Disruption of Gap Junctions in Toxicity and Carcinogenicity

J. Kevin Chipman,1 Angela Mally, and Gareth O. Edwards

School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom

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Although the specific role of connexin-mediated gap junctional intercellular communication in the control of cell homeostasis, proliferation, and death are still not clear, several lines of evidence support these roles. The disturbance of this communication, through multiple mechanisms, may in the short term be a protective mechanism to limit the spread of toxicity in a tissue following chemical or radiation damage. However, sustained downregulation confers a loss of tumor-suppressive action. Consequently, connexin dysfunction has been associated with both the action of many carcinogens and being a feature of cancer per se. Connexins offer not only a target for cancer chemoprevention but also for exploitation in chemotherapy through the “bystander” effect.

Key Words: connexin, gap junction, carcinogenesis, bystander, intercellular communication.

Structure and Function of Gap Junctions

Gap junctions are intercellular membrane channels that allow the direct exchange of small molecules (<1.2 kD) between adjacent cells. They are comprised of two hemichannels (connexons), which are in turn formed by the oligomerization of six protein subunits, termed connexins (Cxs). To date, at least 21 members of this multigene family have been discovered and cloned (Willecke et al., 2002). Cxs consist of four highly conserved membrane-spanning domains, two extracellular loops, a cytoplasmic loop, and cytoplasmic N- and C-terminal regions. While the extracellular loops are thought to be required for connexon docking, the cytoplasmic domains appear to play a role in channel gating and intracellular signaling, and they vary between members of the Cx family. Small ions such as water, Ca2+, cAMP, and inositol triphosphate can pass through the gap junctions from one cell to another, while larger molecules such as proteins, complex lipids, and polysaccharides are retained within the cell (Saez et al., 1989). This process of exchange of molecules between neighboring cells via gap junctions is termed gap junctional intercellular communication (GJIC). A major physiological role of GJIC is the maintenance of tissue homeostasis and proliferation, the regulation of embryonic development and differentiation, and electric coupling of electrically excitable cells such as cardiomyocytes (Loewenstein, 1981). However, more recently, evidence for an intracellular signaling role of connexins in the control of cell proliferation has also emerged (see below).

Mechanisms of Regulation of GJIC

Gap junction-mediated intercellular communication can be regulated at several levels, including transcription, mRNA processing, protein synthesis, and post-translational modification, assembly, trafficking, connexon docking, and gating of the gap junction channel (see Fig. 1).

Several promoter response elements and transcription factors that are involved in the transcriptional regulation of connexins have been identified. For example, the promoter of the rat cx32 gene contains several GC-boxes, and Sp1, HNF-1, YY-1, and NF-1 binding sites have been recognized (Bai et al., 1995; Piechocki et al., 2000). In the human, rat and, mouse cx43 promoters, several sites, including AP1, cAMP and estrogen response elements have been identified (Chen et al., 1995a; Yu et al., 1994). The expression of cx genes can be controlled in a tissue-specific manner. For example, the liver cell-specific expression of cx32 has been shown to be dependent (at least in part) on expression of HNF-1 (Piechocki et al., 2000). Additional promoters have been identified that control the transcription of cx32 in neuronal tissue (Neuhaus et al., 1995). The methylation of CpG islands of promoter regions can lead to gene silencing and methylation of the Cx43 promoter has been reported in MH1C1 rat hepatoma cells, which express Cx32 but not Cx43, while the cx32 promoter was methylated in WB-F344 rat liver epithelial cells, which express Cx43 but not Cx32 (Piechocki et al., 1999).

It appears that connexin mRNA stability also plays an important role in controlling GJIC. Decreased Cx32 mRNA half-life has been observed in regenerating liver (Kren et al., 1993), while sequences located within the 3’ untranslated region of the Cx43 mRNA appear to stabilize this transcript (Lefebvre et al., 1995).
The activity of GJ is also regulated by post-translational modification of connexin proteins. Regulation can be by voltage, calcium and various kinases that act on the intracellular domains (Fig. 1; Holder et al., 1993). Phosphorylation of the C-terminal domain of the connexins (with the exception of Cx26) influences Cx trafficking, channel gating and turnover (Saez et al., 1998). Inhibition of communication through Cx43 hyperphosphorylation has been brought about both chemically (phorbol ester, via MAP kinase and possibly protein kinase C) and by epidermal growth factor through the action of MAP kinase (Rivedal and Opsahl, 2001). Hepatic Cx32 appears also to be inactivated by PKC activation (Elcock et al., 2000). Furthermore a number of oncogene products mediate inhibition of GJIC, such as through the tyrosine phosphorylation of Cx43 by v-src (reviewed by Yamasaki et al., 1999). However, there are multiple phosphorylation sites on different Cx molecules and the balance of the phosphorylation status determines function. It is predictable, therefore, that phosphorylation is mediated by various signal transduction pathways.

Another important mechanism involves the trafficking of connexins from intracellular pools to the plasma membrane. Following protein synthesis a number of the connexin polypeptides have been shown to be inserted into the membrane of the ER and subsequently the Golgi where they oligomerize to form hemichannels. Little is known about what initiates and mediates the transport of connexons to the plasma membrane and the formation of the complete gap junction channel, but cell–cell adhesion appears to be especially important. There is a requirement for E-cadherin expression for Cx43 transport to the cell membrane and for communication to be recovered in a mouse skin papilloma cell line (Hernandez-Blazquez et al., 2001). It is also known that Cxs can associate with other proteins in the membrane, specifically ZO-1 with Cx43 and Cx45 (Defamie et al., 2001; Kausalya et al., 2001). Clustering of gap junction channels into plaques is required for functional cell coupling (Bukauskas et al., 2000). Finally, several mechanisms have been implicated in GJ degradation, including lysosomal and proteasomal pathways.

It is therefore not surprising to find that there are multiple ways by which GJIC can be regulated and disrupted (Saez et al., 1993). Aliphatic alcohols (octanol, heptanol) and anesthetics such as halothane impair gap junction activity by interfering with membrane fluidity that leads to contraction of intercell channels. Other compounds have been reported to physically...
block GJs. Chemical gating is regulated by pH, calcium ions and free radicals (Saez et al., 1993). Thus, toxicant-induced changes in cellular calcium levels and redox state can lead to inhibition of GJIC at least in part through mechanisms that involve disturbance of phosphorylation status (Trosko et al., 1998).

**GJIC and Control of Cell Proliferation and Apoptosis: Multiple Signaling Roles**

GJIC has long been considered to play an important role in the regulation of cell growth and cell death. For example, in the course of liver regeneration following partial hepatectomy, GJ expression is temporarily decreased until liver mass and lobular conformation are restored. Similar effects on GJIC are seen during acute liver injury and tissue restoration after treatment with toxic chemicals such as thioacetamide (Kojima et al., 1994). Studies on liver regeneration after partial hepatectomy, where synchronous initiation of DNA synthesis was preceded by a decrease in the expression of Cx proteins, suggest that downregulation of GJs may be involved as a trigger for cell proliferation (Dermietzel et al., 1987). However, Temme et al. (2000) reported that Cx32-deficient mice showed normal regenerative response following two-thirds hepatectomy, although the extent of initiation and termination of DNA synthesis was slightly altered. Furthermore, hepatocytes completely devoid of gap junctional communication are able to enter the cell cycle upon mitogenic stimulation (Fladmark et al., 1997). Neveu et al. (1990) demonstrated that a decrease of Cx32 in livers of phenobarbital-treated rats was not associated with increased hepatocyte proliferation. Similarly, we have recently shown that downregulation of Cxs by a number of nongenotoxic carcinogens does not necessarily correlate with induction of cell proliferation (Mally and Chipman, 2002). Although a certain stage of mitosis has been associated with decreased levels of Cxs (Dermietzel et al., 1987), loss of GJIC per se does not appear to provide a signal for cells to divide but rather permits cell-cycle progression in the presence of mitotic stimuli (Trosko and Ruch 1998). It has been suggested that downregulation of GJs during mitosis may serve to maintain separate cellular pools of signaling molecules (Fladmark et al., 1997). This would ensure that during critical stages of cell division, proliferating cells remain isolated from potentially damaging signals mediated by neighboring cells.

Just how gap junctions regulate cell growth is poorly understood. Forced expression of Cx43 and Cx32 in neoplastic lung and liver cells restored G1 growth control and was associated with increased p27 (kip-1) and decreased cyclin D1 expression (Koffler et al., 2000). Similarly, overexpression of Cx43 in osteosarcoma cells inhibited cell cycle transition from G1 to S phase by increasing the expression of p27 (Zhang et al., 2001). Chen et al. (1995b) reported alterations in the expression of genes involved in cell cycle control after transfection of Cx43 into a transformed kidney cell line. However, it remains to be established whether the observed decrease in cyclin A, D1 and D2 and the cyclin dependent kinases CDK5 and CDK6 were a direct effect of Cx43 expression or a result of the accompanied density-dependent inhibition of proliferation and prolongation of the G1 and S phases of the cell cycle.

It is becoming increasingly apparent that gap junctions play an important role in intracellular signaling in relation to proliferation in addition to junctional functionality (Duflot-Dancer et al., 1997). Moorby and Patel (2001) reported opposing effects on cell growth in three clones transfected with different Cx43 mutants when grown under noncoupling conditions. In addition, they were able to show that the cytoplasmatic carboxyl domain of Cx43, which includes various phosphorylation sites and has no channel activity, is as effective as wild-type Cx43 in blocking cell growth. These studies suggest that Cxs may regulate cell growth independent of intercellular communication.

As mitosis and apoptosis share some common regulatory features (Kasten and Giordano, 1998), it is not surprising that GJIC appears to play a role in both processes. Wilson et al. (2000) reported relatively high levels of GJIC during early stages of apoptosis and mitosis, which then decreased as each process progressed. During both processes, activation of signal transduction pathways involving Cdc2/cyclin B kinase is an important event (King and Cidlowski, 1995; Pandey and Wang, 1995) and the phosphorylation of Cx43 in vitro is dependent on Cdc2 kinase (Lampe et al., 1998). However, the question remains whether phosphorylation of Cx43 influences apoptosis and mitosis or whether initiation of these pathways results in Cx phosphorylation and inhibition of GJIC.

Since several tumor promoters and oncogenes are known to inhibit both GJIC and apoptosis, while several antitumor promoters enhance GJIC and apoptosis, Trosko and Goodman (1994) hypothesized that a “death signal” may be mediated through gap junctions. The ability of nongenotoxic carcinogens such as the peroxisome proliferators and phorbol esters to disrupt GJIC and at the same time suppress apoptosis suggests a strong link between GJ and apoptosis in homeostatic control of solid tissue. However, although GJIC may be one of the mechanisms contributing to apoptosis signaling, there is little evidence to indicate that functional gap junctions are (generally) required for apoptosis. We have recently investigated the effects of a number of nongenotoxic carcinogens on gap junctions in relation to apoptosis in target tissues in the rat. In this study, the peroxisome proliferator Wy-14,643 decreased both the number of hepatocyte gap junction plaques containing Cx32 and the rate of hepatocyte apoptosis, while 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) appeared to downregulate Cx32 without effects on apoptosis. Most importantly, treatment with methapyrilene led to a dramatic loss of connexins and increased rate of apoptosis (Mally and Chipman, 2002). Similarly, propylthiouracil and low-iodine diet decreased GJIC and at the same time enhanced apoptosis in rat thyroid (Kolaja et al., 2000b). These data clearly show that apoptosis can occur in the absence of functional GJs, despite the fact that with...
certain other compounds a correlation between reduced GJ function, elevated mitogenesis, and reduced apoptosis exists (Kolaja et al., 2000a). It may be that functional GJIC is required for the initial signaling in apoptosis but that this communication is lost at a later stage. In summary, it appears that rather than suppressing growth or signaling a cell to undergo apoptosis, gap junctions may serve to maintain optimal rates of cell growth and cell death. Disruption of GJIC per se may not result in alterations of the rates of proliferation or apoptosis but require the presence of additional stimuli (Trosko and Ruch, 1998). Such a stimulus may include the activation of a cellular oncogene or inactivation of a tumor suppressor in “initiated” or preneoplastic cells (Watanabe and Tonosaki, 1995).

Downregulation of GJIC as an Adaptive, Protective Response to Toxicity

While GJIC can be important in tissue homeostasis and integrated tissue responses, it follows that GJs have the potential to propagate toxic responses also through the transfer of molecules such as superoxide and calcium ions, if elevated in the process of cell toxicity (e.g., in cerebral ischemia; Lin et al., 1998). It is likely, therefore, that the rapid, temporary, and reversible closure of GJ during cellular stress may be an important protective means for preventing the spread of cellular toxicity (Peracchia et al., 2000; Saez et al. 1989). Closure of gap junctions is thought to prevent the spread of apoptotic and inflammatory signals between cells, and furthermore, heart and brain infarction sizes are reduced when cells are uncoupled (reviewed in Brosnan et al., 2001). Studies have demonstrated that when cells are irradiated with 0.3–1.0 cGy doses of α-particles (Azzam et al., 2001; Zhou et al., 2000), stress responses (mutations, micronucleus formation, p53 phosphorylation, p21<sup>WAF1</sup> induction) are observed in more cells than were directly exposed. Upon incubation of cells with GJC blockers such as lindane, the number of responding cells was significantly reduced. However, when communicating cells were irradiated with soft x-rays at sublethal doses in the range of 1 to 5 Gy, GJIC was reduced in a dose-dependent manner (Edwards et al., 2002). Thus, depending on dose, the inhibition of GJIC can limit the spread of stress-inducing molecules through this very same system. This is of importance when considering the dose dependency of effects of exposure to ionizing radiation.

Sustained Aberrant Localization or Downregulation of Cxs Provides a Tumor-Promoting Stimulus

While deliberate, temporary closure of gap junctions serves to protect healthy cells by preventing the spread of toxic molecules and the loss of metabolically important substances, sustained inhibition of GJIC may contribute to carcinogenesis or conditions such as reproductive, neurologological, and cardiovascular dysfunction through loss of homeostatic control. Untimely inhibition of GJIC during critical stages of development may result in embryonic toxicity or teratogenesis. Indeed, many chemicals known to be tumor promoters, teratogens, or neurotoxins modulate GJIC (Elmore et al., 1987; Rosenkranz et al., 1988; Trosko et al., 2002).

There is substantial evidence to suggest that sustained down-regulation of GJIC provides a tumor-promoting stimulus. Normal, contact-inhibited cells exhibit functional GJIC, while transformed cells and many tumors are characterized by reduced expression of GJs involving aberrant localization or downregulation of Cxs and nonfunctional homologous or heterologous GJIC (Loewenstein and Rose, 1992; Trosko and Ruch, 1998; Yamasaki, 1990). Activation of some cellular oncogenes inhibits GJIC and induces malignant transformation (Jou et al., 1995; reviewed in Yamasaki et al., 1999), and tumor-promoting conditions such as wounding and necrosis are associated with decreased GJIC. Moreover, compelling evidence that links loss of GJ function to carcinogenesis has come from the observation that many nongenotoxic carcinogens and tumor-promoting agents inhibit GJIC. A range of nongenotoxic carcinogens has tested positive for inhibitory effect on GJIC in vitro. These include the pesticides DDT, DDE, lindane, hepta-chlor, and dieldrin, the peroxisome proliferators nafenopin, Wy-14,643, clofibrate, and di-2-ethylhexyl-phthalate, and several other classical tumor promoters in rodents, such as phe-nobarbitone, the phorbol ester 12-O-tetradecanoylphorbol 13-acetate, TCDD, and polychlorinated biphenyls (Swierenga and Yamasaki, 1992). Importantly, inhibition of GJIC by tumor-promoting agents has been demonstrated in several in vivo and ex vivo studies (Ito et al., 1998; Kolaja et al., 2000a; Kru-tovskikh et al., 1995; Mally and Chipman, 2002; Tateno et al., 1994). A very important aspect that has received insufficient attention is the need to distinguish between specific inhibition of GJIC and the inhibition that may result secondarily to, or coincidentally with, cell death. In the latter case, such as is seen with carbon tetrachloride in rat liver (Cowles et al., 2000), compensatory cell proliferation is likely to be a more important tumor-promoting influence than the inhibition of gap junctions. However, in many cases, the window between the doses required for GJ disruption and loss of cell viability is substantial both in vitro and in vivo, and the disruption has often been shown to be reversible (e.g., Elcock et al., 2000; Mally and Chipman, 2002; Rivedal and Opsahl, 2001; Swierenga and Yamasaki, 1992).

It is thought that chronic disruption of GJIC may release potentially initiated cells from growth constraints imposed by normal neighboring cells, resulting in clonal expansion and ultimately tumor formation (Fig. 2). Following withdrawal of the tumor-promoting stimulus, GJ function is restored and is associated with reduced size of chemically induced preneoplastic foci (Neveu et al., 1990). Reduced expression of GJs following treatment with nongenotoxic carcinogens appears to be a target organ-specific effect (Mally and Chipman, 2002; Mesnil et al., 1988). Furthermore, the ability of some compounds to modulate GJIC correlates well with sex- and species-
specific tumor incidence (Klaunig et al., 1989; Plante et al., 2002).

Many nongenotoxic carcinogens have been shown to cause gene hypermethylation, and this may be important in the reduced expression of certain tumor-suppressive genes (Sugimura and Ushijima, 2000). It is known that methylation is one important mechanism for the control of Cx expression (see above); and, in particular, the Cx26 gene promoter is found to be hypermethylated (notably at the SP-1 site) in certain breast cancer cell lines (Tan et al., 2002), and treatment with the demethylating agent 5-aza-2’-deoxycytidine resulted in re-expression of Cx26.

Connexin Genes and Tumor Suppression

Although cx gene mutations in tumors are rare, there is a wealth of evidence to suggest that Cxs function as tumor suppressors. As indicated above, the loss of GJIC is a common feature of tumors and appears to be important in the mechanism of action of many nongenotoxic carcinogens. Dominant negative mutants of cx32 and cx26 genes also caused loss of growth control, although this effect in some instances may be independent of GJIC per se (Duflot-Dancer et al., 1997). Numerous studies demonstrate that transfection of Cxs into communication deficient tumor cells can restore growth control in vitro and in vivo (Chen et al., 1995b; Eghbali et al., 1991; Mehta et al., 1991; Omori and Yamasaki 1999; Rae et al., 1998; Yano et al., 2001). Reduction of the malignant phenotype in some of these studies was associated with reduced saturation density, increased doubling times, reduced growth capacity in soft agar, and delayed onset of tumor formation in nude mice. Similarly, treatment of BALB/c 3T3 cells with Cx43 antisense oligonucleotides resulted in inhibition of GJIC and increased saturation density (Ruch et al., 1995). Dominant negative inhibition of GJIC by mutant Cx43 enhanced tumorigenicity of a rat bladder carcinoma cell line (Krutovskikh et al., 1998). In a coculture experiment Esinduy et al. (1995) demonstrated inhibition of growth of neoplastically transformed, communication-deficient cells by cocultured, highly communicating, nontransformed cells. Not only do these studies show potential for the use of connexins in gene therapy but the upregulation of GJIC by drugs or dietary factors may also provide chemoprevention (Trosko and Chang, 2001). Most important will be attention to the relative expression of different Cxs, the balance of Cx43:Cx32 being important in the GJIC of human malignant prostate epithelial cells (Carruba et al., 2002). A clear reduction of malignant potential of human hepatoma cells was indicated by an inhibition of dedifferentiation, reappearance of cell adhesion complex, and reduced proliferation coupled with enhanced apoptosis through transfection of Cx26 cDNA (Maramatsu et al., 2002; Yano and Yamasaki, 2001).

Additional evidence for the role of Cxs as tumor suppressors has emerged from studies on transgenic mice lacking Cx genes. Temme et al. (1997) reported increased susceptibility of Cx32 knockout mice for the development of spontaneous and chemically induced liver tumors. Interestingly, phenobarbitone treatment did not further increase the incidence of liver tumors in Cx32-null animals, supporting the role of Cx32 as a crucial target in tumor promotion (Moennikes et al., 2000). Connexin expression is also inversely correlated to metastatic potential. The loss of cooperation between cells when GJ are disrupted may lead to heterogeneity and cell dissociation, thus supporting metastasis (Carystinos et al., 2001). More specifically, Cx26 expression can reduce invasiveness of a human hepatoma cell line through E-cadherin expression and suppression of matrix metalloproteinase 9 (Yano and Yamasaki, 2001). It needs to be recognized, however, that different Cxs appear to have contrasting effects on metastagenicity (Carystinos et al., 2001).

Role of GJIC in Bystander Effect in Tumor Therapy

GJIC may also play an important role in cancer treatment in both radiotherapy and gene therapy. When radiotherapy is
carried out, the possibility arises that positive effects may be seen in unirradiated areas of organs that may involve bystander effects mediated both by GJIC as well as through the release of signaling compounds into medium (Mothersill and Seymour, 2001).

Furthermore, transfection of a small number of tumor cells with the herpes simplex virus thymidine kinase gene (HSV-TK) followed by treatment with the nucleotide analogue ganciclovir (GCV) leads to phosphorylation of the latter and incorporation into the cells’ DNA, thus terminating DNA polymerization and leading to cell death. Tumors can enter regression even when only a small number of cells are treated, demonstrating bystander killing. This is thought to be GJIC-mediated, as it is believed that phosphorylated GCV may pass through gap junctions as with natural nucleotides. Such bystander killing of tumor cells may be enhanced by transfection with connexin genes (Tanaka et al., 2001) provided that the function of their products can be maintained. Another use of connexins in gene therapy is focused on human glioblastoma. When cultured in vitro and transformed with cx43 (Huang et al., 2001) such cells become sensitive to chemotherapeutic drugs that induce apoptosis (i.e., etoposide, paditaxel, and doxorubicin).

Conclusion

The apparent role of the connexin family of proteins in such a wide range of cell functions relating to differentiation, proliferation, cell death, and migration, and the multitude of potential mechanisms of disruption, not surprisingly leads to the implication of these molecules in the processes of carcinogenesis and renders them potential useful targets in cancer therapy. A major future direction to aid understanding will be through targeted mutagenesis (Plum et al., 2000), dominant negative mutants (Yamasaki et al., 1999), and “knock-in” of specific cx genes in mice.

REFERENCES


