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Pathological Significance of Intracytoplasmic Connexin Proteins: Implication in Tumor Progression

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Abstract A considerable amount of evidence has established that gap junctional intercellular communication (GJIC) suppresses tumor development by halting the stage of tumor promotion. Consistently, GJIC is downregulated in tumors. The downregulation of GJIC is caused by not only the reduced expression level of connexin proteins but also their aberrant cytoplasmic localization. Although it has long been thought that cytoplasmic localization of connexin proteins is merely one of the mechanisms of the downregulation of GJIC, careful studies with human tumor samples have indicated that the expression level of intracytoplasmic connexin proteins correlates well with the grade of malignancy and the progression stage of tumors. Hypothesizing that intracytoplasmic connexin proteins should have their proper functions and that their increase should facilitate tumor progression such as cell migration, invasion and metastasis, we examined the effects of overexpressed connexin32 (Cx32) protein on the phenotype of human HuH7 hepatoma cells, which express a basal level of endogenous Cx32 only in cytoplasm. The cells were retrovirally transduced with the Tet-off Cx32 construct so that withdrawal of doxycycline from the culture medium could induce overexpression of Cx32 protein in cytoplasm. Even when overexpressed, Cx32 protein was retained in cytoplasm, i.e., Golgi apparatuses, and did not induce GJIC. However, overexpression of Cx32 protein in cytoplasm enhanced both the motility and the invasiveness of HuH7 cells and induced metastasis when the cells were xenografted into SCID mice. Taken together, cytoplasmic accumulation of connexin proteins may exert effects favorable for tumor progression.

Keywords Gap junction · Connexin · Aberrant localization · Tumor progression · Hepatocellular carcinoma · HuH7 cell · Metastasis

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Introduction

Several decades have passed since Loewenstein and his colleagues proposed and proved for the first time the tumor-suppressive roles of gap junctional intercellular communication (GJIC) (Loewenstein & Kanno, 1966; Loewenstein & Kanno, 1967). So far, an avalanche of evidence provided by many other groups has reinforced the concept (Yamasaki & Naus, 1996; Trosko et al., 2005), with some exceptions (Ito et al., 2000, 2006). Today, downregulation of GJIC has become one of the hallmarks of neoplastic lesions (Mesnil et al., 2005). The downregulation of GJIC in tumors is caused by different mechanisms, including reduced transcription and/or translation of connexin and its hyperdegradation, hyperphosphorylation, underphosphorylation and aberrant localization into subcellular domains other than the plasma membrane (Leithe et al., 2006).

As described later, aberrant localization of connexin protein is often observed in various cancer cases (Mesnil et al., 2005). Since gap junctions are composed exclusively of connexin protein, it is natural that the aberrant localization

of connexin protein should lead to downregulation of GJIC in the form of ''loss of function.'' However, a few reports state that tumors with higher-grade malignancy tend to exhibit, as revealed by immunohistochemistry, a more intense staining of connexin protein in cytoplasm (Krutovskikh et al., 1994, 1995; Omori et al., 1996; Jamieson et al., 1998; Mehta et al., 1999) and that metastasized tumors restore expression of a certain connexin protein in cytoplasm instead of the plasma membrane in spite of no expression in their primary lesions (Kanczuga-Koda et al., 2006). More recently, we have found that connexin32 (Cx32) protein overexpressed in the cytoplasm of human hepatoma cells enhances cell motility and invasiveness and finally induces metastasis *in vivo* without forming gap junction (Li et al., 2007).

Overall, these facts suggest that connexin protein translocated from the plasma membrane to cytoplasm should exert functions other than cell-cell communication, at least in some pathological conditions. Here, the pathological significance of such cytoplasmic accumulation of connexin protein is described and discussed, especially from the aspects of tumor progression, including cell migration, invasiveness and metastasis.

Cytoplasmic Accumulation of Connexin Protein in Cancer

It is well established that GJIC is severely impaired or abolished in almost all tumors during and after the early stage of carcinogenesis, i.e., tumor promotion. Although downregulation of GJIC observed in tumors results from a decrease in the expression level of connexin mRNA and/or protein in many cases (Leithe et al., 2006), a growing body of careful studies has indicated, as shown in Table 1, that cytoplasmic localization of connexin protein, probably due to a defect of membrane trafficking, is not rare and that it is likely one of the mechanisms for the downregulation of GJIC. We assume that such cytoplasmic localization of connexin could have been observed in human tumors much more frequently than reported. When forming gap junctions properly, connexin proteins give impressive punctate signals, corresponding to gap junction plaques, in cell-cell contact areas, as revealed by immunohistochemical staining (Momiyama et al., 2003). On the other hand, signals for the cytoplasmic connexin proteins are often vague and far from impressive (Omori & Yamasaki, 1998). Although one recognizes that loss of gap junction is not equivalent to loss of connexin protein, connexin proteins localized in cytoplasm tend to be ignored as a kind of background signal. Finally, a certain number of surgical specimens where no gap junction plaques are detected are likely to be categorized as negative for connexin protein regardless of whether or not it is expressed in cytoplasm.

Nobody would doubt that the connexin proteins localized in cytoplasm are nonfunctional as a gap junction. However, do they play any roles while residing in cytoplasm? Several reports have described suggestive observations on intracytoplasmic connexin and tumor progression.

Krutovskikh et al. (1994) examined 20 surgical samples of human hepatocellular carcinoma (HCC) for the expression pattern of Cx32 protein and found that poorly differentiated HCC exhibited stronger signals of Cx32 protein in cytoplasm than well-differentiated HCC. Mehta et al. (1999) immunostained 20 primary and 20 metastatic lesions of human prostate cancer along with normal counterparts to detect Cx32 and Cx43 proteins. While both Cx32 and Cx43 gave punctate signals in cell-cell contact areas of acinar epithelial cells in both normal and well-differentiated adenocarcinoma tissues, both were localized in cytoplasm without forming gap junction plaques in poorly differentiated and undifferentiated carcinoma tissues. Jamieson et al. (1998) examined the immunohistochemical expression of Cx26 and Cx43 proteins in 27 invasive ductal carcinoma (not otherwise specified) cases as well as normal and benign tumor tissues of the human breast and revealed that Cx26 was expressed in cytoplasm in a great majority of the examined cancer samples with grade II or III malignancy while no Cx26 protein was detected in either normal or benign tumor samples. More interestingly, Kanczuga-Koda et al. (2006) clearly showed that the cytoplasmic expression of both Cx26 and Cx43 proteins was much more frequent in tumors that metastasized to lymph nodes than in the primary lesions of human breast cancers.

Table 1 Human cancers where connexin proteins were localized exclusively in cytoplasm in the majority of the examined samples

Histotype of tumor	Connexin	Reference
HCC, moderately and poorly differentiated	Cx32 and Cx43	Krutovskikh et al., 1994; Oyamada et al., 1990
Adenocarcinoma of the prostate, poorly differentiated	Cx32 and Cx43	Mehta et al., 1999
Invasive ductal carcinoma (NOS[a]) of the breast, grades II and III	Cx26	Jamieson et al., 1998
Lymph node metastases of breast cancer	Cx26 and Cx43	Kanczuga-Koda et al., 2006

[a] Not otherwise specified

Table 2 Induced overexpression of cytoplasmic Cx32 protein enhances proliferation, motility and metastatic ability of HuH7 cells (Li et al., 2007)

Cell	Dox	Expression level of intracytoplasmic Cx32 protein	Population doubling time[a] (h ± SD)	Motility[b] (% ± SD)	Metastatic ability of xenografts in SCID mice
HuH7 Tet-off Cx32	+[d]	→	72.8 ± 1.8	10.9 ± 1.6	Nonmetastatic
HuH7 Tet-off Cx32	–	↑↑↑	58.3 ± 1.3*	19.6 ± 2.1*	Metastatic
HuH7 mock[c]	+[d]	→	75.8 ± 0.76	9.1 ± 1.3	Nonmetastatic
HuH7 mock[c]	–	→	75.9 ± 0.51	8.5 ± 1.1	Nonmetastatic

*$p < 0.01$ ($n = 6$) compared with HuH7 Tet-off Cx32 cells in the presence of Dox

[a] Obtained from the growth curves during 480-h incubation

[b] Cell motility was measured by the transwell migration method with a Boyden chamber. Each value represents the percentage of the number of cells in the lower chamber against that in the upper one

[c] Transduced with the construct containing no human Cx32 (*GJB1*) gene. A negative control

[d] Culture medium was supplemented with 4 μg/ml Dox. For *in vivo* experiments, SCID mice were given drinking water containing 2 mg/ml Dox

→ , basal expression of endogenous Cx32 protein; ↑↑↑, fourfold overexpression of both endogenous and exogenous Cx32 protein; SD, standard deviation

Taken together, these results suggest that translocation of connexin protein from the plasma membrane to cytoplasm could lead not only to downregulation of GJIC but also to tumor progression.

Intracytoplasmic Cx32 Protein Enhances Motility and Metastatic Ability of Human Hepatoma Cells *In Vitro* and *In Vivo*

As mentioned above, connexin proteins accumulating in cytoplasm may be capable of promoting tumor progression. Thus, we have recently examined whether cytoplasmic Cx32 protein has the potential to facilitate progression of HCC.

In the liver, normal hepatocytes express both Cx26 and Cx32 and exhibit a high level of GJIC with neighboring counterparts. While the expression of Cx26 mRNA and/or protein is abrogated in both human and rat HCCs, Cx32 protein very often continues to be expressed (Oyamada et al., 1990) not in a cell-cell contact area but in cytoplasm (Krutovskikh et al., 1994) and is, thus, incapable of forming gap junction. Omori et al. (1996) previously scanned the whole coding region of both the human and rat Cx32 genes (*GJB1* and *gjb1*, respectively) in HCC samples and found no mutation besides a silent mutation in a rat HCC sample, suggesting that mutation is unlikely to be a cause for the aberrant localization of Cx32 protein. As reported by Krutovskikh et al. (1994), HCCs with higher-grade malignancy tend to express a larger amount of cytoplasmic Cx32 protein.

In order to assess the effects of cytoplasmic Cx32 protein on tumor progression of HCC, we used human HuH7 HCC cells (Nakabayashi et al., 1982), which were derived from a human HCC with low-grade malignancy and expressed no Cx26 but did express Cx32 protein only in cytoplasm. To avoid a possible influence of clonal diversity, tetracycline-responsive Tet-off Cx32 clones were created so that expression of Cx32 protein could be enhanced by removal of doxycycline (Dox) from the culture medium (Li et al., 2007).

Immunoblotting analyses indicated that the expression of Cx32 protein was upregulated approximately fourfold in our HuH7 Tet-off Cx32 cells in the Dox-free medium compared with in the Dox-supplemented one. However, as revealed by double immunofluorescence with a Golgi marker protein, Cx32 protein was localized in Golgi apparatuses but not in a cell-cell contact area in either the presence or absence of Dox. Subcellular fractionation further proved that no Cx32 protein was contained in a cell surface protein fraction of the HuH7 Tet-off Cx32 cells. Consistently, the HuH7 Tet-off Cx32 cells did not exhibit GJIC in either the presence or the absence of Dox, as revealed by scrape-loading dye transfer assay. Taken together, Cx32 protein could not contribute to gap junction channels in our HuH7 Tet-off Cx32 cells (Li et al., 2007).

Interestingly, the population doubling time of the HuH7 Tet-off Cx32 cells was, however, significantly shorter in the Dox-free medium than in the Dox-supplemented one, while the HuH7 Tet-off mock cells showed similar population doubling times in the two different media (Table 2). Furthermore, when the HuH7 Tet-off Cx32 cells as well as the mock cells were subjected to a serum-stimulated transwell assay in a Boyden chamber, Dox withdrawal enhanced the motility (Table 2) of the HuH7 Tet-off Cx32 cells and promoted their invasion to Matrigel, while Dox did not exert any effect on the HuH7 Tet-off mock cells.

To verify whether overexpression of cytoplasmic Cx32 protein enhances the metastatic ability of the HuH7 Tet-off Cx32 cells *in vivo*, we xenografted the cells into a

subserosal area of the liver of SCID mice. The mice in the Dox-administered (Dox^+) group were given drinking water containing 2 mg/ml Dox. Eight weeks after the implantation, the mice developed tumors at the implanted sites in both the Dox^+ and the Dox^- groups with similar incidences. Macroscopic metastatic lesions were found in all of the tumor-bearing mice in the Dox^- group but in none of the mice in the Dox^+ group, which clearly indicated that the overexpressed cytoplasmic Cx32 protein could impart metastatic ability to HuH7 Tet-off Cx32 cells (Table 2). Our overall results suggest that, while Cx32-mediated GJIC suppresses the development of HCCs, the cytoplasmic Cx32 protein should exert effects favorable for HCC progression such as invasion and metastasis once the cells have acquired a malignant phenotype.

Conclusion and Prospects

Cytoplasmic localization of connexin proteins has long been thought to merely be one of the mechanisms causing downregulation of GJIC in tumors (Omori et al., 1998). Our above recent study demonstrated, however, that an excessive amount of Cx32 protein in cytoplasm enhanced the metastatic ability of HCC cells in a GJIC-independent manner. Cytoplasmic accumulation of connexin proteins is likely not simply a phenomenon coincident with tumor development but rather a rational event contributing to tumor progression (Li et al., 2007).

Several questions remain to be answered. Is the accumulation of connexin proteins in cytoplasm a pathological incident or a physiological phenomenon? We do not have a clear answer at this time. Only a few studies have so far described the intracytoplasmic connexin proteins observed in a physiological condition. For example, Cx43 protein accumulates within the cytoplasm of uterine smooth muscle cells before parturition (Hendrix et al., 1992). On the other hand, aberrant localization of connexin proteins is commonly seen in various pathological situations. Since connexin protein might play some important physiological roles transiently within cytoplasm, we will have to watch and describe the expression and behavior of connexin proteins much more carefully than ever. In addition, it is important to specify the subcellular localization of intracytoplasmic connexin proteins. Although, as we defined, Cx32 protein was retained in Golgi apparatuses in our HuH7 cells (Li et al., 2007), very little information on a specific subcellular localization of connexin proteins is available from published works.

Since the excessive accumulation of connexin proteins in the cytoplasm is most likely due to a disordered formation of connexin hexamers called ‘‘connexons’’ or to impaired sorting of connexons to the plasma membrane, restoration of the proper connexon assembly and membrane trafficking is expected to suppress the progression of tumors expressing intracytoplasmic connexin proteins. What complicates things is that the mechanisms underlying the connexon assembly and membrane trafficking depend on cell types and connexin isoforms (Segretain & Falk, 2004). Three different subcellular domains have so far been proposed as a place of connexon assembly, i.e., endoplasmic reticulum (ER) (Falk et al., 1997; Ahmad et al., 1999), trans-Golgi network (TGN) (Musil & Goodenough, 1993) and ER-Golgi intermediate compartment (Diez, Ahmad & Evans, 1999). Sarma, Wang & Koval (2002) revealed that, while Cx43 assembles in the TGN, Cx32 assembles in the upstream domains of the TGN, probably ER or ER-Golgi intermediate compartment. Different routes taken for the membrane trafficking of connexons have also been observed. In HeLa cells and cardiomyocytes, Cx43 is transported to the plasma membrane via microtubules (Lauf et al., 2002; Shaw et al., 2007). In keratinocytes, both Cx26 and Cx43 take a route mediated by actin bundles instead of microtubules (Hernandez-Blazquez et al., 2001). While Cx32 is retained in the cytoplasm in human HepG2 hepatoblastoma cells, Cx26 successfully forms functional gap junctions in a cell-cell contact area in the same HepG2 cells (Yano et al., 2001). Thus, learning from these studies, we have to unravel complex pathways of connexon assembly and membrane trafficking in each cell type and each connexin isoform to relocate intracytoplasmic connexin proteins to the plasma membrane.

Finally, the mechanism through which intracytoplasmic connexin proteins exert biological effects is a challenging issue to be resolved. Attention has long been paid only to the channel function of connexin. From now on, we will need to observe and analyze connexin molecules from a broader perspective, i.e., cell signaling, membrane trafficking and gap junction.

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