Connexin 32 as an Anti-invasive and Anti-metastatic Gene in Renal Cell Carcinoma

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Cellular homeostasis in many organs is maintained via gap junctions composed of connexin (Cx), a large protein family with a number of isoforms. In fact, gap junctional intercellular communication (GJIC) is actively involved in all aspects of the cellular life cycle, ranging from cell growth to cell death. It has been well known that Cx gene acts as a tumor suppressor gene due to the maintenance of cellular homeostasis via GJIC. On the other hand, recent data show that GJIC-independent function for Cx gene contributes to tumor-suppressive effect of the gene with cell certain specificity. However, the mechanistic aspect of the GJIC-independent function remains largely unknown. In this review, we briefly summarize the tumor-suppressive effects of Cx genes, refer to a new aspect of Cx32 as an anti-invasive and anti-metastatic gene against renal cell carcinoma in a GJIC-independent function and establishment of a new cancer therapy based on the new function of Cx32.

Key words connexin32; anti-invasive effect; anti-metastatic effect; renal cell carcinoma; gap junctional intercellular communication (GJIC)

INTRODUCTION

Among the different types of cell–cell interaction in mammalian cells, gap junctional intercellular communication (GJIC) is considered to be the only route allowing freely direct transfer of ions and hydrophilic molecules of up to 1000 to 1500 Da in size between cells, thereby maintaining the electrical and metabolic cell homeostasis (Fig. 1).1–3) The gap junction is made up of juxtaposed transmembrane hemichannels (connexons) provided by adjacent cells, and each connexon consists of six individual transmembrane proteins called connexin (Cx).4–5) Currently, at least 21 different Cx genes have been cloned in human (hCx23, hCx25, hCx26, hCx30.2, hCx30, hCx31.9, hCx30.3, hCx31, hCx31.1, hCx32, hCx36, hCx37, hCx40.1, hCx40, hCx43, hCx45, hCx46, hCx47, hCx50, hCx59, hCx62; h = human, the number gives the approximate molecular weight in kDa).6) The various connexin isoforms differ with regard to their molecular weight due to different lengths of their C-terminals. Different combinations of Cx genes are expressed in different tissues with temporal specificity during development or tissue differentiation.5) Such rigid regulation of Cx expression may contribute to cell differentiation and cell growth in multicellular organisms by keeping important signals, such as those involved in growth control, at equilibrium among GJIC-connected cells.

There is increasing evidence for gap junction-independent roles of connexins in the control of cell growth and the suppression of tumorigenicity. Our group observed that among cells transfected with various connexin genes, there was no correlation between their GJIC and tumorigenicity. GJIC levels were significantly higher in tumors induced by injecting cells transfected with Cx26 in nude mice although all of the connexin genes (Cx43, Cx40, and Cx26) examined could establish GJIC in HeLa cells.7) Other report shows that transfection of the Cx43 gene into human glioblastoma cells reversed the transformed phenotype of these tumor cells; however, the tumor suppression by Cx43 was unrelated to the activity of GJIC.8) Moreover, tumor-suppressive effect of connexins is more connexin species-specific than their activity in cell coupling.9,10) In one study, three mutated Cx26 genes (C60F, P87L and R143W) were expressed in HeLa cells that contained the wild-type Cx26 gene, and were GJIC-competent and non-tumorigenic. Interestingly, mutants, Cx26-P87L and Cx26-R143W, enhanced the tumorigenicity of the HeLa Cx26 cells in nude mice without any changes in GJIC.11) The study by Moorby and Patel provide direct evidence suggesting that growth regulation by Cx43 is independent of gap junction formation. They show that there was no correlation between the action of Cx43 mutants (S255A, S279A, and S282) on cell growth and Lucifer Yellow dye coupling in 3T3 A31 fibroblasts. They also demonstrates that blockade of gap junction formation by either heptan-1-01 or culturing cells at low density had no effect on the ability of the Cx43 mutants to control cell growth.12) All these data suggest that certain mutant connexins exert dominant negative effects on connexin-regulated growth and that such effects are independent...
of the ability of GJIC.

From the above reports, it is thought that Cxs exert their regulatory functions through both GJIC-dependent and -independent pathways. This dual functionality concept has been clearly demonstrated by Zhang and colleagues. They showed that in human osteosarcoma cells, forced expression of Cx43 suppresses cellular proliferation, which is associated with increased expression of p27. This is, at least in part, a result of re-introduction of gap junctions, which allows intercellular flux of cAMP, being an enhancer of p27 synthesis. On the other hand, Cx43 as such, also promotes the degradation of Skp2, a protein that is involved in p27 ubiquitination, independent of GJIC. The interplay between both mechanisms results in the accumulation of p27, in turn inhibiting cellular proliferation. In a recent review, it has been shown that Cx proteins can interact with several types of proteins, that is, adherens junctions-associated proteins (E-cadherin etc.), tight junction-associated proteins (ZO-1 etc.), protein kinases (PKC etc.), cytoskeletal proteins (tubulin etc.), trafficking-related proteins (caveolins etc.), and other partner proteins (AP26 etc.). At present, it is assumed that Cx can regulate cell growth and maintain cell differentiation through these protein–protein interactions. In this review, we review a new functional aspect of Cx32 as a suppressor gene against invasion and metastasis of renal cell carcinoma (RCC) in a GJIC-independent manner and discuss a possibility on a new cancer therapy based on the Cx32-dependent function.

TUMOR-SUPPRESSIVE EFFECT OF Cx32 IN METASTATIC RCC CELLS AND ITS POSSIBLE MECHANISM

Recent studies including our report have suggested that not all Cx genes are able to exert a tumor-suppressive effect on a given tumor, but rather than that there seems to be a Cx-cell type compatibility for this effect. That is, Cx exerts growth control only in tissues or cell types in which the particular Cx is naturally expressed. At present, this tumor suppressive effect of this gene has been already established in primary cancers, but the role of Cx genes in progressive and metastatic cancers is unclear but seems to be very limited. Of solid tumors, RCC has a very poor prognosis, due to severe metastasis, and it is still unknown which Cx gene acts as a tumor suppressor gene in RCC. In our previous studies, we have shown that Cx32 is expressed in the progenitor cell of renal cell carcinoma (RCC), down-regulated in cancerous regions of human kidneys and acts as a tumor suppressor gene in primary RCC. In addition to the reports, we have recently shown that Cx32 has a potential suppressive effect on the development of a metastatic RCC cell (Caki-1). Cx32 expression almost suppresses the development of Caki-1 cells in a mouse xenograft model and induced a marked regression with hyalinization. This observation indicates that Cx32 acts as a potential tumor suppressor gene in metastatic RCC. In our other study, we have shown that the Src family of kinase (Src) contributes to the appearance of invasive and metastatic phenotypes in RCC cells. Caki-1 cells have a highest Src activity of RCC cells, and Cx32 reduces malignancy of Caki-1 cells, mainly due to the inactivation of Src. In experiments using several deleted mutants of Cx32, we have found that Cx32 interacts with the C-terminal domain of the Cx and regulates the inhibitory effect of Src activity, and that the inhibitory effect of Cx32 is independent of GJIC. In recent studies, there has been increasing evidence that Src plays an important role in tumor cell invasion, angiogenesis and chemoresistance. Of the Src-regulated signal molecules, the signal transducer and activator (Stat3) is considered a key molecule to induce the Src-dependent appearance of malignancy in tumor cells including RCC cells. From these reports, we speculated that Cx32 abrogated the appearance of malignant phenotypes in Caki-1 cells via the inhibition of Src-Stat3 signaling. In fact, we have confirmed that Cx32-dependent inactivation of Src in Caki-1 cells caused the inhibition of Stat3 signaling. Also, we have demonstrated that the inactivation of Stat3 by Cx32 induces down-regulation of vascular endothelial growth factor (VEGF) as an inducer of angiogenesis factor, leading to regression of RCC in vivo via the inhibition of VEGF-triggered angiogenesis. Overall, it is concluded that Cx32 acts as a tumor suppressor gene in metastatic RCC, due to the inactivation of Src in the GJIC-independent manner.

SUPPRESSION OF INVASION AND METASTASIS IN METASTATIC RCC CELLS BY Cx32

In order to estimate if Cx32 could suppress invasive and metastatic phenotypes of metastatic RCC cells, we established a novel metastasis model of human RCC in SCID mice depleted of NK cells. Since NK cells are known to play a critical role in eradication of circulating tumor cells and depletion of the cells leads to enhancement of metastases of solid tumor cells, we deleted NK cells in SCID mice, using anti-IL-2 receptor β-chain antibody (TM-β1), which has potential to reduce NK cells in vivo. In this metastasis model, we found that Cx32 expression in metastatic RCC cells almost abolished metastatic potential of the RCC cells in lung and liver. In linked with this finding, serum levels of type-1 plasminogen activator inhibitor (PAI-1) and VEGF, which were important factors in the process of invasion and metastasis of cancer cells, were reduced by Cx32. Also, we have confirmed that Cx32 reduces in vitro invasion capacity of the RCC cells due to down-regulation of fibronolytic factors including PAI-1 via the inactivation of Src signaling. These results suggest that Cx32 acts as a potential anti-invasive and anti-metastatic gene against metastatic RCC. In general, tumor cells undergo adaptive changes that allow them to survive and proliferate in hypoxic environments, leading to aggressive tumor behavior, and hypoxic tumor cells are resistant to most of anti-cancer agents. A key event of hypoxia adaptation in tumor cells is to induce hypoxia-inducible factor (HIF) via the activation of Src, because HIF acts as a potential inducer of genes required for invasion and metabolism under hypoxia including PAI-1 and VEGF. As mentioned, we have reported that Cx32 reduced productions of PAI-1 and VEGF in metastatic RCC cells via the inhibition of Src signaling. Taken together, it seems that inhibition of hypoxic adaptation of metastatic RCC cells is critical for anti-invasive anti-metastatic effects of Cx32 (Fig. 2).
A POSSIBILITY OF A NEW CANCER THERAPY AGAINST METASTATIC RCC BASED ON Cx32-DEPENDENT TUMOR SUPPRESSIVE EFFECT

Radiotherapy and available chemotherapeutic agents are ineffective for metastatic RCC.\(^{37,38}\) For instance, vinblastine (VBL) is one of the few cytotoxic agents with reproducible activity in RCC, but the outcome of treatment of metastatic RCC with VBL alone is often disappointing.\(^{39,40}\) This poor response rate to chemotherapy has led many physicians to immunotherapy using interferon-\(\alpha\) and interleukin-2, but the therapy has been effective in only a small percentage of patients with metastatic RCC.\(^{41}\) Therefore, establishment of new therapies that would provide more effective responses for a large number of patients is clearly needed for the treatment of metastatic RCC. P-glycoprotein (P-gp), the multidrug resistance (MDR)-1 gene product appears to function as an energy-dependent transport pump capable of decreasing the intracellular concentration of a wide range of anticancer agents such as VBL, which confers a chemoresistant phenotype on cancer cells.\(^{42}\) Since overexpression of P-gp has been found in nearly 80% of RCCs, the chemoresistance of RCC has been ascribed in large part to P-gp.\(^{43}\) Thus, P-gp seems to be an attractive target to improve chemotherapy in metastatic RCC.\(^{44}\) In our recent study, we have reported that Cx32 enhances VBL-induced cytotoxicity against Caki-1 cells via the down-regulation of P-gp within clinical doses in vitro as well as in vivo.\(^{45}\) Additionally, Cx32-dependent GJIC can also enhance VBL-induced cytotoxicity in Caki-1 cells. Due to GJ-dependent cell coupling, dying cancer cells can spread cell death signals into adjacent cells which then also die by apoptosis, and that the death messages which pass through GJ to kill cells are very likely calcium ions.\(^{46}\) These reports suggest that the effective doses of anti-cancer agents are reduced due to the propagation of cell death signals from dying cells to surrounding living cells via GJ. In fact, we observed that inhibition of Cx32-driven GJIC by an inhibitor towards GJ functions (18-glycyrrhetinic acid) partly abrogated VBL-induced cytotoxicity in Caki-1 cells having the expression of Cx32. Therefore, the combination of the GJ-dependent and -independent effects of Cx32, and chemotherapeutic agents such as VBL is a promising procedure to establish a new potential therapy against metastatic RCC (Fig. 3).

In order to establish the combination strategy for clinical usage, an effective procedure to induce expression of Cx32

in metastatic RCC is absolutely required. However, expression of Cx gene in cancer cells using viral vector includes the low efficacy of the viral vector in transferring the gene into the cancer cells, thus preventing efficient expression of the gene in the cells.\(^{57}\) It has been well recognized that methylation of cytosine residues in CpG islands of the gene promoter region is the most common mechanism of gene inactivation in cancers.\(^{48}\) We have also reported that loss of Cx function in RCC depends on methylation of the promoter regions.\(^{49}\) Additionally, loss of Cx function based on mutations or deletion of DNA has been found to be a rare event in cancers.\(^{50}\) These reports mean that demethylation of the promoter regions by DNA methyltransferase inhibitors is effective to gain Cx function in cancers. In fact, we have observed that two DNA methyltransferase inhibitors (decitabine and zebularine) can restore the expression of Cx32 gene in RCC cells in vitro.\(^{51}\) Taken together, it seems that the epigenetic approach leads to establishment of the combination strategy for clinical usage in metastatic RCC.

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