecule is cAMP. This molecule fulfills the above criteria for a gap junction growth regulatory signal:

1. Cyclic AMP, which has a molecular weight of 329, can pass through gap junction channels (165,166).
2. Treatment of cells with cell-permeable cAMP analogues or agonists elevates cAMP levels and inhibits the growth of many types of cells (237). Often, this is due to a block of cell cycle progression in G_1 (237).
3. The level of cAMP normally fluctuates throughout the cell cycle. In many types of cells, cAMP content is highest in G_1 then decreases as cells enter S-phase and this change is essential for the cells to exit G_1 and undergo DNA synthesis (237).
4. In high density, nonproliferating cells, however, cAMP content is more uniform throughout the cell population (238) which suggests that GJIC can dampen cAMP oscillations.
5. Cyclic AMP impedes cell cycle progression in G_1 by at least two mechanisms. First, it decreases the expression of cyclin D1 (239). This cyclin associates with cyclin-dependent kinase (cdk) 4 or 6, depending upon the cell type, and is active in G_1. The active complex phosphorylates the retinoblastoma protein which then releases E2F transcription factors and these activate the expression of genes needed in S-phase (240). In this way, cyclin D_1 is essential for cell cycle progression through G_1 into S-phase. Secondly, cAMP increases the expression of the cyclin-dependent kinase inhibitor, p27/kip-1 (241), which blocks the activity of cyclin D_1/cdk 4/6 and other cylin/cdk complexes (240). Neoplastic cells generally have reduced contents of cAMP and p27/kip-1 and higher cyclin/cdk activity (237,240).

Thus, cAMP fulfills the five criteria listed above for a gap junction, growth regulatory signal molecule. Cyclic AMP levels also fluctuate at other points in the cell cycle besides G_1 in a cell-specific manner and other second messengers similarly oscillate throughout the cell cycle (237). This indicates that the growth regulatory role of cAMP and other potential gap junction signals is undoubtedly very complex. Here we will only further consider the role of cAMP.

Figure 6 illustrates how an oscillating, growth inhibitor such as cAMP might inhibit cell growth via GJIC; this model is based upon previous ones (170,216). First, to inhibit growth, the signal must be sustained above a threshold; periodic oscillations above the threshold would not suppress growth. In cells at low density where cell-cell contact and GJIC would be minimal, the cells would generate these oscillatory, inhibitory signals in an asynchronous manner (panel A). The graph depicts such fluctuation within a single cell and illustrates that the signal would not be sustained above a theoretical growth inhibitory threshold. Growth would result in this cell. Similarly, if cell-cell contacts are extensive but GJIC is low or does not exist (as in neoplastic cells or cells exposed to growth factors or tumor promoters), the signal would still oscillate asynchronously in individual cells and growth would occur (panel B). In contrast, if GJIC is present, the signal oscillations would be dampened by cell-cell diffusion and a steady-state signal level would arise in the cell population (panel C). If this steady-state level is above the inhibitory threshold, growth would be suppressed. Below the threshold, growth would ensue. This situation is analogous to nonneoplastic cells that have extensive GJIC and stop growth when reaching high density (contact inhibition of growth).

Several facets of this model need additional consideration. First, it permits growth in cells that are coupled well by gap junctions, i.e., nonneoplastic cells. Growth in such cells could be achieved by raising the growth inhibitory threshold, by decreasing the steady-state level of the inhibitor, or by reducing GJIC (uncoupling the cells). In the case of cAMP, an increase in the threshold would occur if cyclin D_1/cdk 4/6 activity was activated by another factor. More cAMP might be required to inhibit the complex. A decrease in the steady-state level of cAMP could be achieved by reducing adenylate cyclase activity or increasing phosphodiesterase activity. Finally, gap junction uncoupling of a cell in G_1 (described above)

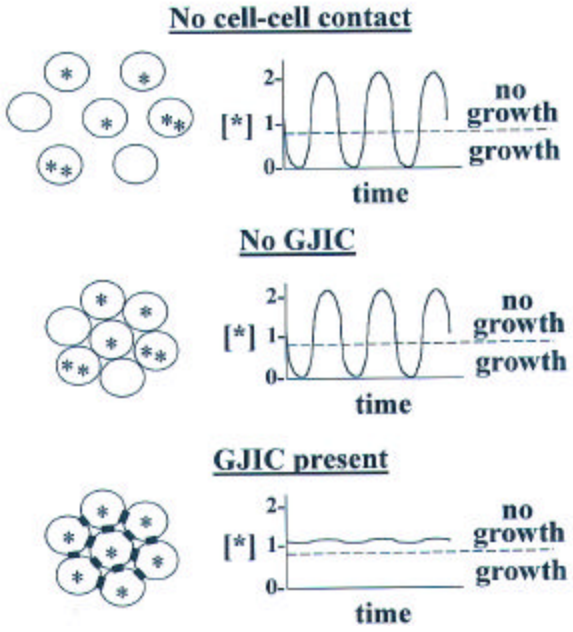

Figure 6. Illustration of how an oscillating, growth inhibitory signal such as cAMP could suppress cell growth only when GJIC was present. (A) in the absence of extensive cell-cell contact (and GJIC), signal levels would oscillate asynchronously in individual cells. The concentration of the signal in an individual cell over time is plotted on the graph. The signal would rise and fall above a growth inhibitory threshold, but because of the lack of sustained signal above the threshold, cell replication would continue. This situation is analogous to low density, proliferating cell cultures. (B) Similar to the case with dispersed cells, the presence of extensive cell-cell contact, but no GJIC would also result in cells with oscillating signal levels and continuous growth. This situation is similar to neoplastic cells that continue to proliferate at high cell density, i.e., they are not contact-inhibited. (C) The signal level in cells that are extensively coupled by gap junctions, however, would reach a steady-state because any oscillation in an individual cell would be dampened (buffered) by the neighboring cells. This steady-state signal level, if above the growth inhibitory threshold, would suppress growth.

would enable the cAMP level to fall because it could not be buffered by neighboring cells. Second, the model also accounts for the dysregulated growth of neoplastic cells. In general, these cells have deficient GJIC, lower cAMP content, and higher cyclin/cdk activities (237,240). This model is consistent with the "Neighborhood Coherence Principle" model of tissue homeostasis mediated by gap junctions (242).

21. MODULATION OF GJIC FOR CANCER THERAPY

Several factors can influence the response of a tumor to chemotherapy (243). One is the vascularization of the tumor. Many tumors have a disorganized, inadequate vascular supply. Many of the cells within such tumors are deficient in oxygen and nutrients. Unfortunately, hypoxic cells may be resistant to certain chemotherapeutic drugs such as adriamycin that generate oxygen free radicals and other reactive oxygen species as a mechanism of cell killing. Poor vascularization may also result in limited penetration of drugs away from tumor blood vessels. This is especially true for water-soluble agents such as methotrexate, adriamycin, and vinblastine. Nutrient-deprived cells in poorly vascularized tumors may also have poor uptake of drugs through active transport and have altered drug metabolism.

One possible mechanism to increase drug penetration and dispersal in tumors would be to increase GJIC. Oxygen and nutrients would theoretically pass through gap junctions from cells adjacent to blood vessels to those further away. This increase in GJIC might be achieved by increasing tumor cell connexin expression pharmacologically (e.g., with forskolin, steroids, and retinoids) or by introducing active connexin genes (gene therapy approach). As demonstrated by the numerous in vitro studies described above, the enhancement of tumor cell GJIC might have the additional benefit of reducing tumor cell growth, besides enhancing chemothrerapy.

GJIC may also improve a more novel type of cancer therapy that involves introducing a lethal gene such as the Herpes Simplex virus thymidine kinase (HSV-TK) gene into tumor cells (244,245). Expression of this gene renders the tumor cells susceptible to the thymidine analogue, ganciclovir (GCV) which is phosphorylated readily by HSV-TK, but not by endogenous TK. The phosphorylated metabolite is incorporated into the DNA of proliferating cells such as cancer cells resulting in cell death. Cells that express the gene are susceptible to GCV, but nonexpressing and nonproliferating cells are very resistant. Introduction of the gene into tumor cells is usually accomplished by infection with a virus that has been genetically engineered to express the gene. Interestingly, however, only a small percentage of the tumor cells take up and express the HSV-TK gene yet a much higher percentage of the cells are killed following GCV treatment. This is known as the "bystander effect" (244,245).

One mechanism for the bystander effect is that phosphorylated GCV can pass between tumor cells through the few gap junction channels that often exist (246-248). It follows then that the enhancement of GJIC in such cells might improve the bystander effect. This has recently been demonstrated using connexin-transfected neoplastic cells (249,250). These results suggest that the enhancement of GJIC might improve the bystander effect in certain types of cancer gene therapies as well as in more conventional chemotherapies.

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