Gap Junctional Intercellular Communication and Cell Proliferation during Rat Liver Carcinogenesis

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During multistage liver carcinogenesis, there is a sequential decrease in gap junctional intercellular communication (GJIC), associated with reduced expression of a major liver gap-junction protein (connexin 32). There are also several lines of evidence indicating that the induction of cell proliferation plays an important role during liver carcinogenesis. The relationship between GJIC and cell proliferation and their roles in liver carcinogenesis are not yet known. Results from various experiments suggest that there is a close relationship between the inhibition of GJIC and stimulation of liver cell proliferation. However, our results also suggest that different stimuli may affect cell proliferation and GJIC differentially by different mechanisms.

Changes in Gap Junctional Intercellular Communication during Liver Carcinogenesis

In multicellular organisms, each cell has various means to communicate with other cells to maintain homeostasis (1). Among such means, gap junctional intercellular communication (GJIC) is the only one by which cells can directly transfer factors from the inside of one cell to the inside of neighboring cells (2,3). Because gap junctions mediate the transfer of important signal-transducing factors such as calcium, cyclic AMP, and inositol triphosphate (4,5), it is believed that GJIC plays a pivotal role in maintaining homeostasis by controlling cellular proliferation and growth (2,3).

Because cancer cells can be regarded as cells that are not subject to homeostatic control, the role of GJIC in carcinogenesis has been extensively studied. Most such studies have been carried out using cultured cells, and the results support the idea that aberrant GJIC may play a crucial role in the process of carcinogenesis as well as in the maintenance of transformed phenotypes (6).

Because rat liver has been extensively used for multistage carcinogenesis studies (7), and because gap junctional proteins and cDNA coding for such proteins have been isolated from the liver (8,9), the relationship between aberrant GJIC and rat liver carcinogenesis has been studied extensively. Several laboratories, including ours, have reported that, during rat liver chemical carcinogenesis, a significant decrease in the level of mRNA or protein of a major liver gap junction, connexin 32 (cx32), occurs in persistent nodules and hepatocellular carcinomas (10-13). Janssen-Timmen et al. (10) found a 71% decrease in the number of gap junctional (cx32) immunofluorescent spots in rat hepatocellular carcinomas induced by N-methyl-N-nitrosourea. Beer et al. (11) observed decreased expression of cx32 mRNA and protein in rat liver tumors induced by an initiation–promotion protocol. Our study also revealed significantly diminished cx32 expression in persistent nodules and hepatocellular carcinoma induced by a Solt-Farber carcinoenic regimen or by continuous exposure to N-ethyl-N-hydroxyethylnitrosamine (12). However, limited information exists

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concerning the implication of decreased cx32 expression in early stages of rat liver carcinogenesis. Thus, Janssen-Timmen et al. (10) did not find any essential change in cx32 expression in the majority of a small, ATPase-deficient, putatively preneoplastic cell population at early stages of liver carcinogenesis. However, Beer et al. (11) reported a decrease of cx32 immunoreactivity in some preneoplastic lesions.

Because GJIC can be modulated at various levels, it is important to measure the function of gap junctions to ascertain the role of GJIC in liver carcinogenesis. It is possible that the level of GJIC can be decreased without changing the expression of connexin mRNA and protein levels, if the modulation occurs at the post-translational level. It is also essential to measure the function of gap junctions if we are to study the involvement of heterologous communication in liver carcinogenesis. For this reason, we have recently developed a dye transfer method for measuring GJIC in slices of liver freshly removed from the rat (13). Using this method and immunostaining of cx32, we studied sequential changes of GJIC during chemical hepatocarcinogenesis in the rat under a modified Solt-Farber protocol. We found a substantial decrease in GJIC in the liver parenchyma, which was free of focal lesions at 4 weeks after the start of the protocol. The decrease in GJIC persisted up to at least the 15th week of treatment (13). The results indicate that the carcinogenic treatment regimen itself led to a reduced communication capacity in the whole cell population of the liver, which might help cells acquire aberrant cell growth control.

Most enzyme-altered focal lesions showed markedly lower GJIC and a significantly lower number of cx32-positive spots than surrounding hepatocytes (13). These results are consistent with previous results from our own and other laboratories. Moreover, we found that most GST-P(pleural glutathione-S-transferase)-positive foci showed a selective lack of GJIC compared to surrounding hepatocytes, with the cells in GST-P-positive foci communicating among themselves but not with surrounding hepatocytes. Hepatocellular carcinomas arising 1 year after the carcinogenic regimen had significantly reduced communication capacity, accompanied by a large decrease in cx32 expression. These results suggest that a progressive decrease in homologous as well as heterologous GJIC in preneoplastic lesions occurs during rat hepatocarcinogenesis and that those preneoplastic lesions with the most prominent disorders in GJIC may be more likely to develop into carcinoma (13).

**In Vitro Evidence for the Role of GJIC in Cell Proliferation Control**

The regulation of cell growth by direct cell contact was first described by Stoker and his colleagues (14, 15). When a few polyoma virus-transformed BHK21 cells were cocultured with nontransformed mouse fibroblasts, there was suppression of growth of the transformed cells (14). Stoker showed that direct cell contact was necessary to obtain this suppression and subsequently provided evidence that there was indeed passage of molecules from surrounding normal cells to transformed cells (15). Growth suppression of polyoma virus-transformed bovine fibroblasts was also seen when they were cocultured with normal fibroblasts (16). Other investigators confirmed this phenomenon using SV40-transformed 3T3 cells and chemically or UV-transformed C3H10T1/2 cells (17, 18). We have recently provided similar evidence using BALB/c 3T3 cells that were transfected with various types of viral oncogene (19). When nuclear oncogene-containing cells were cocultured with control BALB/c 3T3 cells, there was heterologous GJIC, and transformed phenotypes were suppressed in such coculture. On the other hand, cytoplasmic oncogene-transfected cells did not communicate with cocultured normal cells, and they still grew to form distinctly transformed foci, suggesting that GJIC may play a role in growth suppression of certain cells.

To study more directly the effect of GJIC on cell growth control, several laboratories have introduced expression vectors of connexin genes into cancerous cells. For example, Eghbali et al. (20) transfected cDNA encoding rat liver gap junction protein cx32 into communication-deficient human tumor cells. When they compared the growth rate in culture from parental and transfected clones, there was no difference. Eghbali et al. found, however, that tumor development of cx32-transfected cells was slower than that of parental cells (20). In another study, Mehta et al. (21) transfected the expression vector of cx43 into communication-deficient mouse 10T1/2 cells and observed a restoration of intercellular communication, slower cell growth, and reduced population density. When they cocultured transfected cells with normal 10T1/2 cells, there was GJIC between the two types, and there were no transformed foci (21). Before transfection of the cx43 gene, the transformed 10T1/2 cells formed foci in coculture with nontransformed counterparts, and the two cell types did not communicate with each other (21). The Eghbali et al. study thus suggests a crucial role for GJIC in growth regulation. However, this may not be the case for the growth control of other mouse cell lines. When BALB/c 3T3 cells are transformed by various types of carcinogen, transformed cells have GJIC capacity similar to that of normal counterparts (22, 23). The expression of cx43 mRNA was also similar. Because transformed cells grow much faster than normal cells, these results suggest that GJIC per se may not regulate the growth of BALB/c 3T3 cells. However, our studies suggest that establishment of heterologous communication between normal and transformed BALB/c 3T3 cells can suppress the growth of transformed cells (24). The difference, in terms of growth regulation by gap junction, may therefore depend on the cell type.
If GJIC plays an important role in growth control by allowing exchange of growth factors, it is reasonable to assume that GJIC ability may change during the cell cycle. When cultured cells are in mitosis, they appear round in form and seem loosely attached to neighboring cells. The cells look as if they are not communicating with resting cells. However, mitotic cells have been shown to communicate with resting cells (25). If mitotic cells contain factors that are important for cell division and if these factors are transferred by GJIC to resting cells with which they are in contact, why do resting cells not start to divide? One possibility is that such growth control factors are present only just before the cells enter mitosis and that such a cell momentarily does not communicate with neighboring resting cells. This would be consistent with the finding that proliferating hepatocytes in regenerating liver have very few gap junctions (see below). It is also possible that intracellular levels of growth control factors play a key role. Transfer of such factors from mitotic cells to resting cells may occur, but, because of dilution by GJIC among contiguous resting cells, the concentration may not reach the critical level needed to exert a biological effect. A recent study has shown that mitotic cells do indeed have less capacity than resting cells to communicate with resting cells (26).

Changes in GJIC Associated with Increased Cell Proliferation during Rat Liver Carcinogenesis

Cell proliferation is considered to play an important role at various stages of liver carcinogenesis. Partial hepatectomy is often used in liver carcinogenesis studies to stimulate cell proliferation. During cell proliferation after partial hepatectomy, there is a significant change in GJIC. As early as 3 hr after partial hepatectomy, there was a significant decrease in the amount of cx32 protein. Induction of S-phase examined by BrdU labeling was detected only 12 hr after partial hepatectomy, suggesting that blocking of GJIC precedes S-phase induction. The level of cx32 protein continues to decrease until 24 hr after partial hepatectomy, when the BrdU-labeling index reaches its maximum level (Figs. 1 and 2). These results confirm and extend previous observations that partial hepatectomy induces a decrease of GJIC (27–29).

Most studies on the relationship between cell proliferation and liver carcinogenesis have been carried out by inducing with compensatory proliferative stimulators. However, it has been suggested that direct mitogenic cell proliferation may have biologically different effects on liver carcinogenesis. For example, a series of studies by Columbano’s laboratory have shown that direct mitogen-induced liver growth does not support initiation or promotion of chemical hepatocarcinogenesis (30–32). To examine whether mitogenic and compensatory proliferation had different effects on GJIC, we compared the level of connexin mRNA in the livers of rats treated with CCl4 (compensatory proliferation inducer) and Pb(NO3)2 (direct mitogen). Pb(NO3)2 treatment rapidly increases the level of cx32 and cx26 ([unpublished results] cx26 is another major liver connexin (33]), followed by a gradual decrease to almost nondetectable levels 60 hr after treatment. Such a transient increase of cx32 gene expression was not observed in the livers of rats treated with CCl4, suggesting different regulation of gap junction mRNA associated with these two types of cell proliferation. It is now important to examine whether such differences in mRNA of connexin genes are reflected in the function of GJIC.

Many nongenotoxic carcinogens and tumor-promoting agents are involved in liver carcinogenesis and induce cell proliferation (34,35). To see whether cell proliferation induced by tumor-promoting agents is associated with decreased GJIC, we treated rats with different liver tumor-promoting agents, including phenobarbital, chlorofibrate, polychlorinated biphenyl, and dichlorodiphenyltrichloroethane (DDT). All of these agents induced cell proliferation, as determined by staining with antibody Ki67. They also inhibited GJIC as measured in fresh liver slices. The decrease in GJIC was associated with low levels of cx32 protein determined by histochemistry. However, when we examined the relationship between GJIC inhibition and cell proliferation induction by these tumor-promoting agents, there was no quantitative correlation. It is, however, important to note that the cell proliferation was determined by Ki67 antibody staining, which may not accurately reflect the real cell proliferation in the liver.
As described above, the rat liver shows a sequential change of GJIC during multistage carcinogenesis. To see whether the sequential decrease in GJIC is associated with increased cell proliferation in liver carcinogenesis, we examined GJIC and cell proliferation in untreated control cells, foci, hyperplastic nodules, and hepatocellular carcinomas produced in Fischer 344 male rats treated with EHEN, N-ethyl-N-hydroxyethylnitrosamine, (1% in drinking water). When these tissues were stained with cx32 antibody, there was a progressive decrease in staining from untreated cells, treated surrounding cells, cells in foci, cells in hyperplastic nodules to carcinoma cells. When BrdU-labeling index was examined, there was a progressive increase from normal cells, foci, and hyperplastic nodules to carcinomas, suggesting a close correlation between the increase in cell proliferation and decrease in connexin expression.
Conclusion

There is generally a good association between increased cell proliferation and decreased GJIC. Decreased GJIC in relation to cell proliferation must be considered in terms of both homologous and heterologous cell contact. Some lines of evidence suggest that GJIC among homologous cells per se regulates cell proliferation. However, evidence from our own and other laboratories suggest that GJIC with heterologous cell types, i.e., between normal and transformed cells, plays an important role in the regulation of growth of transformed cells. Although an association between cell proliferation and gap junctional intercellular communication is strong, it is not clear whether the decrease in GJIC is the cause of increased cell proliferation. Further studies with expression vectors of various connexin genes will certainly help in establish-
ing whether there is a causal relationship between GJIC inhibition and increased cell proliferation.

GJIC can be controlled by various factors, including extracellular matrix and cell–cell recognition molecules (38–39). Therefore, the effects of these molecules upon GJIC will possibly influence cell proliferation. From this point of view, it is interesting to note that the loss of cell adhesion molecules, notably E-cadherin, has been associated with the invasive ability of tumor cells (40,41). One study has shown that transfection of E-cadherin gene into tumorigenic cells renders them non-tumorigenic (38). These results suggest that various forms of intercellular communication are involved in regulation of cell proliferation as well as in carcino genesis.

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