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Antibodies Targeting Extracellular Domain of Connexins for Studies of Hemichannels

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Abstract

Hemichannels are transmembrane channels composed of either a connexin or pannexin hexamer. The docking of the extracellular domains of connexin hemichannels contributed by neighboring cells forms a gap junction channel that joins the cytoplasm of adjacent cells. Connexins are expressed ubiquitously in different organs, but some subtypes are expressed exclusively in certain tissues and tumors. Both gap junction channels and hemichannels participate in diverse physiological and pathological responses. However, the lack of specific reagents that inhibit only gap junction channels or hemichannels is a challenge that makes it difficult to discern the specific roles of either channel. Fortunately, the available information regarding the connexin sequence, secondary and tertiary structure, and their biochemical and physiological properties permits the development of strategies to block exclusively the hemichannel activity exclusively, with no effect on gap junction activity. This task is accomplished through the use of specific antibodies that target the extracellular sites of desired connexin subtype. However, the underlying mechanism of how antibodies targeting extracellular connexin epitopes actually inhibit hemichannels remains unknown. Although these antibodies are being used for detecting and blocking of hemichannels in normal and tumor cells, they can also be potentially used for tissue-specific treatment and drug delivery in clinical applications. In this article, we will first review the literatures concerning the structure of connexins and the unique properties of extracellular loop domains of the connexins. Furthermore, we will discuss briefly the development of connexin (Cx) 43(E2) antibody, a specific antibody which detects the second extracellular loop of Cx43 and specifically prevents the opening of Cx43 hemichannels. We will then summarize the reported studies of specific reagents used for the inhibition of connexin hemichannels including antibodies developed against extracellular loop domains.

1. Overview of structures of connexins, gap junctions and hemichannels

Gap junctions are intercellular protein channels composed of connexins, which connect the cytoplasm of adjacent cells (Revel and Karnovsky 1967; Goodenough and Paul 2003; Kar et al., 2012). Until now, 21 different types of connexins have been cloned in humans and are named based on their molecular weight. For example, Cx43 means a connexin molecule with a predicted molecular weight of 43 kDa (Söhl and Willecke 2004). These channels permit the passage of short peptides, oligonucleotides and small molecules below 1 kDa,

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such as ions, second messengers and metabolites, which, in turn, allow the coordination between extracellular inputs and intracellular response under normal and stress conditions (kovacs et al., 2007). The intercellular channels are formed by the end-to-end docking of two hemichannels, also termed connexons (Yeager and Harris 2007) (Fig. 1). These structures are formed by the oligomerization of six connexin molecules at the trans-Golgi network and are packed into vesicles to be delivered to the plasma membrane (Saez et al., 2003; Shaw et al., 2007; Batra et al., 2012b). Many gap junction channels are clustered together to form a crystalline structures called gap junction plaques. Gap junction plaques are proposed to undergo two stages of assembly beginning with the loose packing of hemichannels and gap junction channels, followed by full assembly into gap junction plaques (Johnson et al., 2012). In isolated gap junction plaques, the membrane channels are tightly packed in a hexagonal lattice containing a 30° angle with a six-fold symmetry axis. With different isolation conditions and detergents, the lattice constant ranges from 74 to 90 Å (Makowski et al., 1982). Depending on connexin composition, the channel-channel distance in a highly ordered hexagonal lattice varies from about 95–100 Å (Hirokawa and Heuser 1982). This array may restrict the mobility of membrane-bound proteins and exclude other integral membrane proteins, which favors a reduction of the contact surface area necessary for interaction between two neighboring cells (Braun et al., 1984). The packing density is determined by the composition of lipids at spaces between the membrane channels (Henderson et al., 1979). In fact, the cholesterol content of isolated gap junction plaques is high compared to other membranes (Malewicz et al., 1990).

Hemichannels or connexons play important roles in communication between the cell and the extracellular environment (Goodenough and Paul 2003; Bennett et al., 2003). A previous study shows that vesicles packed with Cx43 hemichannels are sorted directly to adherent junctions that are juxtaposed to gap junctions by the microtubule plus-end-tracking protein EB1 and p150 (Shaw et al., 2007). Studies employing tetracysteine tagging and photobleaching of fluorescent-labeled Cx43 support the notion that Cx43 hemichannels are targeted to non-gap junction regions. Some of the hemichannels collapse into gap junction plaques and interact with undocked hemichannels from another cell (Lauf et al., 2002; Cooper and Lampe 2002; Gaietta et al., 2002; Simek et al., 2009; Johnson et al., 2012).

Connexins are integral membrane proteins with four transmembrane (TM) domains, 2 highly conserved extracellular (E) domains, 1 intracellular loop domain, and cytoplasmic N- and C-terminal domains (Saez et al., 2003). The C-terminus and intracellular loop domains are most variable between different connexins and are targets for intracellular regulatory proteins that control the activity and permeability properties of the channels (Saez et al., 2003; Sosinsky and Nicholson 2005). The N-terminus, TM1 and TM2 domains are involved in voltage gating regulation of the channel (Bargiello et al., 2012). Cysteine-scanning (Kronengold et al., 2003; Verselis et al., 2009) and three-dimensional crystal structure (Maeda et al., 2009) studies demonstrate that the N-terminal, TM1 and E1 of Cx46, Cx26 and Cx50 form the pore-proper. However, in other studies, the TM3 domain has been suggested as a major pore lining domain for Cx43 and Cx32 (Unger et al., 1999; Skerrett et al., 2002; Verselis et al., 2009). Additionally, the intracellular regions of the TM1 and TM3 domains form the channel entrance (Maeda et al., 2009). Both the E1 and E2 domains are involved in the docking interaction between hemichannels (Sosinsky and Nicholson 2005; Yeager and Harris 2007) (Fig. 1).

The extracellular region of the Cx26 hemichannel protrudes 23 Å from the membrane surface to interdigitate with the opposite connexon (Maeda et al., 2009). Molecular interactions stabilize the structure of gap junction channels. The crystallographic reconstruction of monomeric Cx26 shows that the secondary structure of the extracellular domains has a double-layered appearance with a double anti-parallel β sheet arrangement

joined by three disulfide bridges (Sosinsky and Nicholson 2005; Yeager and Harris 2007). The docking of the extracellular regions of opposite hemichannels requires an interdigitation of 6 Å and a rotation of 30°. This structural arrangement results in the inter-cellular 'gap' of 40 Å of the apposed extracellular domains (Perkins et al., 1998; Yeager and Harris 2007; Maeda et al., 2009), forming a dual concentric β -barrel configuration with the barrel formed by E1 inside and E2 on the outside (Nakagawa et al., 2010) (Fig. 1). It is proposed that the anti-parallel β sheet from the E1 and E2 domains in each connexin interdigitates with the β sheet of the E1 and E2 domains from the other connexin to form a β -barrel with a tight seal necessary to obtain a functional channel (Foote and Zhou 1998; Yeager and Harris 2007; Maeda et al., 2009) (Fig. 1). The crystalline structure of Cx26 shows a tight double-layered wall formed by the interaction of apposed extracellular domains of Cx26 hemichannels (Maeda et al., 2009). The inner wall is formed by the interaction of all E1 monomers. The molecular interaction is mediated by highly conserved residues of E1, such as Asn-54 that forms hydrogen bonds with the main-chain amide of Leu-56 in the opposite monomer, and Gln-57 forms symmetric hydrogen bonds with the same residue of the diagonally opposite monomer. The outer wall is made by the interactions of all E2 domains, and between each E2 domain, the interaction is predicted to be mediated by two amino acid residues, Lys-168 and Asp-179. The main-chain carbonyl groups of residues Thr-177 and Asn-176 form hydrogen bonds and salt bridges with the opposite monomer. All interactions among connexins connect the apposed hemichannels, allowing the tight seal required to separate the intracellular and the extracellular environment and create a functional gap junction channel (Maeda et al., 2009). The known amino acid sequence of the extracellular loop domains, E1 and E2, suggests that there are segments with hydrophobic residues favoring the hydrophobic interactions, which may play an important role in stabilizing these domains (Sosinsky and Nicholson 2005). A combination of urea, EDTA and high temperature is required to break the interaction between the channels (Ghoshroy et al., 1995). In addition, extracellular loops contain three aspartic acids and/or glutamic acids, and Asp-178 is highly conserved. These anionic amino acid residues are possibly involved in binding Ca^{2+} required for calcium-induced closure of Cx32 hemichannels (Gomez-Hernandez et al., 2003).

2. A challenge in the development of reagents that blocks hemichannels but not gap junction channels

It is now accepted that connexin hemichannels operate independently of gap junctions and also serve different roles. Hemichannels serve as a pathway for the interchange of chemical signals in an autocrine/paracrine manner between the intracellular and extracellular compartments (Goodenough and Paul 2003; Saez et al., 2003; Kar et al., 2012). Under resting conditions, hemichannels have a low opening probability and a low permeability to small molecules (e.g., ATP, NAD^+ , prostaglandins, small peptides, small fluorescent dyes, such as ethidium bromide, YoPro, DAPI, propidium, Lucifer yellow and sulphorhodamine) (Bruzzone et al., 2001; Cherian et al., 2005; Neijssen et al., 2005; Orellana et al., 2011a). Hemichannel activity is enhanced by covalent modifications of connexin subunits (e.g., S-nitrosylation, phosphorylation and dephosphorylation) (Contreras et al., 2003; Retamal et al., 2006), variations in membrane physiochemical conditions (e.g., membrane positive voltage, high pH, mechanical stimulation and low concentrations of monovalent and divalent cations) (Siller-Jackson et al., 2008; Schalper et al., 2010; Burra et al., 2010) and by extracellular signaling molecules (ATP, FGF1/2 and lipophilic molecules) (Garré et al., 2010; Retamal et al., 2011; Schalper et al., 2012; Kar et al., 2012). In contrast to the aforementioned stimuli which increase hemichannel activity, the endogenous aminosulphonates, like taurine, decrease hemichannel activity either through disruption of the interaction between the C-terminal tail and cytoplasmic loop or through occlusion of these channels.

One of the major challenges for connexin hemichannel research is the development of reagents specific for hemichannels, but not gap junctions. So far, short peptides with specific sequences homologous to extracellular connexin domains, termed connexin mimetic peptides, have been widely used to demonstrate involvement of connexin hemichannel in diverse cellular responses (Evans et al., 2006). Since extracellular loops are conserved between connexins, these mimetic peptides, which are believed to selectively bind to a homologous segment, are not unique to a specific connexin and thus can bind to the surface of several connexins (Evans et al., 2006; Wang et al., 2007). Furthermore, the mechanisms of action for connexin mimetic peptides remain unknown. The most studied connexin mimetic peptides are GAP26 and GAP27, which are homologous to the E1 and E2 loop domains, respectively. The binding of mimetic peptides to the extracellular domain of connexins still remains to be demonstrated empirically. Using atomic force microscopy-based signal molecule spectroscopy, it is shown that GAP26 recognizes and interacts with the extracellular loop domains of Cx43 reconstituted in lipid bilayer (Liu et al., 2006). This mimetic peptide affects the voltage gating properties and thus reduces hemichannel activity and permeability in about 3 minutes, likely through decreasing the flexibility and size of the vestibular space (Evans et al., 2006; Desplantez et al., 2012). The study from GAP26 supports the notion that these peptides do not affect the docking interaction and plaque formation, and have no significant effect on gap junction coupling if treated for less than 30 min (Sosinsky and Nicholson 2005; Evans et al., 2012; Desplantez et al., 2012). In addition, GAP26 does not affect the unitary conductance of Cx43 hemichannels (Wang et al., 2012). GAP27 also reduces the voltage sensitivity of Cx43 hemichannels in about 4 min (Wang et al., 2012). The slow inhibition kinetics of mimetic peptides (~ 3 to 4 min) suggests that the interaction site is hard to gain access to either because it is located deeper in the channel pore in a place that does not form part of the lumen and/or it becomes accessible only when the channel opens (Desplantez et al., 2012). The reduction of voltage sensitivity of Cx43 hemichannels induced by Gap26 or Gap27 mimetic peptides could be mediated by the interaction of the peptide with the charged amino acid residues, changing the voltage sensor and thus altering the activation threshold (Wang et al., 2012). A large number of studies demonstrate specific effects of these peptides on connexin hemichannels by using scrambled peptide as controls in conjunction with hemichannel blockers (Schalper et al., 2008). However, in the oocyte expression system, high doses (~1 mM) of GAP26 and GAP27, a Cx32 mimetic peptide, GAP24 and a pannexin 1 mimetic peptide, Panx1, cause a non-specific blockade of hemichannels formed by Panx1, Cx46, and a Cx32 chimera carrying the E1 domain of Cx43 (Cx32E143) (Wang et al., 2007). In addition, high concentrations of scrambled mimetic peptides inhibit Cx43 hemichannel activity in HeLa cells transfected with Cx43 (Wang et al., 2012), possibly by means of a steric occlusion mechanism (Wang et al., 2007; Wang et al., 2012). Further studies are required to identify the mechanism of action of these blocking peptides so that the specificity of these pharmacological tools may be improved and the role of functional hemichannels may be dissected. Development of antibodies to bind directly to extracellular epitopes could be a promising tool to understand the unique role of hemichannels without affecting the gap junction function. In this review, we will briefly discuss the development and current application of an antibody targeting the second extracellular loop of Cx43, which specifically blocks the hemichannels.

3. Overview of antibodies against the Cx43 extracellular loop domains and their applications

Antibodies are an important component of the immune system, which defend higher vertebrates against foreign intrusion. Antibodies are stable secreted proteins that act in extracellular fluids where they interact with specific epitopes of target proteins on the cell surface. The specific characteristics and available sequence information of target proteins

permit the development of a large number of antibodies for research applications (Wayner and Hoffstrom 2007). For example, antibodies against soluble epitopes, such as growth factors, cytokines or virus particles are used widely as neutralizing antibodies (Chames et al., 2009; Scott et al., 2012; Dinarello et al., 2012; Burton et al., 2012). Some extracellular antibodies developed against plasma membrane proteins including receptors, integrins, adhesion proteins, etc., can induce conformational changes, resulting in the activation or inhibition of the specific protein target and corresponding cellular responses (Meyer et al., 1992; Wayner and Hoffstrom 2007). Currently, about 30 monoclonal chimeric or humanized antibodies are commercialized as therapeutics, mainly for cancer and immune disorders (Chames et al., 2009; Scott et al., 2012).

The antibodies against the Cx43 E1 or E2 domains have been used in controlled digestion, electron and fluorescence microscopy to reveal the extracellular topology of Cx43. These experiments corroborate the predicted membrane topology of Cx43 (Laird and Revel 1990). Initially, the antibody raised against the E2 domain of Cx43 was used to detect the expression of Cx43 protein in cell lysates by western blotting and in fixed cells by immunolabeling (Meyer et al., 1992; Hofer and Dermietzel 1998; Baklaushev et al., 2009). Meyer and coworkers (1992) (Meyer et al., 1992) were the first to illustrate the effects of antibodies against the Cx43 E2 domain. Incubation with antibodies for the extracellular domains of Cx43 during the re-aggregation of disaggregated Novikoff cells impede cell-cell dye transfer and inhibit the assembly of gap junction structures. This study demonstrates that a functional interaction between the extracellular domains of the corresponding hemichannels is necessary for the formation of gap junction channels, and cellular interactions mediated by N-cadherin are required for gap junction assembly. Furthermore, Fab fragments of antibodies for N-cadherin or the Cx43 extracellular domains block adherens junction formation (Meyer et al., 1992).

Since two extracellular loop domains of connexins are highly conserved among various types of connexins and across mammals, it is rather difficult to develop a single connexin-specific antibody against these domains. aEL2-186, an antibody reported by Hofer and Dermietzel (1998) (Hofer and Dermietzel 1998), not only has a high affinity for the Cx43 E2 domain, but also has a reduced reactivity against Cx32. Cx43 and Cx32 share about 70% homology at the E2 domain. Aiming to detect and disrupt specifically the Cx43 gap junction function, Baklaushev and coworkers (2009) (Baklaushev et al., 2009) developed a monoclonal antibody against the Cx43 E2 segment, called Mabcx43E2.

All antibodies directed to the Cx43 extracellular loop domains would specifically detect hemichannels that have these domains facing extracellularly (Hofer and Dermietzel 1998; Siller-Jackson et al., 2008; Baklaushev et al., 2009). In contrast to smaller molecules, such as chemical compounds and peptides, an immunoglobulin is a voluminous protein with a molecular weight of 150 kDa, which makes it almost impossible to gain access into the gap junction plaques (with channel distance ~ 100 Å) and disrupt already formed gap junctions (Hirokawa and Heuser 1982) (Fig. 2). Indeed, assembled gap junctions do not interact with the aEL-46 or aEL-186 antibodies directed against Cx43 E1 or E2, respectively, unless the junctions in myocytes and astrocytes were disrupted (split) with alkali/urea, which break hydrogen and hydrophilic bonds between hemichannels of gap junction channels (Laird and Revel 1990; Lin et al., 2002). However, incubation with aEL-46 (directed to the E1 loop domain), aEL2-186 and Mabcx43E2 for a longer time blocks dye coupling activity in astrocytic cells and reduces plaque size, possibly because the dimensions of the antibody-hemichannel complex disrupt the docking and incorporation of the channels into the tight gap junction plaques (Meyer et al., 1992; Hofer and Dermietzel 1998; Baklaushev et al., 2011). Moreover, aEL-46 or aEL-186 Fab fragments inhibit the aggregation of freshly

prepared suspension cultures of C6-Cx43 cells, which shows the importance of these domains for hemichannel docking and cell-cell adhesion (Lin et al., 2002).

The oligomerization of connexins to form hemichannels occurs at the trans-Golgi network; therefore, the chance to detect monomeric forms of Cx43 at the cell surface has not been described to date. Incubation of live cells with antibodies targeting the connexin extracellular domains at low temperatures (to avoid antibody endocytosis) facilitates the visualization of only hemichannels on the extracellular surface of membranes (Batra et al., 2012a). The use of the aEL2-186 antibody for detection and hemichannel blocking also supports the notion of the presence of a free hemichannel pool at the plasma membrane domains (Hofer and Dermietzel 1998). Free Cx43 hemichannels in the tips of filopodia are detected by electron microscopy using aEL2-186 as a primary antibody followed by a secondary antibody labeled with gold particles. The mechanism responsible for the presence of concentrated hemichannels at the tips are unknown, however, it is suggested that cytoskeletal elements play a crucial role in either the clustering effect of hemichannels in a particular domain (Shaw et al., 2007; Simek et al., 2009) or the targeted transport of hemichannels directly to membrane apposition zone (Shaw et al., 2007). The reactivity at unopposed domains such as filopodia support the idea that filopodia and growing tips might play a pivotal role in the assembly and pairing mechanism of hemichannels in order to form functional gap junction channels (Hofer and Dermietzel 1998).

4. Development of the antibody against the second extracellular domain of Cx43, Cx43(E2)

Connexin hemichannels have extracellular loops exposed to the extracellular environment and could be an ideal target to develop inhibitory antibodies. We have recently reported the development of a polyclonal antibody to the E2 domain of Cx43 (Siller-Jackson et al., 2008), which can specifically detect cell surface-expressed Cx43 and block only hemichannels formed by this connexin. This antibody is called Cx43(E2). Since its development, Cx43(E2) antibody is widely used by various groups to determine the function of Cx43 hemichannels and the roles of Cx43 expressed on the cell surface, but not at the junctional plaques (Bao et al., 2011; Orellana et al., 2011a; Orellana et al., 2011b; Batra et al., 2012a).

The glutathione S-transferase (GST) gene fusion system was used to produce the antigens of the Cx43 extracellular loop domains. Three GST-Cx43 loop domain (E1, E2, and both E1 and E2 (E1-E2)) fusion protein expression constructs were made by the insertion of PCR amplicons into pGEX-2T plasmid (Amersham Biosciences) and were expressed (GST-E1 (A43-V75), GST-E2 (Y185-K206), and GST-E1-E2 (A43-V75+Y185-K206)) in *E. coli*, DH5 α cells. The fusion proteins were isolated using glutathione-sepharose 4B beads (Amersham Biosciences) and were further purified from degraded products by electro-elution of excised SDS-PAGE gel strips. These fusion proteins were conjugated with ovalbumin, which serves as a carrier protein, and were injected into rabbits to generate antiserum following the standard protocol (Pocono®). Both preimmune and anti-Cx43 sera were collected from the same rabbit. Cx43 antibodies against the extracellular loop domains were immunopurified by using two affinity columns: the first was a GST-conjugated column to remove antibodies against GST protein; the second column was conjugated with GST-Cx43 extracellular loop domain fusion proteins to affinity purify antibody specific for the 2nd extracellular loop domain of Cx43 (Siller-Jackson et al., 2008) (Fig. 3). Preimmune sera were collected and purified as described above. Three antibodies were developed and were used to test for immune reactivity, specificity and hemichannel-blocking function. Of the three antibodies, only the anti-Cx43 antibody against the E2 domain showed high immune specificity by western blotting and immunostaining. This antibody could block the function

of the Cx43 hemichannel but not the gap junction channel (Siller-Jackson et al., 2008). The antibodies generated targeting the E1 or E1+E2 domains showed a low immunoreactivity compared with the Cx43(E2) antibody. Interestingly, a similar outcome was also described in a previous paper by Hofer and Dermietzel (1998) (Hofer and Dermietzel 1998). Antibodies generated from peptides covering the E1 segment of Cx43 (amino acids 42–54 (EL1-42)) have a reduced immunoreactivity as compared with those made with the E2 domain (amino acids, 186–206 (EL2-186)). The latter yields a robust signal on western blotting of brain tissues, almost identical to the pattern obtained by serum against the Cx43 C-terminal domain. Therefore, this antibody (EL2-186) has been used for in vivo labeling of astrocytic hemichannels and for hemichannel blocking experiments (Hofer and Dermietzel 1998).

5. Application of the Cx43(E2) antibody in detection and inhibition of hemichannels

The epitopes of the Cx43 hemichannel recognized by the Cx43(E2) antibody are hard to elucidate because it is a polyclonal antibody generated against the entire second extracellular loop domain of Cx43 (Siller-Jackson et al., 2008). In permeable and fixed osteocytes, this antibody gives strong perinuclear reactivity with vesicular, fine intracellular punctate staining and a slight increase of reactivity in the cell border that was not detected with the pre-immune serum. Intracellular immunoreactivity distribution is comparable to Cx43 antibodies targeting the Cx43 C-terminus, except that the Cx43(E2) antibody does not detect junctional plaques in osteocytic cells (Siller-Jackson et al., 2008). The immunoreactivity of the Cx43(E2) antibody in impermeable cells is detected at the cell surface via confocal imaging with the distribution in the periphery of osteocytic cells. Co-immunolabeling shows that Cx43 co-localizes with integrin $\alpha 5$, in agreement with a distribution at the extracellular side of the plasma membrane (Batra et al., 2012a). In live osteocytic cells, Cx43(E2) antibody can block the Cx43 hemichannel activity without affecting the docking and function of the gap junction channels, even after 4 hrs of treatment with higher concentrations of the antibody (Siller-Jackson et al., 2008). Due to the bulky size of the antibody, the binding of Cx43(E2) to the E2 domain could sterically block the conformational changes that induce the opening and/or occlude the channel pore. This antibody has been shown to inhibit the permeability of these channels to small molecules including anionic, cationic dyes and endogenous ligands (ATP and PGE₂) (Siller-Jackson et al., 2008; Orellana et al., 2011a; Orellana et al., 2011b; Batra et al., 2012a). Cx43(E2) antibody is also shown to block electrical current with a unitary conductance compatible with Cx43 hemichannels (~ 220 pS) in microglia, tanicytes and astrocytes. Similar lack of conductance is observed in these cells pretreated with GAP27 or an Cx43(E2) mimetic peptide, and also in isolated astrocytes from glial fibril acidic protein (GFAP)-cre Cx43^{-/-} flox mice, a conditional knockout in astrocytes (Orellana et al., 2011a; Orellana et al., 2011b). Cx43(E2) antibody blocks the aggregation and adhesion between normal and tumor cells seeded into non-adhesive agarose gels (conditions that allow microtissue self-assembly in a three-dimensional cell culture) by a disruption of hemichannel docking, function and reduction of N-cadherin based interactions (Bao et al., 2011). Therefore, in addition to its effect on the blockage of hemichannels, this antibody might also block cell-cell interactions that require the close apposition of cells.

6. Research advances and future perspectives of development and application of blocking antibodies specific to hemichannels

Connexins are present in almost all tissues and organs except in healthy adult skeletal muscle fibers and erythrocytes (Cea et al., 2012; Kar et al., 2012). Connexins show tissue-

specific expression as well, i.e. hepatocytes preferentially express Cx32 and 26, and lens fibers primarily express Cx46 and 50 (Kar et al., 2012). This characteristic makes the extracellular epitopes of connexins suitable targets for drug development towards tissue-specific pathologies as speculated by Baklaushev et al. 2009 (Baklaushev et al., 2009). As summarized in Fig. 4, the extracellular domains of connexins can be used as potential targets for the delivery of antitumor therapies and genetic material to the target cells. This approach can resolve problems of undesired effects of chemotherapy. Nanobiotechnological approaches employing selective nanoparticles (NPs) and nanocontainers such as liposomes can be loaded with polar and apolar substances, i.e. chemotherapeutic drugs. Nanoparticles coated with Mabcx43E2 are used to deliver fluorescent and paramagnetic tracers. This reagent was injected in rats under control condition or gliomas that overexpress Cx43. Fluorescent-labeled liposomal nanocontainers or paramagnetic immunoliposomes were detected at the periphery of the glioma 48 or 6 hours after injection, respectively. These data suggest that immunoliposomal nanocontainers containing antibodies to E2 domain of Cx43 can be made suitable for targeted delivery of diagnostic and therapeutic drugs to the peritumoral invasion zone of high-grade gliomas (Baklaushev et al., 2009). These results increase the possibilities for use of connexin extracellular antibodies as a magic bullet for treatment. It is encouraging to further investigate the role of connexin hemichannels under physiological and pathological conditions, like ischemia or chronic inflammation, both with an exacerbated hemichannel activity (Schalper et al., 2009), and to develop new and innovative therapies to pathologies with unknown etiology.

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- Overview of structures of connexins, gap junctions and hemichannels
- A major challenge in development of reagent that block hemichannels, but not gap junctions
- Review antibodies against Cx43 extracellular loop domains and applications
- Process of generating a specific antibody against 2nd extracellular loop of Cx43 and application
- Research advances and future perspectives of development and application of blocking antibody

Figure 1.

Membrane topology and organization of a Cx protein, hemichannels and gap junctions. This diagram was drafted based on a structural model of gap junctions by Nakagawa et al. 2010 (Nakagawa et al., 2010). The connexin protein has 4 transmembrane domains (TM), 2 extracellular loop domains (E) and at the cytoplasmic side, it contains the N-terminus (NH₃⁺), C-terminus (COO⁻) and intracellular loop domain (IL). The assembly of six connexin molecules forms a hemichannel. The black box shows the region represented at the top right with a top view of one hemichannel from the extracellular side. The diagram illustrates the disposition of extracellular loops in the hemichannel topology with each of six connexins that form the hemichannels presented in different colors. The E2 and E1 are bounded by 3 disulfide bridges, E2 of 6 connexin molecules are surrounded by 6 E1, forming part of the vestibule of a hemichannel. The double layer barrel makes a tight seal separating the interior from the extracellular ambient.

Figure 2.

Diagram illustrating an antibody that recognizes Cx43 (blue) extracellular loop epitopes. Since gap junctions are tightly packed arrays of channels with a limited interchannel space, the IgG molecule cannot get access to epitopes located in the gap junctional plaques. In this diagram, Cx43(E2) antibodies might interact with homomeric (formed with the identical connexin molecules,) or heteromeric (composed of different kinds of connexin molecules) hemichannels present at the extracellular cell surface. In theory, even the Cx43(E2) antibody might interact with committed hemichannels, which can form gap junction channels, before the docking event. This interaction, in particular, may block hemichannel functions such as opening, adhesion and docking.

Figure 3.

Schematic diagram illustrating the development of Cx43(E2) antibody. A DNA fragment containing the E2 domain of Cx43 (amino acids: 185–206) was cloned into the pGEX-2T vector. The fusion protein, GST-Cx43(E2), was expressed in *E. coli* and then was isolated using a glutathione-sepharose bead column. To generate the anti-serum, the fusion protein was conjugated and then injected into rabbits. The anti-serum was purified using two affinity columns; first conjugated with GST protein to remove antibodies against GST protein and second conjugated with the GST-Cx43(E2) domain to affinity purify Cx43(E2) antibody.

Figure 4.

Current and future applications of Cx43(E2) antibody. Cx43(E2) antibodies have been used for immunoblotting of cell lysates, and immunofluorescence or immunohistochemistry of live and fixed cells (Siller-Jackson et al., 2008; Batra et al., 2012a). This antibody has also been used to block hemichannel function in two ways: preventing the Cx43 homomeric or heteromeric hemichannel opening or blocking the docking interaction of Cx43 hemichannels (Siller-Jackson et al., 2008). On the right are examples for potential future applications. Monoclonal Cx43(E2) antibody coated liposomes were successfully tested in rats with glioma tumors with high efficiency of cargo delivery on the tumor cells (Baklaushev et al., 2009). Injection with monoclonal Cx43(E2) antibody labeled with radioisotopes or fluorescent molecules is used for detecting and labeling glioma cells rich in Cx43. This approach can be used for detection of other tumor cells that express Cx43 in high levels on the cell surface. Cell-cell interaction and aggregation are important for stress protection, concentration of soluble protecting/growth factors and proliferation. It is shown that Cx43(E2) antibody can block docking interaction, inhibit N-cadherin interaction and reduce gap junction plaque size and cell-cell communication (Bao et al., 2011). Additionally, Cx43(E2) antibodies could be used to block Cx43 hemichannels in cells, that require hemichannels for the release of small molecules to carry out cellular function.