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Invited Review

The role of gap junctional intercellular communication (GJIC) disorders in experimental and human carcinogenesis

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Summary. There is a growing body of evidence supporting the etiologic implication of gap junctional intercellular communication disorders in carcinogenesis. Substantial progress has recently been made both in molecular biology of gap junction and in the field of cancer research. They provide new insights and conceptions of gap junctional disorders in tumor pathology. Modern understanding of the structure, function and regulation of gap junctions, as well as putative mechanisms of its disorders in human and experimental carcinogenesis are discussed in this review with particular emphasis on fast-moving aspects of this problem.

Key words: Gap junction, Connexin, GJIC, Hepatocarcinogenesis

Introduction

Since the control of cell cycle plays a pivotal role in cell growth and its deregulation is considered as a major etiologic factor in carcinogenesis, genetic alterations of cell-cycle regulatory genes during carcinogenesis are being studied intensively. While most of such genes are direct intracellular regulators of cell cycle, it is getting more and more obvious that cell-to-cell interaction also plays an important role in the growth control and thus, in tumorigenesis as well. Recently a number of genes involved in cell adhesion, namely integrins, cadherins, APC and DDC genes, α- and ß-catenins, γ-catenin (plakoglobin), vinculin, α-actin (Behrens et al., 1989; Frixen et al., 1991; Gluck et al 1993; Rodríguez-Fernández et al., 1993; Tsukita et al., 1993; Simcha et al., 1996) have been shown to possess tumor suppression activity. Cell-cell interaction machinery is rather complex and include functionally different components. One of them is gap junctional intercellular communications (GJIC), and its possible role in tumor pathogenesis has been a matter of intensive investigations during the last decade.

The principal body of evidence for etiologic implication of GJIC disorders in tumorigenesis was based upon numerous *in vitro* studies demonstrating its prominent reduction in most of rodent and human cancer cell lines and on the capacity of a wide range of tumor promoters to inhibit GJIC in cultured cells. Both of these phenomena were intensively reviewed in scientific literature (Klaunig and Ruch, 1990; Holder et al., 1993; Hotz-Wagenblatt and Shalloway, 1993; Yamasaki et al.,1995)

Only recently, the role of aberrant gap junctions in carcinogenesis was addressed using *in vivo* materials, namely, to experimental and human tumors. Although nearly all observations obtained on tumors so far were very much in line with early finding on cultured cells, the mechanisms of GJIC disorders occurred in tumors and its etiological role in sporadic and chemical carcinogenesis remain to be understood. In this review, we will try to concentrate on aspects of this subject which are not yet well established.

Gap junctions - their structure and function

The structure and function of gap junction has been extensively reviewed (Beyer et al., 1990; Paul, 1995; Severs, 1995; Kumar and Gilula, 1996); we present here only a brief overview of the points which are pertinent to the topic of this review.

Gap junction was originally characterized by its appearance in electron microscopy as a pair of membranes separated by a 2-nm «gap» (Robertson, 1963; Revel and Karnovsky, 1967). Although the «gap junction» was thus discovered by its structure, it has later become clear that this is the means for cells to directly exchange small hydrophilic molecules.

Structurally, gap junctions are relatively simple; they are plaque-like clusters of intercellular aqueous channels that mediate communication between cytoplasms of contiguous cells; to form the junction, each of two

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adjoining cells contributes a connexon unit which makes up half of the whole channel (Fig. 1). Therefore, to successfully establish a gap junction, equal contribution of both contiguous cells is required. Each connexon consists of six transmembrane protein molecules named connexin. Connexins are a multigene family. At least 13 different mammalian connexins are currently known. They have specific sequences, molecular weight and biochemical features, but all connexins share similar transmembrane topology: they have four membrane-spanning domains intermediated by two extracellular and one intracellular loops and two intracytoplasmic tails. Schematically, connexins can be presented in a shape of "W" (Fig. 2).

Up to now, there is no convincing evidence to suggest the direct interaction of gap junctions with any intracellular structures or organelles. However, they appear to be controlled by intercellular contacts mediated by cell adhesion molecules (CAM), particularly cadherins. To establish and maintain GJIC, a close physical interaction of lateral plasma membranes of juxtaposed cells provided by CAM's and cadherins appear to be required. It may be that connexon-connexon interaction is not strong enough itself to keep plasma membranes in tight contacts and to form gap junctions. As was shown recently, abrogation of cell adhesion function by site directed mutagenesis of N-cadherin disrupted well-established GJIC (Hertig et al, 1996). Conversely, transfection of cadherin genes into communication-deficient tumor cells restored their GJIC (Jongen et al., 1991). The fact that several genes related to CAM superfamily were found to be tumor suppressors and therefore are disactivated in tumors, indicates their possible implication in gap junction impairments occurred in tumors (see review: Mesnil and Yamasaki, 1993)

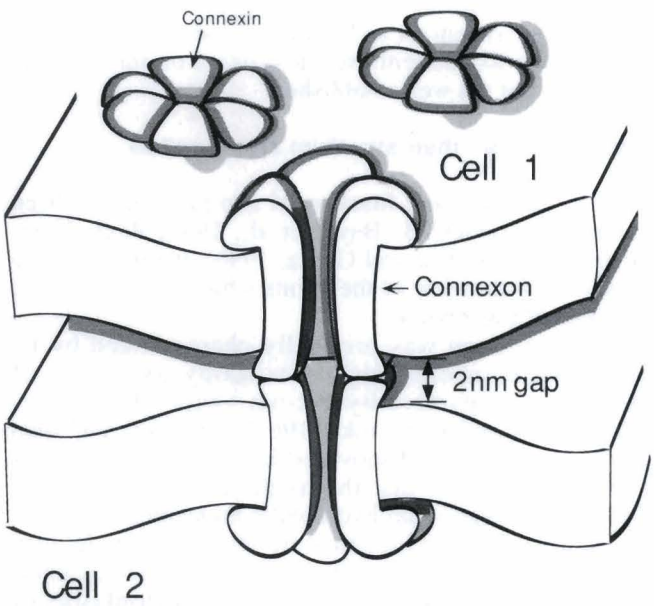

Fig. 1. Structure of gap junction. (See details in the text).

Since a great variety of biologically active molecules are potentially capable of passing through gap junctions, it is not surprising that GJIC is involved in virtually all vital cellular processes. Among generally accepted gap junction-diffusible messengers are ions, water, Ca^{2+}, cAMP, inositol phosphate, as well as oligonucleotides, different metabolites, nutrients (Saez et al., 1989). The principal physiological role of GJIC is to control possibly important messengers to be at similar concentrations within a group of cells so that they are organized into a tissue as syncytium. In other words, the main function of GJIC is a maintenance of tissue homeostasis. Besides, there is a number of other physiological functions attributed to gap junctions; they conduct electrical waves in excitable cells, keeping their contractions coordinated. In certain tissues without blood vessels, such as cornea, gap junctions are the only mean to nourish cells. Gap junctions are also playing a role in tissue response to hormones, by means of release through gap junctions a wave of second messengers from hormonally activated cells to those contiguous ones which are nonactivated or nonresponsive to hormones and thus increasing overall hormone response in tissue. Another physiological function of gap junctions is regulation of embryonic development, by spreading of morphogenic signals in embryos and by defending the boundaries of developmental compartments. As it was recently shown, the impairment of GJIC during embryogenesis introduced by knock-out mice technique leads to severe developmental anomalies (Reaume et al., 1995).

GJIC is also considered as an important, albeit not unique, cell growth control pathway; this function of gap junctions is of our particular interest in view of its putative etiological implication in carcinogenesis. The conclusion about involvement of GJIC in cell proliferation is based upon three following principal groups of experimental observations: (i) inverse relationship between communicational and growth capacities in tumor/transformed cell lines, (ii) restoration of GJIC in tumor cells by transfection of connexin genes inhibits

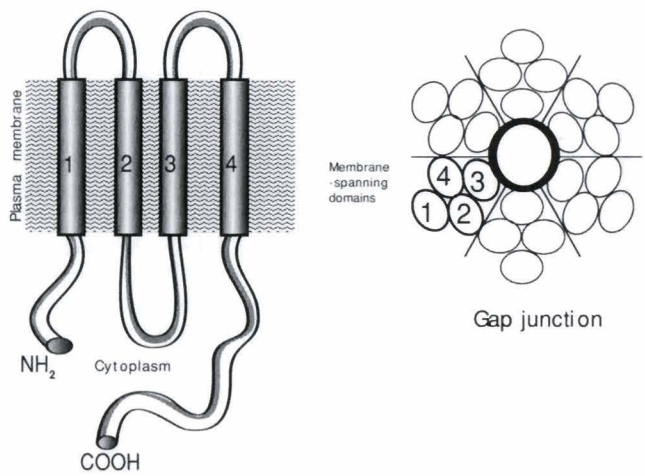

Fig. 2. Topology of connexin protein.

their growth, and (iii) growth stimulation by different types of mitogens dramatically decrease GJIC; conversely, growth inhibition increase gap junctional cell coupling. The mechanism by which GJIC regulates cell proliferation is not yet fully understood and "could conceivably be manifested at the level of gap junctions itself through intercellular passage of information, or at the level of membrane connexin proteins performing functions related to cellular behavior including indirect effects on cell adhesion and/or gene expression." (Nonner and Loewenstein, 1989). Recent connexin gene transfection experiments essentially corroborated suggestion that connexins may induce, besides intercellular communica-tion, a number of other effects, which are required for temporal expression of certain cell cycle regulating genes. Thus, Cx43 transfection into TRMP cells (transformed dog kidney epithelial cells) not only just restored their GJIC, but also normalized their growth and tumorigenicity and altered proliferation rate associated with doubled duration time of G1 and S phases of their cell cycle. These Cx43-induced effects were coupled with decreased expression of specific cell cycle regulatory genes critical to cell cycle progression, including cyclin A, D1, D2 and cyclin-dependent kinases CDK 5 and 6 (Chen et al., 1995).

There is some recent, albeit rather indirect, evidence also suggesting possible implication of GJIC in another component of cell proliferation control system, namely, apoptosis (Trosko and Goodman, 1994).

Mechanisms of GJIC regulation

The knowledge of physiological machinery of GJIC regulation may help to understand mechanisms of GJIC disorders implicated in different pathological conditions including cancer.

The establishment of GJIC depends on the coordinated execution of a series of events, including: (i) connexin synthesis; (ii) oligomerization of connexin monomeres into a connexon; (iii) translocation of connexons to the plasma membrane and accumulation at the site of cell-to-cell apposition; (iv) interaction with the partner connexon of the adjacent cell; and (v) gating of the complete intercellular channel between open and closed states.

Correspondingly, there are at least three levels of GJIC modulation, as suggested by Holder et al. (1993): (i) fast control, or gating, when the pore of gap junction channel quickly, in milliseconds, close by means of either changing of connexin proteins allosteric configuration, or rotating of connexins within connexon along pore axis, much like the aperture of camera lens. Owing to its quick transitory nature, this type of GJIC regulation does not contribute essentially to carcino-genesis; (ii) intermediate control, required minutes or hours, when redistribution of connexin proteins pool between plasma membrane, where their assembling in gap junctions is going on, and intracytoplasmic depot buffers the connexins supply, occurred. For some connexins (Cx43) the cellular location is determined by degree of their phosphorylation (Musil et al., 1991). Apparently, majority of tumor promoters disrupt GJIC, when certain part of total amount of connexins could be sequestered from plasma membrane in different subcellular compartments; aberrant intracytoplasmic connexin localization also is a common immunohisto-chemical finding in different tumors; and at last, the type (iii) of GJIC control - long-term - occurs on the level of Cx mRNA synthesis rate and stability. Apparently, this type of GJIC regulation is operating in long-term autonomous communicational failure observed in some tumors.

How GJIC can be studied *in vivo?*

One of the most impressive experimental attraction of GJIC is that one can study their function directly in living cells, both *in vivo* and *in vitro*.

Basically, there are two principal ways to approach GJIC: functional intensity of intercellular coupling could be estimated either electrophysiologically, by metabolic cooperation assay, or by means of visualization with artificial tracers, usually Lucifer Yellow fluorescent dye, spreading in cell monolyer or tissue selectively through gap junctions, after its introduction into cells by microinjection (so-called dye-transfer technique); and biochemically, by measurement of intensity of connexin gene expression on different levels. Actually, the principal methods to study GJIC function directly have been developed and intensively and successively used long time before discovery of gap junction proteins. Generally, functional assays provide more relevant

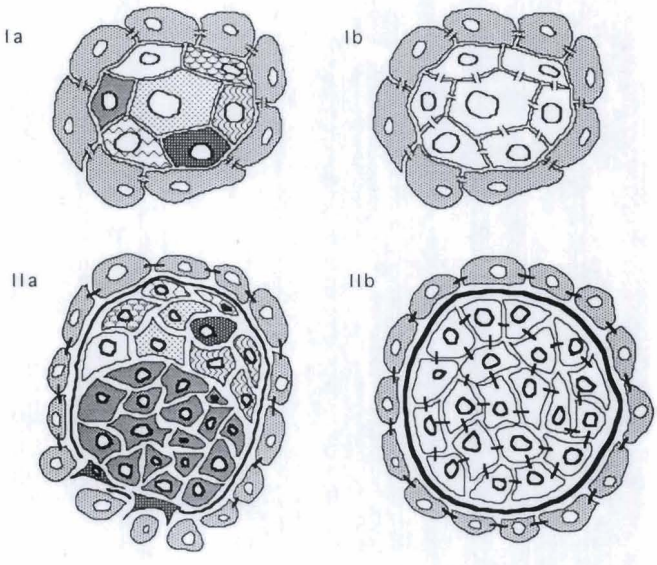

Fig. 3. Putative role of the lack of heterologous type of GJIC in tumorigenesis. Foci of tumor cells without **(A)** or with **(B)** capacity in coupling homologously, that is among themselves, both do not communicate heterologously with surrounding normal counterparts, resulting in their autonomous selective outgrowth and malignisation.

information, but in a number of situations, only biochemical approach is possible and may provide useful information. It is becoming clear that both functional and biochemical assays should be used in parallel as much as possible so that not only functional behavior, but also molecular mechanisms, of GJIC be studied.

What is known about alteration of GJIC during carcinogenesis?

The principal concept of putative etiological role of GJIC disorders in carcinogenesis was formulated long time ago, mostly on a basis of *in vitro* findings (Loewenstein and Kanno, 1966), that tumor cells usually have very low capacity to communicate between each other. Later, the lack of GJIC in cancer cells was extended to cancer/normal cell communication, i.e. heterologous GJIC (Yamasaki, 1990). Therefore, it was suggested that such selective communicational isolation of tumor cells from surrounding normal tissue helps them to escape from signals keeping proliferation in normal tissues under negative control (Fig. 3). This idea was strongly supported by finding that tumor promoters specifically and efficiently inhibit GJIC in cells *in vitro*, regardless of their prominent difference in structure and mode of tumor promoting action. A huge database of tumor promoters inhibitory action on GJIC *in vitro* was created and reviewed recently (Budunova and Williams, 1994). Later, tumor suppressive role of gap junction proteins - connexins - have been postulated, mostly upon a basis of *in vitro* transfection studies, usually resulted in reversing of malignant cell phenotype into rather normal one (Naus et al., 1992). However, any *in vitro* experimental system, even thoroughly designed and sophistically developed, could not be considered as a full equivalent of carcinogenic model *in vivo*. In order to validate the hypothesis of etiological implication of GJIC disorders in carcinogenesis, direct evidence from *in vivo* carcinogenic models needs to be obtained.

In order to elucidate the etiologic implication of GJIC disorders in experimental carcinogenesis, several

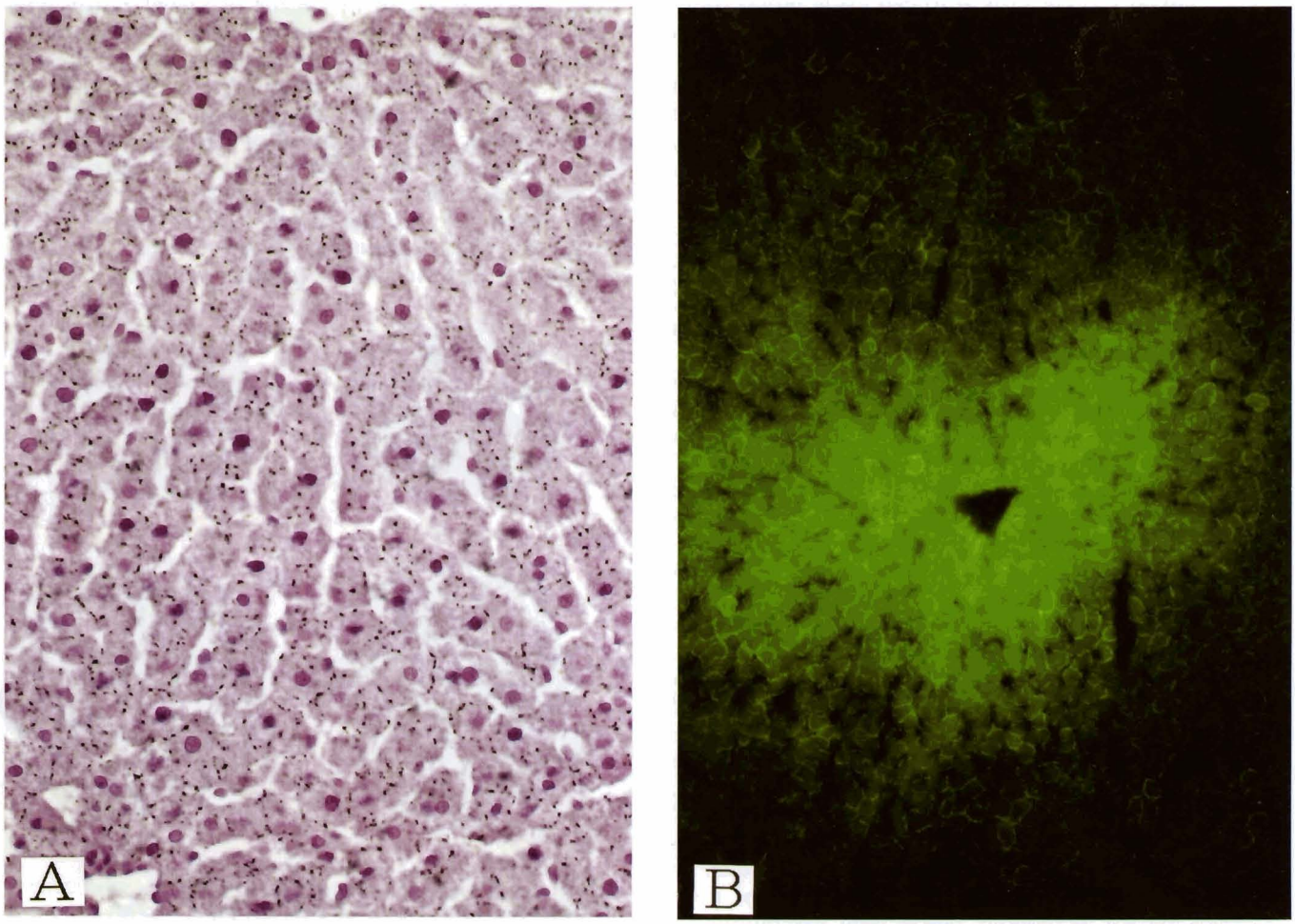

Fig. 4. GJIC in normal rat liver.**A.** Immunohistochemical localization of connexin32 in gap junctions in rat liver, revealed as discrete tiny black spots in lateral membrane of hepatocytes. x 350. **B.** The pattern of Lucifer Yellow dye spread in normal rat liver after microinjection. x 90

studies were carried out, employing rat hepatocarcinogenic model. There is a number of advantages in this carcinogenic model to study GJIC disorders. It represents a useful multistage carcinogenic model, in which discrete populations of cells can be identified to study initiation, promotion and progression. Rat liver is the main target tissue for overwhelming majority of non-genotoxic carcinogens, many of which may act by inhibiting GJIC. Hepatocytes abundantly express well characterized Cx32 and Cx26 and corresponding antibodies and cDNA probes are widely available. And at last, unique parenchymal liver structure allowed to measure GJIC function directly in freshly-removed livers, by Lucifer Yellow dye microinjection (Krutovskikh et al., 1991, 1994; Krutovskikh and Yamasaki, 1995) (Fig. 4).

Thus, it was shown, using our *in vivo* microinjection assay and different indirect techniques, that GJIC is strongly inhibited already at the early stage of rat experimental hepatocarcinogenesis, such as in liver enzyme altered preneoplastic foci (EAF) (Neveu et al., 1990; Krutovskikh et al., 1991). It was also found that some, but not all EAF, considered as earliest morphologically detectable liver tumor precursors, do not express Cx32 mRNA or proteins. As it was found by direct Lucifer Yellow dye transfer technique, certain EAF's also do not communicate heterologously, with their surrounding normal hepatocytes. Inhibition of connexin32 expression was particularly prominent in promoter independent lesions.

It was suggested that Cx32 may be a more biologically appropriate biomarker of liver preneoplastic lesions, than any other commonly used enzymes, such as GGT and GST-p, expression of which appears to reflect rather toxic nature in chemical carcinogen-initiated cells. The strongest evidence in favor of such an opinion came from SV40 large T antigen transgenic rat model of hepatocarcinogenesis. In these rats, liver preneoplastic and tumor lesions were negative on any enzymatic markers mentioned above, but displayed Cx32-negative phenotype (Hully et al., 1994).

Communicational failure detected at early stages of rat liver tumor formation was found to persist during further steps of tumor progression. Thus, connexin32 immunostaining of rat hepatocellular carcinomas in overwhelming majority of cases demonstrated drastic reduction of Cx32 positivity (Janssen-Timmen et al., 1986, Sakamoto et al., 1992; Neveu et al., 1994). And even in a few tumors in which Cx32 is expressed at relatively high level, these gap junction proteins were usually immunolocalized abnormally, mostly intracytoplasmically, suggesting their functional impairment (Krutovskikh et al., 1991, Omori et al., 1996a,b). It is interesting to note that the expressions of Cx32 and Cx26 in rat liver lesions are differentially effected; while Cx32 was found downregulated already in some preneoplastic foci, Cx26 was often even upregulated at that stage of hepatocarcinogenesis. Only in hepatocarcinomas expression of Cx26 was reduced (Sakomoto et al., 1992; Neveu et al., 1994). Subsequent study of

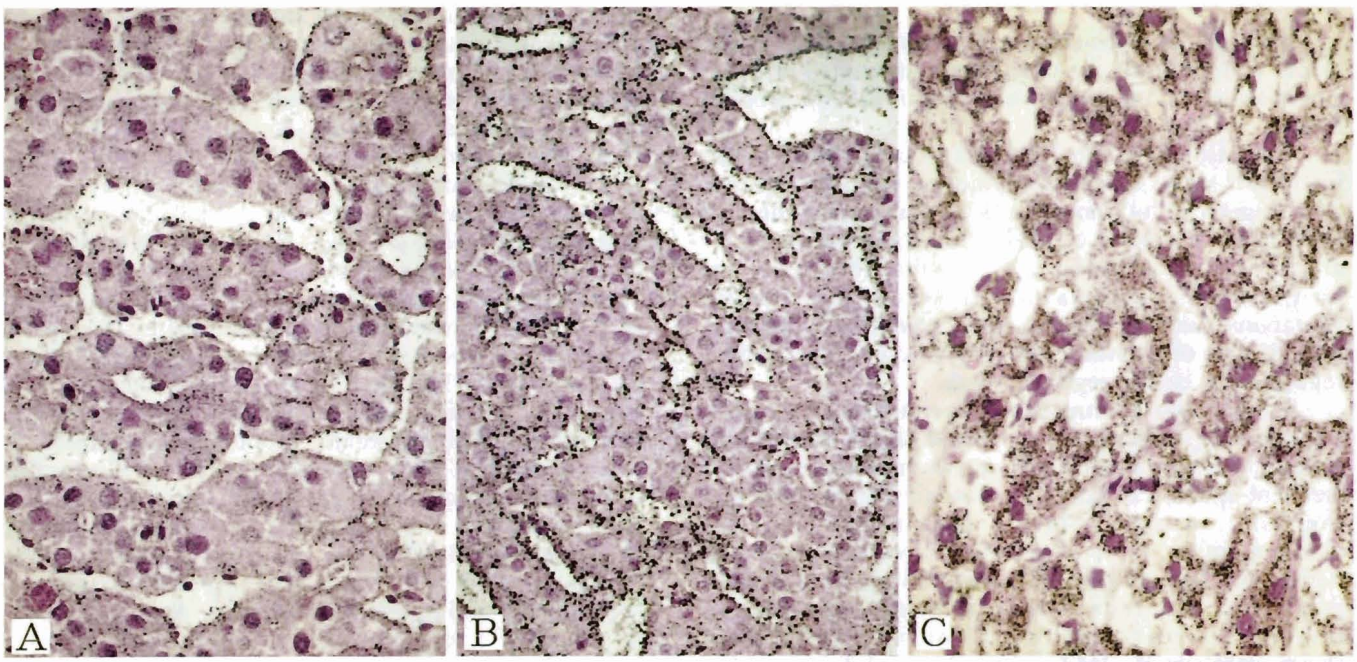

Fig. 5. Immunohistochemical localization of connexin32 in different types of human hepatocellular carcinomas (HCC). **A.** Acinar type of well differentiated human HCC. Cx32-positive spots are rather in parts of tumor cell plasma membrane faced basal membrane around glandular-like tumor structures than between individual cells. x 230. **B.** Trabecular type of well differentiated human HCC. Cx32-positive spots are mostly in parts of tumor cell lateral membrans around trabecular tumor structures. x 230. **C.** Moderately differentiated human HCC with predominant solid structure. Cx32 mostly detected intracytoplasmically. x 230

human liver tumors revealed the difference compared to rat hepatocarcinomas in terms of pattern of alteration of Cx32 expression. Unlike in the rat model, human hepatocellular carcinomas almost always had a high level of Cx32 protein, although immunohistochemically it was nearly always abnormally localized inside tumor cells (Krutovskikh et al., 1994), as it was found in some rat liver tumors (Omori et al., 1996a,b) (Fig.5). Direct measurement of GJIC in human liver tumors indeed confirmed a functional impairment of cytoplasmic Cx32.

Interesting observations concerning GJIC implication in carcinogenesis have been made on other carcinogenic models. Thus, unlike hepatocarcinomas, it was found that N-ethyl-N-(4-hydroxybutyl)nitrosamine (EHBN) induced rat bladder carcinomas abundantly expressed Cx26 and Cx43 (Asamoto et al., 1994). Initially, it was postulated that increased connexins expression may give growth advantage in rat bladder carcinomas. However, further transfection of Cx43 antisense sequence into bladder cancer cell lines unexpectedly enhanced their malignancy, therefore confirming again tumor suppressive property of connexin proteins (Asamoto, unpublished data).

Progressive and differential downregulation of Cx26, Cx43 and Cx31.1 expression was described immunohistochemically in mouse skin tumors as well, in parallel with tumor progression (Budunova et al., 1995; Kamibayashi et al., 1995)

Putative mechanisms of GJIC disorders in carcinogenesis

Basically, two principal mechanisms of communicational failure could be considered as contributing to carcinogenesis - at genetic and functional (epigenetic) levels.

Genetic alteration GJIC may occur either at the initiation stage of carcinogenesis, when genotoxic carcinogens induce mutations in connexin genes, or other genes related to cell-cell interaction machinery, or at later stages of tumor progression, as a result of progressive accumulation of secondary genetic alterations due to general genetic instability. Mutational disactivation of connexin genes may occur either in their regulatory or coding region, or both.

Since most of tumor suppressors are usually mutationally disactivated on initial stages of carcinogenesis, one may suggest that connexin genes could be a frequent specific target for genotoxic carcinogens and therefore, mutated during the initial stage of carcinogenesis. Several attempts have been made to verify this suggestion, but no mutations in structural regions of different connexins in different human tumors, including hepatocarcinomas, were found (Krutovskikh et al., 1994, 1996). Meanwhile, in one rat hepatocarcinoma, induced by genotoxic carcinogen N-ethyl-N-hydroxyethylnitrosamine (EHEN), a missense mutation of Cx32 gene at codon 220 substituting from Arg to His was found (Omori et al, 1996a,b). Some rat liver tumors in which Cx32 proteins are immunohistochemically aberrantly localized, also revealed altered electrophoretic mobility of Cx32 proteins, suggesting possibility of changes either in its primary structure (mutation) or by post-translational modifications (Neveu et al., 1994). Frequent occurrence of stable prominent decrease of Cx32 expression in rat liver tumors (Janssen-Timmen et al., 1986; Fitzgerald et al., 1989) suggested a high feasibility of genetic alterations in regulatory regions of this gene. Therefore, if one examines this region of connexin genes for the presence of mutations, it may be possible that the frequency of mutations of connexins genes in tumors is much higher; however, such analysis was not done so far due to the unavailability of these sequences.

Overall, we may conclude, that mutation of connexin genes, at least in their coding region, occurs rather rarely in experimental and sporadic human carcinogenesis. However, since only Cx32 and Cx37 gene mutations have been seriously examined, we need results on other connexin genes.

As discussed before, the functional establishment of gap junctions is a rather elaborate process which heavily depends on complex machinery of cell-cell interactions. It is worthwhile to remember that virtually all proteins involved in intercellular adhesion, such as cadherins, integrins, catenins, vinculin and plakoglobin have recently been characterized as strong tumor suppressors and correspondingly their expression was found to be severely damaged in different tumors. Therefore, it is conceivable that the lack of GJIC found in tumors is rather a consequence of primary genetically damaged cell-cell interaction machinery than a result of direct alteration of connexin genes. In fact, mutations of catenins, APC, integrin and cadherins have been found in several types of tumors (Backer et al., 1994; Risinger et al., 1994; Aberle et al., 1995; Rimm et al., 1995; Muta et al., 1996; Oda et al., 1996). However, very few attempts have been made so far to characterize direct dependence of GJIC disorders in tumors from alterations of cell-cell adhesion machinery.

Contrary to rare genetic alterations of connexins, epigenetic mechanisms are apparently playing an essential role in GJIC disorders occurred during carcinogenesis. The first supportive evidence came, a long time ago, from *in vitro* studies, when reversible inhibition of GJIC by tumor promoters were found. It was suggested that non-genotoxic carcinogenic compounds, being applied repeatedly, could induce long lasting inhibition of GJIC resulting in chronic abrogation of circulation in target tissue of cell proliferation control signals, which in turn would eventually promote initiated cells to grow and progress into tumors. As it was shown by means of direct estimation of GJIC function in rat liver, chronic administration of different hepatopromoters did inhibit GJIC in this organ (Tateno et al., 1994; Krutovskikh and Yamasaki, 1995). Again, immunostaining with anti-Cx32 antibody revealed, that tumor promoter induced inhibition of GJIC in rat liver

was associated with sequestering of Cx32 proteins from lateral membrane of hepatocytes with its subsequent accumulation intracytoplasmically, very much the same way as found in liver tumors (Krutovskikh et al., 1995). Apparently, mechanisms responsible for connexin protein trafficking into plasma membrane are most vulnerable during the promotion phase of carcinogenesis. However, to understand the significance of this observation, a better knowledge of basic mechanisms of connexin protein trafficking is required.

Another rather common mechanism of connexin downregulation repeatedly observed in experimental tumors is prominent connexin mRNA instability (Neveu et al., 1994; Budunova et al., 1995). It was found that the high level of connexin mRNA in tumors often does not correspond to the intensity of connexin immunostaining.

Taken together, the observations obtained from *in vivo* studies indicate that GJIC disorder is an essential etiologic factor of tumor formation. It must be underlined that apparently, multiple mechanisms (mostly epigenetic) are responsible for GJIC disturbance during carcinogenesis and only few of them are so far understood.

It must be mentioned that cancer is not the only disease associated with GJIC failure. As it was recently shown, mutations of some connexin genes may be responsible for some heritable diseases; thus, multiple mutations in Cx32 was found in case of neurodegenerative X-linked Charcot-Marie-Tooth disease, (Bergoffen et al., 1993; Chance and Fishbeck, 1994; Fairweather et al., 1994; Ionasescu et al., 1994; Bone et al., 1995) or Cx43 was found mutated in patients suffer with atrio-visceral heterotaxia syndrome (Britz-Cunningham et al., 1995). Information concerning biological meaning of connexin mutations obtained from these patients may shed light on the not yet known basic functions of connexins, and therefore, could help to better understand the mechanisms of GJIC disorders in carcinogenesis (Omori et al., 1996b).

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