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COMMENTARY

Role of connexin genes in growth control

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I. Introduction

During multistage carcinogenesis, multiple genetic changes progressively accumulate in a cell until it acquires a complete set of altered genes necessary for malignant phenotype expression (1). Those altered genes often found in human cancers are understandably those involved in cell growth control. Thus, so far, the most abundantly changed tumor-suppressor gene and oncogene in human cancers are the p53 and ras genes, respectively (2,3). The p53 gene plays a central role as a cell cycle checkpoint (4) and the ras is placed at the crossroads of signal transduction (5).

In addition to those conventional genes involved in the control of cell cycle/signal transduction of individual cells, there is a second group of cell growth control genes whose job is to control growth so that individual cell growth is in harmony with surrounding homologues and partners. Since most cancer cells do not behave in harmony with their normal neighbors, it is not surprising that the function of those genes involved in such intercellular communication mechanisms are also disrupted in many tumors. Thus, in the past, several oncogenes have been identified as those genes involved in humoral (mediated by growth factors or hormones) intercellular communication such as c-erb, c-erb B2 and c-sis genes (6). More recently, however, it has become clear that cell–cell contact mediated intercellular communication plays a crucial role in cell growth control and acts as a tumor suppressive element (7,8).

There are two groups of molecules involved in direct cell–cell contact mediated communication which are considered to be involved in cell growth control, i.e. cell adhesion molecules and gap junction proteins. Unlike growth factor-related genes which are considered as oncogenes, these direct cell–cell communication genes appear to form families of tumor-suppressor genes (7,8). Recent advances in molecular characterization of gap junctional intercellular communication (GJIC*) identified a family of connexin genes as its channel proteins (9,10).

In this article, we review recent advances made in the role of connexin genes in carcinogenesis.

I.1 Connexin gene family

The gap junction is the site of the intercellular membrane channels which provide for direct cytoplasmic continuity between adjacent cells (9). The structural unit of the gap junction is the *connexon*, a proteinaceous cylinder with a hydrophilic channel; connexons spanning the plasma membranes of closely-apposed cells align end-to-end, forming intercellular channels (Figure 1). Gap junction channels provide for the exchange of small molecules (<1000–2000 Da) between cells to allow metabolic cooperation (11); they also transmit developmental signals involved in cell patterning (12). Among the metabolites which pass through gap junction channels are agents involved in cellular regulation, including such second messengers as inositol 1,4,5-trisphosphate and Ca^{2+} (13–15). Since the initial report of gap junctions by Furshpan and Potter (16), describing a novel form of synaptic transmission in the giant motor synapse of the crayfish which involved the rapid transfer of electrical activity from the pre- to post-synaptic fibre, the complexity of the family of gap junction proteins and their constitutent channels has increased dramatically (10).

Following the isolation of gap junction proteins and the cloning of their mRNAs, it has been established that these proteins, the *connexins*, are encoded by a multi-gene family consisting of at least 12 members in mammals (10,17) (Table I). Although it is widely accepted to name connexin genes based on the predicted Mr of the protein (18), some investigators have proposed a different system utilizing the greek letters α and β to differentiate between two main groups of gap junction genes (19,20). This system is based on genetic origin and sequence similarities at the nucleotide and amino acid level.

Comparisons of the connexin cDNAs reveals regions of high homology and other regions of little or no homology (17,21). Alignment comparisons of connexins show that the transmembrane regions are highly homologous whereas the amino terminus, cytoplasmic loop and, particularly the carboxy terminus, are divergent (Figure 1).

The channels composed of these different connexins display differences in unitary conductances and voltage sensitivity (22). Furthermore, it is now becoming apparent that the channels encoded by the different connexin genes exhibit some selectivity (23,24) and directionality (25,26) in molecular transport. These differences may underlie the observed specificity of some functional aspects of GJIC, including their role in tumor suppression (see Section III).

I.2 Expression in tissues and cells

Gap junctions can be found in almost all mammalian tissues except circulating blood cells and adult skeletal muscle, attesting to a likely homeostatic role for these channels in cell function (27). With the recent isolation of several novel connexin genes, investigators have examined the tissue specific expression of many gap junction proteins. Several connexins exhibit a characteristic tissue and cellular distribution in the adult animal, implying functional differentiation among the different types of channel. However, many tissues and their constituent cells have been shown to express multiple connexins (Table I). In addition, dramatic changes in expression of connexin genes have been reported in many tissues during

*Abbreviations: GJIC, gap junctional intercellular communication; Cx32, Cx43 etc..., connexin32, connexin43, etc...; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; DDT, dichlorodiphenyltrichloroethane; PCB, polychlorinated biphenyls.

Table I. Expression of connexin genes

Connexin	Class	Tissue	Cell type
Cx26	β	liver, kidney, spleen, testes, lung, stomach, intestine, brain, pancreas, skin, pineal gland	hepatocyte, neuron, keratinocyte, pinealocyte
Cx30.3	β	skin	
Cx31	β	skin, testes	keratinocytes
Cx31.1	β	stratified squamous epithelia, skin, testes	keratinocytes
Cx32	β	liver, brain, kidney, spleen, uterus, testes, lung, stomach, intestine	hepatocyte, oligodendrocyte, neuron, Schwann cell, thyroid epithelial cells
Cx33	α	testes	Sertoli cells
Cx37	α	vasculature, heart, brain, stomach, intestine, testes, liver, kidney, spleen, uterus, ovary, lung, skin	endothelial cells, myocytes, keratinocytes
Cx40	α	vasculature, heart, kidney, uterus, ovary, lung, intestine	endothelial cells, Purkinje fibers
Cx43	α	heart, brain, smooth muscle, kidney, uterus, ovary, testes, lung, stomach, intestine, skin, lens, cornea, bone, placenta	myocytes, smooth muscle, astrocytes, endothelial cells, fibroblasts, keratinocytes, ependymal cells, Leydig cells, macrophages, osteocytes, pancreatic β-cells, thyroid follicular and epithelial cells, trophoblast giant cells
Cx45	α	lung, brain, kidney, skin, heart, intestine	
Cx46	α	lens, heart, kidney, peripheral nerve	lens fiber, Schwann cell
Cx50	α	lens, cornea, heart	lens fiber, epithelial cell, atrioventricular valves

This table summarizes data obtained from several studies describing the distribution of connexin mRNAs and proteins. For details, see recent reviews in refs 9,10,17.

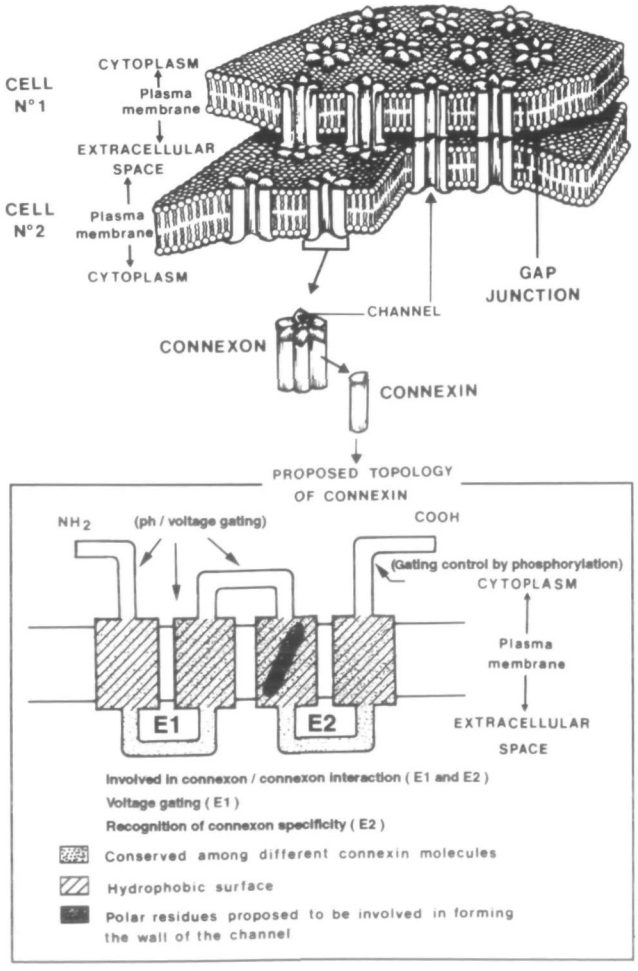

Fig. 1. Schematic view of gap junctions: connexons and connexins (modified from ref. 65).

development (28–30), as well as in response to a number of pharmacological treatments (see below). While many studies have also reported the expression of various connexins in several different established cell lines, the relevance of this expression to the normal endogenous expression patterns is unclear. For example, the majority of established cell lines express connexin43 (Cx43), while very few cell lines express readily detectable amounts of other connexins.

I.3 Assembly into connexons and gap junctions

As cells come into contact with each other, they develop various intercellular junctions between their apposed membranes, including gap junctions. One of the initial steps prior to formation of intercellular junctions involves cell contact mediated by cell adhesion molecules (CAMs). Investigators have shown a correlation between the expression of CAMs and gap junction proteins (31–33). Transfection of E-cadherin into communication deficient cell lines that did not express CAMs resulted in an induction of GJIC in E-cadherin expressing clones (34). Similarly, transfection of LCAM into communication-deficient cells also induced GJIC, this change correlating with changes in phosphorylation of endogenous Cx43 and translocation of the protein from cytoplasm to plasma membrane (32). Meyer *et al.* (33) demonstrated a relationship between intercellular communication and cell adhesion with the use of function blocking antibodies directed against extracellular epitopes of N-cadherin. This not only abolished intercellular adhesion, but also GJIC between the cells. Presumably, intercellular contact induces events related to the formation of gap junction channels. The association of cell adhesion with GJIC is of particular significance in cell transformation, given the evidence for the involvement of both of these processes in tumor suppression (7,8).

The phosphorylation of gap junction proteins appears to be important for the establishment of functional intercellular communicaton. Phosphorylation of connexins was first shown by Saez *et al.* (35) who demonstrated that cAMP increased the incorporation of radiolabeled phosphate into Cx32 protein and enhanced junctional conductance, indicating the involvement of a cAMP-dependent protein kinase. Further studies examined other kinases and detected similar phosphorylation events by protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (36–38). Synthetic peptides corres-

ponding to the cytoplasmic region of Cx43 were phosphorylated only by PKC and not cAMP-dependent protein kinase nor Ca^{2+}/calmodulin dependent protein kinase II (39). Cx43 has also recently been shown to be phosphorylated on serine by mitogen-acitvated protein kinase (40). Recent analysis of the effects of activators and inhibitors of various protein kinases has led to the suggestion that connexin-specific sequences may determine distinct phosphorylation patterns for different connexins, and thereby may be reflected in differences in regulation of expression and function of different connexins (41). Evidence is accumulating for a role of phosphorylation in connexin assembly (32,42,43) or as a regulator of channel gating (39,44,45). Thus abnormalities in connexin phosphorylation could lead to perturbation of normal GJIC, which may result in aberrant cell growth control (see Section II.4).

With respect to connexin trafficking, there is evidence that connexin proteins pass through cellular organelles on route to the plasma membrane. Cx43 has been shown to pass through the endoplasmic reticulum to enter the Golgi apparatus in studies using protein trafficking inhibitors (46,47). Furthermore, oligomerization of connexins into connexons is inhibited if transport from the endoplasmic recticulum to the Golgi apparatus is blocked (48). Once in the membrane, connexons must associate with their counterparts in the adjacent cell. This may involve disulphide bond formation either at the intramolecular or intermolecular level (49,50). Disruption of trafficking of gap junction protein to the cell membrance results in lack of GJIC. For example, cells which express Cx43 that fails to reach the plasma membrane are communication deficient (32). However, stimulation of cell adhesion by transfection with LCAM leads to translocation of Cx43 to the plasma membrane and the subsequent establishment of GJIC. Therefore, although gap junction proteins may be present in various cancer cells, they may not be appropriately processed and/or transported to form functional gap junctions.

Gap junction proteins are rapidly turned over in the cell, having relatively short half lives, ranging from 1–3 h (37,43,51,52). During disassembly of gap junctions, it is believed that one cell separates taking the whole gap junction complex, that is internalized in the form of annular gap junctions which may be associated with vesicles (53,54). In some cases, these vesicles have been shown to contain acid phosphatase activity, suggesting that gap junction proteins are broken down rather than re-utilized (53,55). More recent evidence suggests that proteolysis of Cx43 is mediated by the ubiquitin proteasomal pathway (56). It seems reasonable to assume that other connexin proteins are also degraded in this manner.

I.4 Connexin and connexon interactions

Given the emerging complexity of the gap junction gene family, it has become important to assess the compatibility of interaction between the connexin proteins, as well as the functional characteristics of homomeric and heteromeric associations. Evidence from STEM mass analysis of gap junction plaques isolated from liver suggest that homomeric connexons (i.e. connexons composed of the same connexin protein) composed of Cx26 or Cx32 self-associate in the plasma membrane (57). However, coinfection of Sf9 insect cells with baculovirus expressing both Cx32 and Cx26 led to the formation of heteromeric connexons (i.e. connexons composed of different connexin proteins), where the two proteins were localized in the same connexon oligomer (58). Further support for the formation of heteromeric connexons comes from results of dominant negative mutations of connexins. Coinjection of RNA transcribed from a chimeric connexin43/connexin32 construct with either Cx43 or Cx32 RNA into Xenopus oocytes blocked junctional conductance of both species of gap junction channels, suggesting this chimera was able to participate in the formation of heteromeric connexons (59,60). In addition, co-expression of multiple connexins can lead to modulation of gap junctional coupling, presumably through the formation of heteromeric connexons. Thus, expression of transfected Cx45 was found to interfere with endogenous Cx43 function when transfected into rat osteosarcoma cells (61).

With regard to homomeric connexons, evidence supports the concept of selectivity in the formation of heterotypic gap junctions (i.e. gap junctions formed by two different connexons in two different cells) (62,63). Recent studies have clearly established that the extracellular domains, particularly in the second extracellular loop, are critical in determining the specificity of connexin interactions between two cells (62,63). A remarkable degree of selectivity has been demonstrated with regard to the functional interactions of various connexins. It has now been clearly established that connexon–connexon interactions are dependent upon the connexin proteins involved. Thus certain connexins can form functional channels with some connexins and not others (10).

Given the multiple steps controlling connexin gene transcription, translation and post-translational modification, connexin trafficking and degradation, as well as the variety of interactions of connexins and connexons at the molecular level, it is becoming apparent that modulation of GJIC can occur at multiple points. In addition, perturbations at many of these stages have been correlated with loss of GJIC and cell transformation (Figure 2).

II. Aberrant control of connexin expression and function in tumors or after carcinogen exposure

II.1 Down-regulation of homologous and heterologous GJIC in tumors

Since unharmonized cell growth is a hallmark of tumors, it is not surprising that altered GJIC is found in almost all solid tumors. The first example of down-regulated GJIC in cancer was reported by Loewenstein and Kanno (64). Following this original finding, a number of investigators have examined various types of cancers and confirmed that cancer cells show aberrant GJIC. However, it has become clear that aberrant GJIC of cancer or transformed cells can manifest itself in two distinct types of cell–cell interaction, i.e. homologous and heterologous GJIC (65).

Homologous GJIC involves communication between like cells. Among tumor cells this type of GJIC is frequently disrupted. On the other hand, some tumor cells do maintain a level of homologous GJIC similar to that found in their normal counterparts. For example, when BALB/c 3T3 cells were transformed *in vitro*, GJIC level among transformed cells was not changed. However, heterologous GJIC was disrupted since the transformed cells did not communicate with surrounding normal cells. Such a specific lack of heterologous GJIC was observed with all transformed BALB/c 3T3 cells, regardless of the carcinogens used, including chemicals, UV light and oncogenes (66,67). Lack of heterologous GJIC between cancer and normal cells was also observed *in vivo*; some rat liver

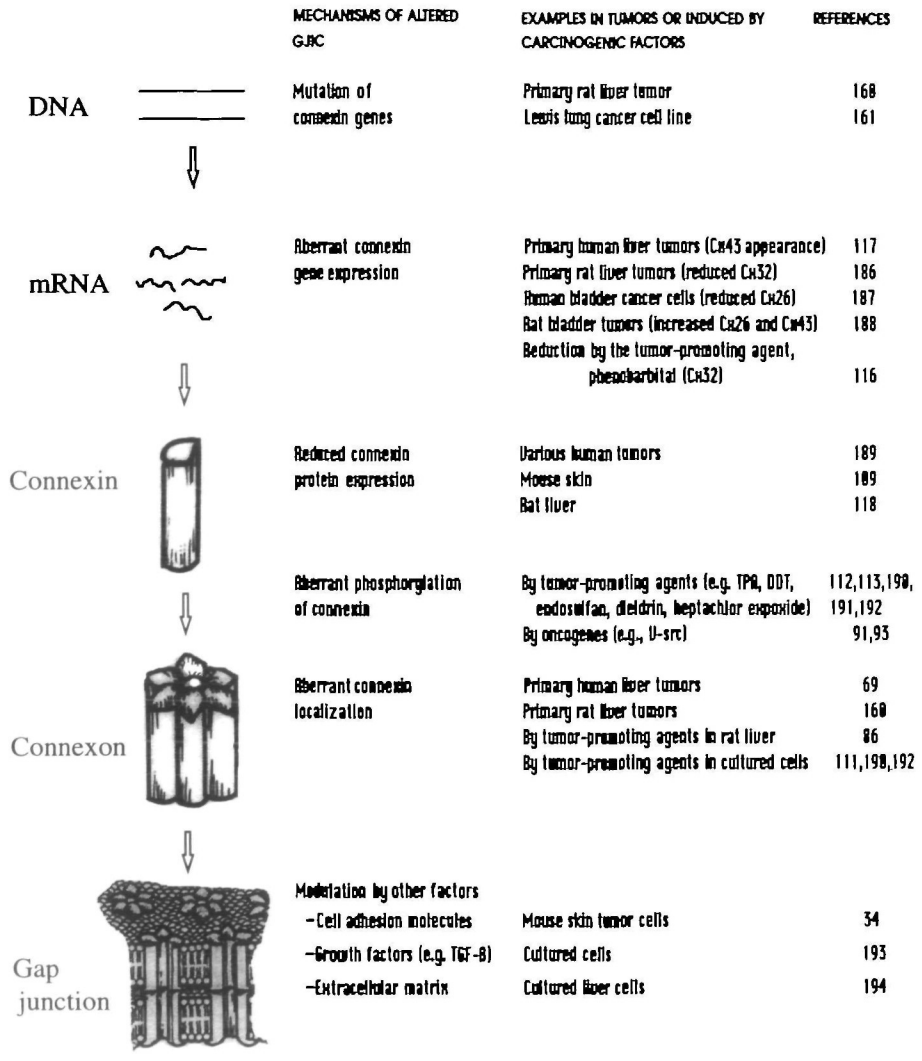

Fig. 2. Examples of mechanisms involved in GJIC modulation observed in tumors or induced by tumor-promoting agents.

preneoplastic foci showed clear lack of communication with surrounding normal parenchymal cells (68). Similarly, human hepatocellular carcinoma cells were clearly not coupled with surrounding normal cells (69). However, in this case, carcinomas were physically separated from the surrounding cells by encapsulization. Aberrant homologous and heterologous GJIC are not mutually exclusive; both types of GJIC were decreased in cultured rat liver epithelial cells (70,71), rat liver tumors (68) and human liver tumors (69).

For cancer cells to grow without constraint from surrounding normal cells, it may indeed be advantageous to avoid communicating with them by creating a mechanism to block heterologous GJIC. In addition, loss of homologous GJIC among cancer cells themselves may contribute to increase their heterogeneity and those cells with more malignant phenotypes may gradually dominate the tumor cell population during the progression process.

II.2 Down-regulation of GJIC by tumor-promoting agents, oncogenes and growth factors

In addition to defective GJIC of cancer cells, another line of evidence which suggests the role of aberrant GJIC in carcinogenesis has come from the finding that many tumor-promoting agents inhibit GJIC. It has first been demonstrated by Yotti *et al.* (72) and Murray and Fitzgerald (73) that GJIC of cultured cells can be reversibly inhibited by phorbol ester tumor promoters, such as TPA. Subsequently, Enomoto *et al.* (74) have shown that TPA and its active congeners not only inhibit intercellular transfer of molecules, but also ion transfer.

The discovery of GJIC inhibition by phorbol esters not only provided a clue to understand cellular mechanisms of tumor-promotion, but also triggered an interest to use this as an endpoint to detect tumor-promoting activity of environmental agents (75). Many such agents have thus been tested for their ability to inhibit GJIC in cultured cells. As reviewed by Swierenga and Yamasaki (76) and Budunova and Williams (77), many, although not all, tumor-promoting agents have been reported to inhibit GJIC. Since there is no appropriate endpoint to study nongenotoxic activity of carcinogens, disrupted GJIC may help identify carcinogens which escape detection by conventional genetic toxicology tests (78). While there are three major methods to measure GJIC, i.e. metabolic cooperation, dye-transfer and electrical coupling, the dye-transfer assay is being used more widely than others for screening chemicals (76,77). This method not only enables investigators to determine GJIC of various types of cell, but also several variations of the method have been developed

(79–81). In combination with molecular probes available for connexins, i.e. cDNAs and antibodies, the mechanisms of action of carcinogens on GJIC can thus be studied in detail.

Most studies on the effect of carcinogens on GJIC have been performed in cultured cells. However, since a complicated network of intercellular communication cannot be mimicked *in vitro*, it is necessary to obtain information on how *in vitro* results reflect *in vivo* situations. Several attempts have been made to examine GJIC *in vivo* before and after exposure of animals to tumor-promoting agents. The application of TPA to mouse skin has been reported to both inhibit (82), and stimulate (83) GJIC. It is possible that the difference in methods employed to measure GJIC in these two studies is a reason for this apparent discrepancy. Several liver tumor-promoting agents have been shown to decrease gap junctions and inhibit GJIC in the rat liver *in vivo* (84–86). These results strengthen the idea that the lack of GJIC plays an important role in the clonal expansion process of potential cancer cells, i.e. tumor promotion.

In addition to tumor-promoting agents, certain oncogenes and growth factors have been shown to inhibit GJIC. Those oncogenes reported to down-regulate GJIC include retroviral oncogenes (v-src, v-Ha-ras, v-raf, v-fps), DNA viral oncogenes (polyoma-middle T, SV-40 T, HPV 16-E5) and cellular oncogenes (c-src, c-Ha-ras, c-erbB2) (65,87–92). While some of these oncogene products, such as $pp60^{src}$, appear to target connexin genes directly (93), it is not clear whether others affect the function of connexins directly. It is possible that GJIC inhibition is the result, rather than cause, of oncogenic transformation induced by some oncogenes. Growth factors and hormones which have been reported to inhibit GJIC include fibroblast growth factors, platelet derived growth factor, transforming growth factor β, epidermal growth factor and testosterones (40,94–96). These results support the link between disrupted GJIC and abnormal cell growth control. Furthermore, some anti-tumor-promoting and chemopreventive agents, such as retinoids, are known to increase GJIC, providing another line of evidence for this link (see Section V.1).

II.3 Relationship between down-regulated GJIC and the process of carcinogenesis

We described above three major events which link aberrant GJIC and cancer: (i) aberrant GJIC in tumors, (ii) down-regulation of GJIC by cancer-causing agents or genes, and (iii) up-regulation of GJIC by inhibitors of carcinogenesis. These findings, however, do not necessarily provide information on whether and how disruption of GJIC is involved in the process of carcinogenesis. Earlier studies with *in vitro* cell transformation have provided evidence for and against the role of blocked GJIC in the transformation process. The enhancement of transformation and inhibition of GJIC in BALB/c 3T3 cells with a series of phorbol esters and their endogenous homologue, diacylglycerol, correlated very well (97,98). Similarly, several anti-tumor-promoting agents enhanced GJIC and inhibited the transformation of BALB/c 3T3 cells (99). However, other agents which enhanced growth of BALB/c 3T3 cells, okadaic acid and TGF-β, did not affect their GJIC (100,101). A genetic variant line of BALB/c 3T3 cells which shows up to 500-fold higher sensitivity to carcinogen-induced cell transformation lost its GJIC capacity when they reached confluency, suggesting a role of blocked GJIC in its higher cell transformation capacity (102). However, when these cells are hybridized with the BALB/c 3T3 cells with normal susceptibility to cell transformation, their hybrid showed lower GJIC at their growth confluence but lower susceptibility to transformation induction, demonstrating a dissociation of these two phenomena (103).

In Syrian hamster embryo (SHE) cells, there was also a good relationship between GJIC inhibition and transformation enhancement by phorbol esters (104). However, no such correlation was found with non-phorbol type promoting agents (105). On the other hand, SHE cell transformation was recently found to be facilitated by lowering the pH of culture media and that GJIC was also inhibited at lower pH, showing a good relationship between GJIC block and cell transformation enhancement (106).

In vivo information provides more supportive evidence for the involvement of GJIC disruption in carcinogenic process. In the rat liver, four liver tumor-promoting agents which are considered to act through different mechanisms inhibited GJIC; these are phenobarbital, clofibrate, PCBs and DDT (86). Progressive decrease of GJIC was found during rat liver tumor progression (107). In mouse skin carcinogenesis, cell lines established from papillomas are usually communicating less than normal cells, but more than those cells from carcinomas (108). Reduction of Cx26 and Cx43 expression was also observed in mouse skin squamous carcinomas (109).

These results indicate that GJIC block is an important contributing factor, but not a necessary event, in carcinogenesis. On the other hand, as described later in this review, there is rather strong evidence that connexin genes form a family of tumor-suppressor genes.

II.4 Molecular mechanisms involved in GJIC inhibition in tumors or by carcinogens

Since GJIC is modulated by a number of factors, such as tumor-promoting agents, oncogenes and growth factors, it is reasonable to assume that various control mechanisms of GJIC are targets for such insults. In fact, GJIC can be modulated by various mechanisms. In addition to usual regulatory mechanisms which apply for most proteins, such as transcription, mRNA stabilization, translational control and post-translational phosphorylation, the function of connexin proteins can also be modulated at other control levels. For example, connexins are assembled into connexons in the trans-Golgi apparatus and their translocation to cytoplasmic membrane may also be regulated (42). Moreover, even when gap junctions are formed between two cells, it is known that gap junctional channels can be closed or opened under certain circumstances (110). More indirectly, GJIC can be influenced by cell adhesion molecules as well as extracellular matrix and by growth factors. Mutations of connexin genes or those genes which are involved in the control of connexin function may also be involved in the regulation of GJIC. These various regulatory points appear to be vulnerable to carcinogenic insults and/or during the transformation process (Figure 2).

A few carcinogenic agents have been studied for their mechanisms of action on GJIC. It appears that post-translational modulation is a major mechanism of aberrant GJIC induced by carcinogens. For example, TPA-treated cultured rat liver epithelial cells did not show any change in their mRNA level for the Cx43 gene (111). Cx43 protein was also present at a similar amount before and after TPA treatment. However, the phosphorylated forms were changed. Associated with such modified phosphorylation, connexin protein was localized in the cytoplasm rather than in the plasma membrane. Therefore,

it seems that TPA phosphorylated connexin proteins probably through protein kinase C activation, and thus translocated them from plasma membrane to cytoplasm (112,113). On the other hand, it has recently been proposed that TPA does not affect Cx43 localization, but it simply disturbs connexon assembly (114). Since connexin proteins can function only when they are in the cytoplasmic membranes, this internalization of connexins may be responsible for inhibition of GJIC. Similarly, GJIC down-regulation by certain oncogenes is considered to be due to phosphorylation of connexin proteins by such oncogene products. The direct phosphorylation of Cx43 proteins by $pp60^{src}$ has recently been demonstrated by Loo *et al.* (93). There is a good correlation between the phosphorylation of tyrosine 265 of Cx43 by $pp60^{v-src}$ and GJIC inhibition (115). Similarly, Cx43 phosphorylation by the $p130^{gag-fps}$ has been reported by Kurata and Lau (91).

In vivo studies also suggest that post-translational modulation of connexins and/or aberrant localization is a common mechanism for carcinogen-induced down regulation of GJIC. In our own study, groups of Fischer 344 rats were submitted to repeated chronic treatment of phenobarbital, PCB, DDT, and clofibrate for 5 weeks. All four tumor-promoting agents decreased dye coupling in rat liver. This decrease was associated with a reduced number of gap junctions and aberrant localization of some Cx32 protein in hepatocytes; Cx32 was often observed in the cytoplasm of hepatocytes instead of at gap junctions in the plasma membrane. Western blot analysis showed only slight changes in the level of Cx32 protein. Although Cx26 protein at gap junctions was usually decreased by tumor promoters in rat liver, local induction of Cx26 protein expression in centrolobular groups of hepatocytes after PCB and DDT treatment was observed. The expression of Cx43 was induced in hepatocytes after PCB, DDT and clofibrate exposure, but this protein was also localized intracytoplasmically, suggesting no functional role (86).

It has previously been shown that the treatment of rat with phenobarbital lowers the level of Cx32 mRNA (116). It has also been shown that in many rat liver tumors, Cx32 mRNA level was decreased. However, in human hepatocellular carcinomas, the level of Cx32 mRNA was not changed although the level of Cx43 mRNA was significantly increased (117). In another study of rat liver carcinogenesis, Sakamoto *et al.* (118) reported the decrease of Cx32 protein expression in preneoplastic foci and hyperplastic nodules, whereas Cx26 protein expression was increased in these lesions. Interestingly, both Cx26 and Cx32 proteins were markedly reduced in hepatocellular carcinomas (118). During mouse skin tumor progression, Kamibayashi *et al.* (109) observed no quantitative changes in Cx26 or Cx43 protein expression in papillomas induced by 7,12-dimethylbenz[*a*]anthracene and TPA painting. However, these two connexin proteins appear to be more colocalized in papillomas than surrounding non-tumorous lesions. In squamous cell carcinomas, there was a significant decrease of Cx26 and Cx43 protein expression.

There is a close functional relationship between connexins and cell adhesion molecules. It has been reported that E-cadherin expression is essential for Cx43 to form functional GJIC in mouse keratinocytes (34). This explains why GJIC of mouse keratinocytes is Ca^{++}-dependent; E-cadherin is a Ca^{++}-dependent cell adhesion molecule (119). Stoler *et al.* (120) further reported that E-cadherin expression is lost in mouse skin tumors during their transition to a more invasive, metastatic phenotype. Furthermore, E-cadherin has been shown to be an invasion suppressor gene of various carcinomas by transfection experiments (121,122). Decreased expression or mutations of the E-cadherin gene are also associated with several types of human cancers (123,124).

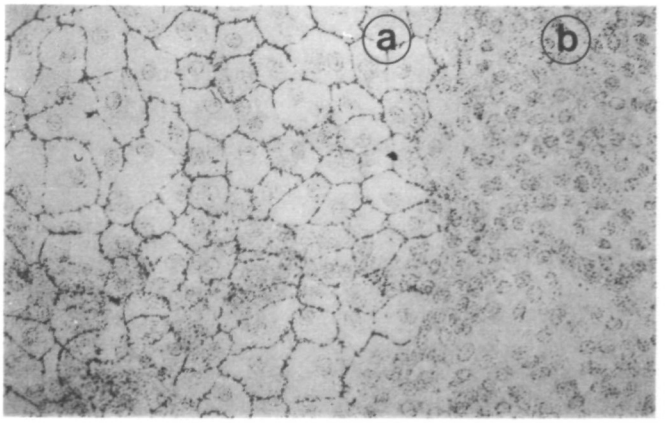

Fig. 3. Absence of connexin molecules in Cx43 expressing nontumorigenic cells at the border with their tumorigenic counterparts. Co-culture of nontumorigenic IARC 20 (**a**) and tumorigenic IAR 6–1 (**b**) cells showing the localization of Cx43 by immunoperoxidase staining. Cx43 is well distributed at the edges of IAR 20 cells which are in close contact with other identical cells. In contrast, Cx43 is mostly detectable in the cytoplasm of IAR 6–1 cells. Note the selective absence of Cx43 in the plasma membranes of IAR 20 cells that are in contact with IAR 6–1 cells (reproduced from ref. 113).

That GJIC requires appropriate cell recognition mechanism is in line with the observation that certain tumorigenic cells do not form heterologous GJIC with non-tumorigenic counterparts even if both of them express the same type of connexin gene. When tumorigenic and non-tumorigenic rat liver epithelial cell lines were cocultured, no heterologous GJIC was established (113). The immunohistochemical staining of the coculture with anti-Cx43 antibody revealed that Cx43 protein was abundantly present in non-tumorigenic cells but not in tumorigenic ones. As shown in Figure 3, Cx43 protein was, however, specifically absent at the boundary of 'tumorigenic/nontumorigenic' cells. Interestingly, these same non-tumorigenic cells at the boundary show Cx43 protein only at the sides contacting other non-tumorigenic cells. These results suggest that homologous cells have their own mechanism enabling them to recognize each other and that such a mechanism is not present between tumorigenic and non-tumorigenic cells.

III. Tumor suppression by connexins

Since there is substantial evidence implicating loss of GJIC in transformation, a variety of approaches have been used to up-regulate intercellular communication to restore growth control (reviewed in 7,65,125,126). For example, many reports describe effects of retinoids on increasing GJIC and decreasing cell growth and transformation (99,127–129). With the recent cloning of many of the connexin cDNAs, a direct approach to increasing connexin expression and subsequent GJIC has allowed a more thorough examination of the role of intercellular communication on tumor suppression. While functional analyses have been carried out either by injection of specific connexin RNAs into Xenopus oocytes, or by transfection into a variety of cell lines, because of the variety of interests of investigators, only some of the studies transfecting connexin cDNAs into mammalian cells have reported on effects on cell growth.

Table II. Transfection of connexin genes

Cell line	Transfected connexin	Endogenous connexin	Growth *in vitro*	Growth *in vivo*	Reference
SkHep hepatoma	Cx32	Cx32	↔	↓	(134,137)
WB-F344 liver epithelial cells	Cx43	Cx32,26	↓[a]	nd	(148)
Rat-1 fibroblasts	Cx43as[c]	Cx43	↓[b]	nd	(144)
transformed 10T1/2	Cx43	Cx43	↓	↓	(132)
MCA-10	Cx43		↓	↓	(133)
HeLa	Cx26	Cx26	↓	↓	(131)
	Cx40	Cx26	↔	↔	
	Cx43	Cx26	↔	↔	
C6 glioma	Cx43	Cx43	↓	↓	(130,135)
	Cx32	Cx43	↔	↓	(139)
rhabdomyosarcoma	Cx43	Cx43	↓	nd	(141)
transformed kidney epithelial cells	Cx43	Cx32	↓	nd	(156)

[a]Suppressed growth of cocultured transformed cells.
[b]Lost ability to suppress growth of cocultured transformed cells.
[c]Transfected with antisense Cx43 cDNA.

III.1 Growth suppression in vitro and *in vivo*

The introduction and overexpression of connexin cDNAs in tumor cells by transfection has shown that the presence of functional gap junctions can suppress growth and/or tumorigenicity of some types of transformed cells (summarized in Table II). Transfection and expression of Cx43 (130–136), Cx32 (137–139), Cx26 (131,140) and Cx40 (131) in various cells resulted in increased communication. However, only some connexins caused a slower growth *in vitro* and *in vivo* which correlated with the level of expression. The unique presence of certain connexins in different cell types suggests functional cell specificity for connexins. This concept is supported by the finding that chemically transformed mouse fibroblasts (132), rat glioma cells (135) and human rhabdomyosarcoma cells (141), which are deficient in Cx43 expression in comparison to their nontransformed counterparts, exhibit reduced growth and tumorigenicity following transfection of Cx43 cDNA. A similiar observation was seen following transfection of human hepatoma cells with Cx32, which is normally present in hepatocytes (134), and following Cx26 introduction into HeLa cells which were derived from cervical cells which normally express Cx 26 *in vivo* (131). From these observations, it is clear that the establishment of GJIC in tumor cells does not always lead to effects on cell growth. There appears to be selective effects on cell growth mediated by specific connexins.

As an alternative approach to examine the role of GJIC in cell growth and differentiation, several investigators have sought to interfere with endogenous connexin expression with antibodies in nontransformed cells (142,143). However, in such studies, an estimation of effects on subsequent growth of injected cells is not feasible. More interestingly, Goldberg *et al.* (144) transfected Rat-1 fibroblasts with a Cx43 antisense vector, significantly reducing the expression of functional gap junction channels. While there was no effect on cell growth or saturation density, these cells had reduced ability to inhibit formation of foci of $pp60^{v\text{-}src}$ transformed fibroblasts in a coculture assay. This provides strong support for the function of heterologous GJIC between normal and transformed cells (see Section II.1 above).

Given the accumulation of evidence supporting a role for GJIC in growth control and differentiation, one might expect to see evidence of transformation *in vivo* in transgenic mice with null mutations of connexin genes. Targeted Cx43 gene knockout through homologous recombination resulted in lethality at birth, due to cardiac malformations (145). Preliminary studies have also been carried out with Cx26 and Cx32. Unfortunately, Cx26 knockout mice die at 10–11 days *in utero*. In contrast, connexin32 knockout mice show no apparent phenotype (146). These transgenic mice provide interesting models in which to test the transformability of cells in tissues with reduced or absent GJIC.

III.2 Possible mechanisms of negative growth control by connexins

The earliest hypothesis concerning the role of GJIC in growth control suggested that gap junctions provide a conduit between cells for the dispersion of a diffusible intracellular factor (i.e. negative growth regulator) to control cell growth (64). Furthermore, it has been suggested that this factor passes through heterologous gap junctions formed between normal and transformed cells, imposing growth restrictions on the latter (147,148). The nature of this transferable growth inhibitor remains to be determined.

If GJIC mediates effects on cell growth, it might be expected that there would be changes associated with the cell cycle. The clearest demonstration of an association of modulation of GJIC with the cell cycle occurs following partial hepatectomy, where loss of GJIC and decreased expression of Cx32 and Cx26 occurs during nearly synchronous mitotic activity (149,150). Furthermore, this decreased expression of connexins was shown to be due to alterations in mRNA stability (151). GJIC has also been shown to be reduced between mitotic and nonmitotic immortalized rat granulosa cells (152), as well as between cells in developing Xenopus embryos (153). On the other hand, GJIC has been shown to occur between mitotic and interphase fibroblasts in culture (154), and the level of connexin transcripts has been shown to remain unchanged or even to be increased during S-phase (155). Thus the role of GJIC during the cell cycle remains unclear.

More recently, Chen *et al.* (156) reported changes in the expression of genes involved in regulating the cell cycle, namely decrease in cylins A, D1 and D2 and cyclin-dependent kinases CDK5 and CDK6, in transformed cells transfected with Cx43. These changes were accompanied by density-dependent inhibition of proliferation and prolongation of the G_1 and S phases of the cell cycle. These findings support the notion that GJIC can affect gene expression.

As a possible mechanism of connexin-induced tumor suppression, we have evidence for a diffusible extracellular factor which correlates with the degree of GJIC among Cx43 transfectants. We tested glioma cells transfected with Cx43 for their ability to form gap junctions with nontransfected glioma cells and thus their ability to regulate tumor cell growth through heterologous coupling (157). Glioma cells did form gap junctions with cocultured transfected cells, and a decrease in proliferation was noted. However, transfected cells also appeared to secrete some growth inhibitory factor(s), since conditioned medium alone could also decrease glioma cell growth. Alterations in expression of IGF and IGF-binding proteins following transfection of C6 glioma cells with Cx43 further supports the notion that GJIC can modulate cell growth via alterations in gene expression (158).

IV. Mutations of connexin genes in cancer and other human diseases

Certain connexin genes suppress malignant cell growth after their transfection as described above. If connexin genes are indeed tumor-suppressor genes, it is expected that they are deleted and/or mutated in certain cancers, similar to many other known tumor-suppressor genes. There is increasing evidence that connexin gene mutations are not only involved in carcinogenesis, but also in other human diseases.

IV.1 Connexin gene mutations in primary tumors and cell lines

The first attempts to examine possible connexin gene mutations in primary tumors focused on the Cx32 gene. The analysis of 20 human liver and 22 human gastric tumor samples, showed no Cx32 gene mutations in its coding sequence (69,159). On the other hand, we found one mutation in the Cx32 gene among 13 rat liver tumors induced by a nitrosamine (160). The mutation was a G to A transition at codon 220 and changed the amino acid from Arg to His. Immunohistochemical analysis of this sample showed perinuclear concentrations of Cx32 protein, suggesting that it was not contributing to functional GJIC. However, when we cloned and transfected the mutant Cx32 gene into HeLa cells, which do not express any known connexin gene and thus are communication-deficient, there was good recovery of GJIC. Therefore, this mutation may be either a polymorphism at the level of amino acid sequence, or it manifests only in response to some modulating factors. These results are indicative that Cx32 gene mutations are rare events in carcinogenesis. This is not very surprising given the location of the Cx32 gene on the X-chromosome, which does not often contain LOH (loss of heterozygosity) or deletions in cancers.

More recently, Cx37 gene mutations have been reported in the murine Lewis carcinoma cell line (161). The mutations were identified at the amino acid sequence level and thus no transfection experiment has been carried out. We have recently found that there is a polymorphism in this gene in the sequence coding for the cytoplasmic loop of Cx37. Furthermore, our preliminary results suggest mutations of this gene in some hepatic angiosarcomas (unpublished observation). These results indicate that Cx37 may be mutated in some tumors and play a role in carcinogenesis.

IV.2 Connexin gene mutations in other human diseases

Since GJIC is considered to play a key homeostatic role in multicellular organisms, connexin gene defects may manifest various pathological phenotypes besides cancer. Germ line mutations of the Cx32 gene have indeed been identified in families of X-linked Charcot-Marie-Tooth disease (CMTX). So far, over 35 different mutations have been reported including cytoplasmic, extracellular and transmembrane domains (162–166). It is interesting to note that the carriers of these mutant genes generally do not show visible malformations but manifest their neuropathological defects only in adulthood, suggesting that malfunction of the Cx32 gene has no effect on normal development. Since the Cx32 gene is on the X-chromosome, men with a mutation are essentially Cx32-null; in fact, some mutations involve the deletion of most parts of Cx32, resulting in the production of a truncated protein. Therefore, it appears that the Cx32 gene is dispensable for most physiological functions except for normal myelination of peripheral nerves. This is compatible with the fact that Cx32-null transgenic mice display no apparent phenotype (146).

Contrary to that of Cx32 gene, germ line mutations of the Cx43 gene have been reported to result in heart malformation in humans (167). Similarly, Cx43-null mice die from heart malformation (145). In humans, Cx43 gene mutations at serine residues of the C-terminal have been found in the patients with visceroatrial heterotaxia (167). Since these serine residues are within the motifs of several kinases, their mutations would probably result in aberrant phosphorylation and disturbance of Cx43 functions. The Cx43-null mice survived to term but died shortly after birth, probably due to obstruction of the pulmonary artery which caused neonatal cyanosis (145). These results from visceroatrial heterotaxia and Cx43-null mice suggest that Cx43 is dispensable for synchronous beating of myocardiac cells but is rather essential for heart architecture. They also indicate that the Cx43 gene which is expressed in a number of tissues may not be essential for the development of organs other than heart.

These results suggest that connexin gene mutation is one mechanism to down-regulate GJIC, which in turn, may be induced by mutagenic carcinogens and contribute to carcinogenic process.

IV.3 Dominant negative effects of mutated connexins

Connexin proteins form hexamers called connexons, which are transferred and inserted into the cell membrane to form gap junctions. Mutant connexins which do not form functional GJIC can form oligomers with wild type connexins. If these resultant heteromeric connexons fail to function as a gap junction, they can be considered to inhibit the wild-type's function in a dominant negative manner. This possibility has been examined in both Xenopus oocytes (59,164) and mammalian cells (168) since mutant and wild-type connexin proteins would co-exist *in vivo* when one of two alleles of connexin genes is mutated.

Mutants studied were those of the Cx32 gene found in CMTX. In Xenopus oocytes, two missense mutations (Arg 142 Trp and Clu 186 Lys) and a frameshift at position 175 resulting in a premature stop codon, completely abolished GJIC forming ability (164). Similarly, three other missense mutations (Cys 60 Phe, Val 139 Met and Arg 215 Trp) could not restore HeLa cell GJIC (168). On the other hand, deletion of the C-terminal did not affect the formation of GJIC in Xenopus oocytes (169) and in HeLa cells (168). These results suggest that the C-terminal is not essential for intrinsic ability of connexins to form functional gap junction channels, but rather affect GJIC ability in response to endogenous or exogenous factors.

When those missense Cx32 mutant constructs which failed to form functional gap junctions were injected into Xenopus oocytes pairs which had been communicating with Cx26, their communication was abolished (164), suggesting dominant-negative effect of mutants. Similarly, when HeLa cells communicating with transfected wild-type Cx32 gene were transfected with Cys 60 Phe, Val 139 Met or Arg 215 Trp mutant genes, their GJIC was inhibited (168). It can be hypothesized that the dominant negative effect of mutant Cx32 genes on GJIC is due to the formation of heteromeric non-functional connexons composed of wild-type and mutant connexin subunits. It is possible that the resultant connexons are not able to function, since they cannot be properly inserted into the membrane. In fact, when the three base substitution mutant Cx32 genes were singly transfected into HeLa cells, their proteins were not detected in the plasma membrane. By immunohistochemical analysis, we saw strong staining for Cx32 proteins in HeLa cells transfected with the wild-type Cx32 gene. However, in cells doubly transfected with Val 139 Met mutation, much less Cx32 protein was detectable at the plasma membrane, suggesting that mutant and wild-type connexins indeed formed heterotypic connexons which were not properly inserted into the membrane (168).

The dominant negative effect of connexin gene mutations could be achieved very efficiently and therefore contribute significantly to the down-regulation of GJIC. When equal numbers of wild-type and mutant connexin molecules are present, the probability of the formation of a connexon composed of exclusively wild type or mutant connexin is as low as one in 2^6. Thus, it is possible to reduce the number of intact homomeric connexons by 98.5%, if one allele of the Cx32 gene is mutated in a given cell. Thus, a point mutation on one allele of the Cx32 gene may have a much more dramatic effect on GJIC than other changes, such as complete deletion of one allele. This is supported by our data, which show that mutant Arg 215 Trp reduced wild-type-Cx-mediated GJIC capacity significantly in HeLa cells, in spite of its much lower level of expression than the wild type. In mammals, such a dominant negative effect of point mutations will be more pronounced with connexin genes other than Cx32 gene; since the Cx32 gene is on the X-chromosome, no such effect will be seen in males and one of the two alleles is silent in females. In most cell types, several species of connexin genes are expressed. It has recently been reported that two different types of connexins can form heterotypic connexons in insects (58). Taken together with the finding of Bruzzone *et al.* (164), that several mutations of Cx32 produce a dominant negative effect on wild-type Cx26, the dominant negative effect of mutant Cx32 or any other connexin may have much more significant consequences.

V. Role and application of connexins or GJIC in cancer chemoprevention and therapy

GJIC is being exploited in two separate applied cancer research activities, i.e. as an endpoint for identification of chemopreventive agents and its use to enhance cancer therapy.

V.1 Detection of cancer chemopreventive agents with GJIC enhancement as an endpoint

Since the disruption of GJIC is considered to play an important role in carcinogenesis and since restoration of normal GJIC in tumors appears to work as a tumor suppressive element, it became a good candidate endpoint to look for chemopreventive agents. In the past, those agents which antagonize the tumor-promoting effect of TPA have been examined. Retinoids, glucocorticoids and cAMP, for example, are known to exert such anti-tumor-promoting activity and they have been shown to up-regulate GJIC (102). In BALB/c 3T3 cells, these agents up-regulated GJIC and suppressed transformation induced by carcinogens (99). The effect of retinoids on GJIC is studied in depth and they appear to up-regulate the expression of Cx43 at the transcriptional level (170). However, Bex *et al.* (171) reported that retinoic acid enhances Cx43 expression at the post-transcriptional level in rat liver epithelial cells. In the case of cAMP, it appears that both transcriptional as well as post-transcriptional control mechanisms are involved (172,173); in fact, Cx43 protein has a phosphorylation motif for cAMP-dependent kinase.

More recently, some food- and drink-related chemicals or mixtures have been studied for their potential chemopreventive activity. Green tea (*Canellia sinensis*) and other teas have been reported to inhibit tumor promotion *in vivo* and *in vitro* (174), and subsequently Sigler and Ruch (175) have shown that its aqueous extract and several constituents antagonized the inhibitory effect of DDT on GJIC of cultured rat liver epithelial cells. Takahashi *et al.* (176) examined the effect of a Japanese traditional soybean-fermented food, Natto. After extraction and fractionation of Natto, they observed that a fraction showed antagonistic effect on TPA-mediated inhibition of GJIC in BALB/c 3T3 cells. They further have shown that the straight-chain saturated hydrocarbon, hentriacontane, may be an active principle, since it antagonized the TPA effect at a concentration as low as 0.65 ng/ml. Chaumontet *et al.* (177) have shown that two flavones, apigenin and tongeretin, enhance GJIC of cultured rat liver epithelial cells.

Further studies on this line will determine whether GJIC enhancement is a good marker for cancer chemopreventive activity of chemicals. At least, there is enough scientific information to stimulate this line of research.

V.2 Use of GJIC for enhancement of efficacy in cancer therapy

Since GJIC is a means to transfer water-soluble molecules directly from one cell to another without passing through plasma membranes, it can serve as a local transporter of molecules. This may be exploited to deliver cancer therapeutic agents only to cancer cells without spreading them to normal cells. The killing of neighboring cells through GJIC has been demonstrated almost 25 years ago (178). When $HPRT^-$ cells are cultured in the presence of thioguanine, they die only if they are in contact with $HPRT^+$ wild-type cells. Thioguanine-derived nucleotides are presumably transferred from $HPRT^+$ to $HPRT^-$ cells. Such a metabolic cooperation effect was called 'kiss of death' (178).

The idea of using such a phenomenon for an effective cancer cell killing was first developed by Yamasaki and Katoh (179) when they observed that microinjection of Lucifer Yellow followed by blue-light exposure produced bystander cell killing. In this way, transformed rat liver epithelial cells and BALB/c 3T3 cells, which did not commmunicate with their normal counterparts, could be selectively eliminated by Lucifer Yellow microinjection: microinjection into a few cells accomplished the killing of many cells, suggesting that the bystander effect seen may be mediated by gap junctions.

More recently, possible use of GJIC in cancer therapy gained a renewed interest owing to the evidence that gap junctions may play an important role in enhancing gene therapy of cancer. In gene therapy to treat cancer, typically only a fraction

of the tumor cells can be successfully transfected with a gene. However, in the case of brain tumor therapy with the thymidine kinase gene from Herpes simplex virus (HSV-tk), not only the cells transfected with the gene, but also the neighboring others can be killed in the presence of ganciclovir. Such a 'bystander' effect has been proposed to occur due to the metabolic activation of ganciclovir by HSV-tk, which can be transferred to neighboring non-gene carrier cells through GJIC (180).

Recently, Mesnil *et al.* (181) have shown from *in vitro* studies that connexin genes are indeed responsible for such a bystander cancer cell killing. Thus, when GJIC-deficient HeLa cells were transfected with HSV-tk gene and cocultured with non-transfected cells, only HSV-tk transfected HeLa cells (tk^+) were killed by ganciclovir. However, when HeLa cells transfected with a gene encoding for the gap junction protein, Cx43, were used, not only tk^+, but also tk^- cells were killed, presumably owing to the transfer, via Cx43-mediated GJIC, of toxic ganciclovir molecules phosphorylated by HSV-tk to the tk^- cells. Such a bystander effect was not observed when tk^+ and tk^- cells were cocultured without direct cell–cell contact between those two types of cells (181). Similar conclusions have also been obtained by Fick *et al.* (182), who used the combination of HSV-tk^+ murine fibroblasts, and various rodent and human cell lines which show different degrees of GJIC ability. Thus, these results give strong evidence that the bystander effect seen in HSV-tk gene therapy may be due to connexin-mediated GJIC.

Many tumor cells have little or no detectable GJIC. Since connexin genes are likely to be tumor-suppressor genes, it is possible that the transfection of connexin genes themselves may prove to be an efficient cancer therapy, providing dual effects, bystander killing and cell growth control. Possible combination of connexin genes and other genes, such as HSV-tk gene, also needs to be exploited.

VI. Perspectives and hypotheses

During the past 10 years, since the first cloning of connexin cDNAs, we have seen a rapid progress in the field of GJIC. However, many more unanswered questions have also emerged. While most scientists believe that connexins are the major functional component of gap junctions, Finbow and his colleagues consistently proposed that the 16 K protein 'ductin' is the major component of gap junctions (183). Ductin is considered to be multifunctional; it is a component of the vacuolar H^+-ATPase and at the same time, a component of gap junctions. It is fair to say that most available data on connexins are consistent with the idea that they are the functional component of gap junctions. However, it is also fair to say that there is no convincing evidence against a role of ductin in GJIC. Recent studies started to reveal an interesting association between ductin, GJIC and cell growth control. Goldstein *et al.* (184) reported that HPV E5 oncoprotein specifically binds to ductin and such an interaction was proposed to be a mechanism of HPV-mediated cell transformation. On the other hand, Oelze *et al.* (92) reported that the E5 protein down-regulates GJIC in the human keratinocyte cell line HaCaT. Moreover, it has been reported that the ductin mutants transform NIH3T3 cells (185). These results are consistent with the idea that the ductin is associated with GJIC and cell transformation. Further studies will help us understand if there is a relationship between ductin and connexins.

Why does a single cell express multiple species of connexin genes? Biological redundancy or mutiple utility? From recent observations that heteromeric connexons (composed of, for example, Cx26 and Cx32) can exist in a given cell (58), we can propose that the presence of multiple connexin species may be important for cell growth control. Given that different connexin species form connexons with different GJIC abilities (23), it is reasonable to assume that GJIC mediated by heteromeric (chimeric) connexons show even more divergent characteristics. Accordingly, cell growth control may be affected based on the composition of connexin molecules in gap junctions. For example, Cx26 and Cx32 expression in hepatocytes may be differentially regulated and thus connexin compositions in connexons may also vary in response to endogenous and exogenous factors. Based on the composition of connexons in hepatocytes, their GJIC is influenced and thus, their growth is regulated. This is a testable hypothesis and answers may become available soon.

As for many other genes, attempts in deleting specific connexin genes in rodents and studying their physiological roles are yielding some interesting results (see above). However, this approach has its own severe limitations. Since most connexin gene species are critically involved in development and in fundamental physiology of multicellular organisms, some of these 'knock-out' mice (i.e. Cx43, Cx26) are not viable and thus are not available for long-term studies such as examining their susceptibility to carcinogenesis. It would thus be important to create such 'knock-out' mice which are viable. One approach available now is to create tissue-specific connexin 'knock-out' rodents, utilizing dominant-negative effects of mutant connexins. For example, if a Cx32 mutant gene driven by the albumin gene promoter is expressed in the liver, in mice, the mutant Cx32 would delete the function of endogenous Cx32 by the dominant-negative mechanism. Since this would theoretically occur only in the liver, due to the liver-specific albumin promoter, these mice should be viable and could be used to study the role of Cx32 in liver carcinogenesis; such mice are currently being created (Mesnil *et al.*, unpublished).

Both intra- and intercellular growth control mechanisms would finally be reflected in effects on the cell cycle. In spite of unprecedented speed of progress in identification and characterization of molecules involved in cell cyle control mechanisms, very few studies have linked connexin genes and cell cycle genes. In such studies, it is important to design experiments to address whether connexin genes causally alter the cell cycle. Most results so far can be interpreted that cell cycle changes are causes of changes in GJIC or connexin gene expression. Cell culture studies with those cells lacking expression of specific connexin genes (i.e. knock-outs) may be a good tool for such studies. It will be of importance to see whether connexin genes are also involved in cell cycle checkpoint regulation in response to exogenous factors including carcinogens.

Connexin molecules are considered to be a major player of homeostasis in multicellular organisms and thus, their defects are seen in several human diseases besides cancer. While certain malfunctions of the nervous system and heart have so far been reported to be due to mutated connexins (see above), it is likely that some other diseases will be associated with aberrant GJIC and thus with connexin defects. With respect to cell growth control, more attention is being paid to mechanisms involved in intracellular signal transduction systems, such as the role of p53 and $p21^{WAF1}$. As discussed in this article, intercellular control of cell growth, especially that mediated

by connexins and cell adhesion molecules, appears to play a critical role in carcinogenesis. In the future, cross talk between intercellular and intracellular growth control mechanisms needs to be studied further.

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