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Aberrant Expression and Function of Gap Junctions during Carcinogenesis

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Gap junctional intercellular communication plays a key role in the maintenance of homeostasis in multicellular organisms. Reflecting deranged homeostasis in cancer cells, most transformed or cancerous cells show aberrant gap junctional intercellular communication; they have decreased junctional communication between each other and/or with surrounding normal cells. Studies with *in vitro* cell transformation and animal carcinogenesis models suggest an involvement of blocked intercellular communication in later stages of carcinogenesis. Analysis of expression of gap junction proteins (connexins) and corresponding mRNA indicates that a number of regulation sites are involved in aberrant function of gap junctions during carcinogenesis. Suppression of transformed phenotypes is often seen when transformed cells are physically in contact with their normal counterparts. Some studies suggest that gap junctional intercellular communication is involved in such tumor suppression.

Introduction

The major role of gap junctional intercellular communication (GJIC) is considered to be the maintenance of homeostasis in multicellular organisms. Gap junctions mediate the transfer of factors of molecular weight less than 1000 from within one cell into adjacent cells. It is believed that through GJIC the level of second messages which are important for growth control is harmonized among cells in a given tissue and that the homeostasis of a tissue can be maintained (*1,2*). Aberrant growth control *vis-à-vis* surrounding normal cells is an essential feature of cancer cells. Therefore, it has long been considered that altered GJIC might play an essential role in carcinogenesis (*2–4*).

Recent studies on the structure of gap junction proteins have begun to reveal the fine structure and membrane topology of gap junctions. As depicted in Figure 1, a gap junction consists of two connexons, each coming from one cell. Each connexon consists of six subunits called connexins. The connexin membrane topology is also shown in Figure 1, and it is believed that the chain traverses the membrane four times with the C-terminal and N-terminal in cytoplasmic areas and two loops in the extracellular space (*5–7*).

Although the schematic view presented in Figure 1 represents common features of connexins, there are several different proteins that are expressed in different tissues. For example, connexin 32 codes for the connexin molecule with a molecular weight of 32 kDa; it was originally isolated from the liver and is also expressed in kidney and stomach cells but not in, for example, heart cells (*8–10*). On the other hand, connexin 43, which was isolated from heart cells (*11*), is not expressed in hepatocytes; however, it is expressed in biliary epithelial cells and various fibroblasts. In addition to these two connexins, connexin 26 has recently been isolated as a second connexin of the liver (*7*). While the structure of these connexins appears to be similar, their regulation may be different. For example, connexin 26 has a shorter carboxy terminus, and therefore phosphorylation sites for cAMP-dependent protein kinase are absent in connexin 26 (*7*). Antibodies and cDNA for these three different connexins have been prepared (*7–13*) and much used in studies of the mechanisms of function of gap junctions. While it has been reported that connexin 43 and 32 can together form functional gap junctions in Xenopus oocytes (*14*), it is still not known whether such heterologous combination of connexins can occur in somatic cells.

GJIC can be modulated at various levels (*15*). In addition to the usual mechanisms for modulation of protein synthesis, it is thought that GJIC can be modulated after the formation of functional gap junctions. Under certain circumstances, it is believed that the hole in a gap junction can be closed by twisting six connexin subunits (*16*). Recent studies also suggest that cell-to-cell recognition may be a prerequisite for two adjacent cells to form gap junctions. The cell-cell recognition molecules include extracellular matrix and cell adhesion molecules (*15*). The existence of different points of regulation of GJIC is reflected by the fact that cancer cells have GJIC alterations at different levels and that such

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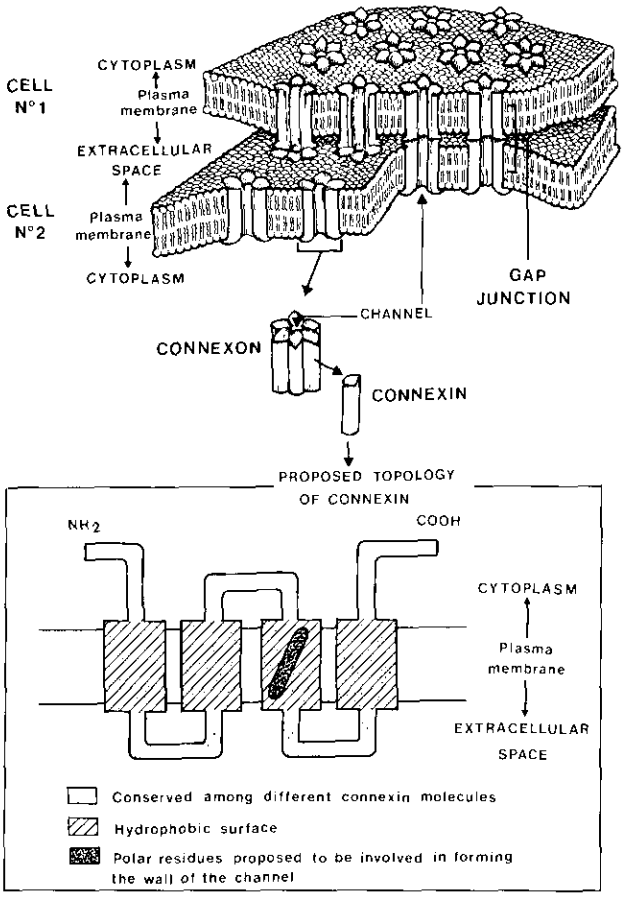

FIGURE 1. Schematic view of gap junctions in membrane lipid bilayers and topology of connexins. While the structure and topology are generally considered common to various connexin molecules, there are important differences between them which may be related to the regulation of their function (see text for details).

alterations occur during carcinogenesis and in various cancer cells, as discussed below.

Cancer Cells Have Altered Expression or Function of Gap Junctions

The most convincing evidence for the involvement of aberrant GJIC during carcinogenesis has come from the fact that most, if not all, cancer cells have aberrant GJIC. The loss of GJIC in cancer cells was first demonstrated by the group of Loewenstein and Kanno (*17–19*). Many ensuing studies have confirmed that various cancer cells have lost or decreased GJIC capacity (*20–24*). However, we have recently shown that not all tumorigenic or transformed cells have decreased GJIC. For example, BALB/c 3T3 cells transformed by various carcinogens maintained their GJIC at levels similar to that of nontransformed counterparts (*25–27*). However, these transformed cells did not communicate with surrounding normal cells (*25–27*). Similar selective lack of GJIC was observed with tumorigenic and nontumorigenic rat liver epithelial cells (*28*). From these results and others, we postulated that, for cells to become cancerous, they may need to lose GJIC with their surrounding normal cells rather than losing their homologous GJIC (*29*). Such a loss of heterologous intercellular communication can occur by one of the following two ways. Either *a*) the tumor cells may lose the means of homologous communication between each other, in which case it is natural to consider that they could also not communicate heterologously with normal cells, or *b*) normal cells and tumorigenic cells communicate among themselves but not heterologously. These two hypotheses are represented schematically in Figure 2.

While most evidence for aberrant GJIC has come from cell culture studies, it is now possible to analyze tumor cells taken directly from animals or humans using molecular probes. For example, it has recently been shown that rat liver hepatocellular carcinoma, as well as preneoplastic nodules, show a drastic decrease in connexin gene and protein expression (*12,30,31*). On the other hand, analysis of six human hepatocellular carcinomas surgically removed from patients revealed no decrease in levels of the mRNA for connexin 32 (the connexin expressed in hepatocytes), but there was increased expression of connexin 43 (*32*); connexin 43 is not usually expressed in hepatocytes in humans. While it is not clear whether such aberrant expression of connexin 43 in hepatocellular carcinoma contributes to aberrant function, such an aberrant expression of connexins may be associated with the malignant growth of hepatocellular carcinomas, since all tested hepatocellular carcinomas

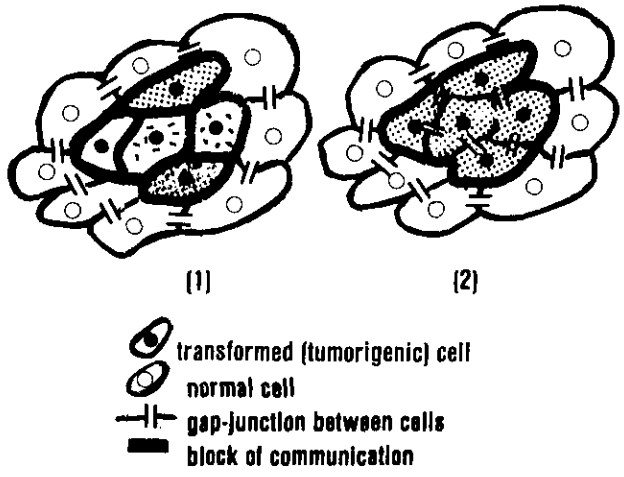

FIGURE 2. Selective intercellular communication and maintenance of transformed phenotypes—schematic view. Scheme (1) shows a tumor in which individual cells cannot intercommunicate and thus cannot communicate with the surrounding normal cells. In scheme (2), the cells in the tumor intercommunicate among themselves but not with surrounding cells. In neither case is there communication between the tumor and the surrounding normal cells.

have shown the increased expression of this particular connexin (*32*).

Involvement of Aberrant Gap Junctional Intercellular Communication in Carcinogenesis

Although ample evidence suggests that GJIC is aberrant in cancer cells, it is not clear when such an alteration occurs during the process of carcinogenesis. This question was first asked by the groups of Trosko and Murray; they found that the tumor-promoting phorbol esters can reversibly inhibit GJIC between cultured cells (*33,34*). This finding suggested strongly that the block of intercellular communication plays an important role in the tumor-promotion stage of carcinogenesis. The hypothesis is that, at the initiation phase, there is a genetic alteration that creates a dormant, initiated cell. Such an initiated cell may be dormant because surrounding normal cells can suppress the expression or expansion of the initiated cells through control by GJIC. A block of GJIC by tumor-promoting agents liberates the initiated cells from this growth control so that they expand clonally. It has subsequently become apparent that many, though not all, tumor-promoting agents can inhibit GJIC. This has been found using different methods for measurement of intercellular communication and different types of cells. These findings are summarized in Table 1.

Table 1. Inhibition of gap-junctional intercellular communication by tumor-promoting stimuli.

Method of communication measurement[a]	Examples of promoting stimulus
Metabolic cooperation	
[^{3}H]uridine metabolites transfer	Phorbol esters, chlordane
HGPRT$^+$/HGPRT$^-$	Phorbol esters and many other tumor-promoting agents
ASS$^-$/ASL$^-$	Phorbol esters, DDT
AK$^+$/AK$^-$	Phorbol esters
Electrical coupling	Phorbol esters Skin wounding
Dye transfer	
Microinjection	Phorbol esters, cigarette smoke condensate, PCB, diacylglycerol, and certain other tumor-promoting agents Partial hepatectomy
Photobleaching	TPA, dieldrin, PBB
Scrape loading	TPA, dieldrin, and other tumor-promoting agents
Gap junction structure analysis	
Electron microscope	Phorbol esters, mezerein Phenobarbital, DDT
Gel electrophoresis analysis	Phorbol esters
Analysis with gap junction antibody	Partial hepatectomy
Gap junction gene expression	
Connexin 32 mRNA level	Phenobarbital Partial hepatectomy

[a]HGPRT, hypoxanthine guanine phosphoribosyltransferase; ASS$^-$, argininosuccinate synthetase-deficient; ASL$^-$, argininosuccinate lyase-deficient; AK, adenosine kinase.

Although the molecular mechanisms by which tumor-promoting agents block GJIC are largely unknown, some information is available on how phorbol esters block communication. It has been suggested that phorbol-ester-mediated block of GJIC involves the activation of protein kinase C (*35,36*). While connexin molecules have phosphorylation sites for protein kinase C (*37*), it is not known whether phosphorylation of connexin by this enzyme is related to the inhibition of the function of connexin. We have recently shown that phorbol ester treatment of cultured cells does not decrease the level of connexin mRNA. The level of connexin mRNA was not changed during the inhibition and the downregulation period of 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) effects. On the other hand, there was a drastic decrease in gap junction structures after TPA treatment, as demonstrated with connexin-specific antibody staining. The gap junction structures revealed by connexin antibody reappeared when the TPA effect on gap junctional communication diminished. In other words, there was a close correlation between the loss of function of gap junctions and disappearance of immunostainable connexin molecules, suggesting posttranslational regulation of gap junctions. These findings are consistent with our previous results which suggested that regulation of gap junctional communication by phorbol esters is a posttranslational event (*38*).

Using a BALB/c 3T3 cell transformation system, we have provided several lines of evidence that suggest that decreased GJIC is indeed related to the later phase of cell transformation. For example, *a*) enhancement of cell transformation mediated by phorbol esters or diacylglycerol is related to inhibition of GJIC in these cell lines (*39,40*); *b*) antitumor-promoting agents such as retinoic acid, cAMP, and glucocorticoids can antagonize phorbol-ester-mediated inhibition of intercellular communication and can inhibit cell transformation in these cell lines (*41,42*); *c*) a BALB/c 3T3 cell variant that is susceptible to chemicals or UV light-mediated cell transformation lost GJIC when the cells reached culture confluence, while other cell lines did not, suggesting that the loss of GJIC in the cell transformation-susceptible cell line is related to its high transformation frequency (*43*).

On the other hand, there are also several lines of evidence that suggest that block of GJIC is not necessarily related to enhanced cell transformation. For example, transforming growth factor (TGF)-β, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCCD), and okadaic acid have been shown to enhance cell transformation of BALB/c 3T3 cells or C3H10T1/2 cells (*26,44,45*). These agents, however, did not block GJIC in the same target cells. While it is possible that these tumor-promoting agents block GJIC only locally, including initiated cells, it is more likely that they act by different mechanisms and that these agents enhance transformation by mechanisms not involving block of GJIC. Analysis of samples during tumor progression suggests that block of GJIC is also a later event in animal carcinogenesis. For ex-

ample, the analysis of cell lines established from different stages of mouse skin carcinogenesis revealed that there is a progressive decrease of GJIC as the stage of carcinogenesis advances (*24*). Similarly, analysis of gap junction proteins and mRNA levels during liver carcinogenesis has revealed the progressive decrease in their expression from normal to preneoplastic foci, preneoplastic nodules, and finally to hepatocellular carcinoma (*31*; unpublished results).

Gap Junctional Intercellular Communication and Oncogenes

Considering the importance of signal transduction within cells, it is conceivable that a membrane event such as GJIC and nuclear factors such as cellular oncogenes influence each other and that such an interaction may play an important role in carcinogenesis. Earlier studies on the relationship between oncogenes and GJIC examined whether oncogenes can modulate GJIC. Such studies have shown that certain viral oncogenes such as the v-*src* gene can indeed inhibit GJIC (*46–48*). Other oncogenes have also been shown to inhibit GJIC. However, some laboratories, including ours, have found no inhibitory effect of some of these oncogenes on gap junctional communication. These results are summarized in Table 2.

In collaboration with M. Bignami and F. Tato, we examined the relationship between v-*onc* expression, focus formation, and GJIC in NIH 3T3 cells (*49*). When cells containing v-*myc* or v-*fos* genes were co-cultured with normal cells, they communicated heterologously and did not form distinct foci on the monolayer of the normal cells. When a GJIC blocker, namely, a phorbol ester, was added to the co-culture, transformed foci appeared. On the other hand, *ras*- or *src*-containing cells did form distinct foci over a monolayer of normal cells and did not show heterologous communication with surrounding normal cells.

These results suggest that GJIC between normal cells and oncogene-containing cells can influence oncogene-mediated expression of transformed phenotypes. However, we could not determine whether GJIC was modulating oncogene expression or viral oncogenes were modulating GJIC. Since viral oncogenes by themselves did not modulate homologous communication in transfected cells, it is more likely that heterologous communication modulated oncogene expression.

Possible Role of Gap Junctional Intercellular Communication in Tumor Suppression

If aberrant GJIC plays an important role in carcinogenesis, it means, from the other side of the coin, that normal GJIC can act in a tumor-suppressive role. As summarized in Table 3, there are several lines of evidence that direct contact of transformed cells with normal cells can indeed suppress transformed phenotypes. Earlier studies did not relate this direct cell contact-mediated suppression of transformed phenotypes with GJIC *per se*. However, some recent studies have revealed a close correlation between tumor suppression and GJIC between transformed and normal cells. As described above, we have shown that transformed BALB/c 3T3 cells do not communicate with surrounding

Table 2. Effect of oncogenes on homologous and heterologous gap junctional intercellular communication.

Oncogene	Cells	Homologous communication[a]	Heterologous communication[b]	Reference
v-*src*	NRK	↓	NT	(*46*)
	NIH 3T3	↓	NT	(*48*)
	Quail and chick embryo fibroblasts	↓	NT	(*47*)
	NIH 3T3	→	−	(*49*)
c-*src*	NIH 3T3	↓	NT	(*56*)
v-*ras*	NIH 3T3	→	−	(*49*)
EJ-*ras*^{H}	BALB/c 3T3	→	−	(*27*)
	Rat liver epithelia cell line IAR20	→	+	(*57*)
	Rat liver epithelia cell line	↓	NT	(*58*)
v-*myc*	NIH 3T3	→	+	(*49*)
v-*fos*	NIH 3T3	→	+	(unpublished data)
v-*mos*	C3H10T1/2	→ or ↑	NT	(*59*)
PyMT	Rat F cells	↓	NT	(*60*)
	NIH 3T3	→	−	(unpublished data)
PyLT	NIH 3T3	→	+	(unpublished data)
SV40T	Human hepatocytes	↓	NT	(unpublished data)
	Human keratinocytes	↓	NT	(unpublished data)
	Human fibroblasts	↓	↓	(*61*)

[a]Homologous communication is the communication among oncogene-containing cells, and their communication capacity was compared with that of normal counterparts. (↓) decreased; (→) no change; (↑) enhanced.

[b]Heterologous communication is the presence (+), absence (−), or decrease (↓) of communication between oncogene-containing cells and normal cells measured in co-culture of these two types of cells. NT, not tested.

Table 3. Suppression of transformed phenotypes by contact with normal counterparts.

Cell type	Evidence of GJIC[a] involved in the suppression	Reference
Polyoma virus-BHK21 cells	—[b]	(*62*)
SV40-Swiss 3T3	Rescue of transformed foci by croton oil	(*63*)
Chemically transformed C3H10T1/2	—	(*64*)
UV + TPA-transformed C3H10T1/2	Rescue of transformed foci by TPA	(*65*)
Tumorigenic rat tracheal epithelial cells (*in vivo* transplant)	—	(*66*)
Harvey sarcoma virus-transformed mouse epidermal cells (+ dermal fibroblasts *in vivo* grafting)	— —	(*67*)
Chemically transformed mouse epidermal cells	— —	(*68*) (*68*)
Chemically and virally transformed C3H10T1/2 cells	Dye transfer	(*50*)
Chemically transformed BALB/c 3T3 cells (with dbcAMP, retinoic acid, glucocorticoids)	Dye transfer	(*42*)
C-*myc*- or N-*myc*-transformed 3T3 cells	—	(*69*)
v-*myc*-transformed NIH 3T3 cells	—	(*70*)
v-*myc*-, v-*fos*-, PL-LT-transformed BALB/c 3T3 cells	Dye transfer	(*49*)

[a]This was obtained either by adding gap junctional intercellular communication (GJIC) blocking agents (croton oil or TPA) to rescue transformed foci or by direct measurement of GJIC between transformed and normal cells.
[b]No attempt was made to relate the suppression to GJIC.

normal cells, although the transformed cells communicate among themselves (*25–27*). However, when agents such as cAMP, retinoic acid, and glucocorticoids were added to culture dishes that contained already transformed foci, many transformed foci disappeared (*42*). There was also a resumption of GJIC between transformed cells and surrounding normal cells (*42*). Although the evidence is indirect, these results suggest that suppression of transformed phenotypes by surrounding normal cells may be due to resumption of GJIC. Similarly, Loewenstein's group has shown that in co-cultures of transformed and nontransformed C3H10T1/2 cells, transformed cell growth was suppressed when there was good heterologous GJIC between transformed and nontransformed cells (*50*).

Studies on genetic mechanisms of tumor suppression have revealed several genes as candidates for involvement in tumor suppression. Some of these candidate genes seem to be linked to cell-cell interaction. For example, a gene believed to have a tumor-suppressive role in relation to human colorectal cancers and located on chromosome 18 has recently been shown to have sequence homology to N-CAM, a cell adhesion molecule (*51*). Similarly, Saito et al. (*52*) have determined the structure of a putative tumor-suppressive molecule, phospho-tyrosine phosphatase, and suggested that the structure is very similar to that of N-CAM. Since cell adhesion molecules are thought to play an important role in the control of GJIC (*53*), it is possible that these putative tumor-suppressive genes may be involved in functional regulation of GJIC.

Conclusion and Future Perspectives

Available evidence suggests that aberrant GJIC is associated with the tumor promotion phase of carcinogenesis and with maintenance of transformed and/or tumorigenic phenotypes. There is also evidence that normal GJIC may act in a tumor-suppressive role.

Since molecular probes to study GJIC have only recently become available, there remains much scope for further research on the role of GJIC in carcinogenesis and in tumor suppression. One of the fundamental questions we have to ask is what kind of molecules are going through gap junctions and which of those molecules are important in the regulation of cell growth and differentiation. So far, calcium, cAMP, and inositol triphosphate have been shown to pass through gap junctions (*54,55*). It is believed that many other molecules are also exchanged between cells through gap junctions. Identification of such molecules is difficult. It is even more difficult to identify those molecules that pass through gap junctions and are also important in growth control, since these are likely to be second messengers in signal transduction and are probably present in low amounts.

The molecular mechanisms of regulation of GJIC are not yet known. However, it is important to emphasize the possible involvement of cell adhesion molecules. We found a selective intercellular communication between transformed and nontransformed BALB/c 3T3 cells. Since both transformed and nontransformed cells communicate among themselves, but not with each other, it appears that a cell-to-cell recognition mechanism is altered. Recently, we have introduced a gene for a cell adhesion molecule into cells that were otherwise incapable of gap junction communication and found that the transfected cells did express the cell adhesion molecule and started to communicate, suggesting the direct control of GJIC by cell adhesion molecules. This also implies that it is not necessary to directly alter GJIC itself in order to block communication; instead, blocking or al-

tering the cell-cell recognition mechanism may modulate GJIC. By this means, alteration of cell-cell recognition may be an important aspect involved during carcinogenesis.

As emphasized above, most available evidence for the involvement of GJIC in carcinogenesis is circumstantial. It is important now to investigate more directly the relationship between GJIC and carcinogenesis. Molecular probes such as expression vectors of gap junction proteins (connexins) and cell adhesion molecules have recently become available, which should make this possible. We expect to see rapid progress in our understanding both of fundamental aspects of GJIC regulation and of its implication in multistage carcinogenesis.

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