Commentary

Cytokine Regulation of Gap Junction Connectivity

An Open-and-Shut Case or Changing Partners at the Nexus?

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In this issue, Chanson et al¹ present data that document the failure of airway cells expressing the mutant form of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene to down-regulate gap junction connectivity after treatment with the cytokine tumor necrosis factor- α (TNF- α). However, this response could be restored by transfecting in the wild-type CFTR gene, suggesting that a functional CFTR gene is necessary for regulation of gap junctional communication by TNF- α . The authors postulate that the persistence of gap-junction connectivity in the inflamed CF airway epithelium permits the intercellular diffusion of signaling molecules that serve to activate neighboring cells, thus contributing to the excessive inflammatory response characteristic of the CF airway epithelium. Although these conclusions are based only on estimation of gap-junction connectivity using transfer of the gap-junction permeant dye Lucifer Yellow, the data fit well with several studies that have documented regulation of gap junction connectivity in a number of different cell types by proinflammatory cytokines such as TNF- α and interleukin-1 β (IL-1 β).^{2–8} These interesting observations raise the questions of how inflammatory cytokines might regulate gap junction connectivity and in what way expression of the mutant CFTR gene could influence this response.

Evidence for Loss of Gap Junction Connectivity in Vivo

Alterations in gap junction expression have been noted in a number of different disease conditions with an underlying inflammatory process. So, for example, in the liver, a rapid loss of Cx32 mRNA and protein was observed after the induction of an acute inflammatory state by injection of endotoxin.^{6,9} Ischemia, which is known to up-regulate the expression of cytokines such as IL-1 β and TNF- α in many tissues, has also been associated

with down-regulation of gap junctions composed of Cx32 in the liver¹⁰ and Cx43 in the heart.¹¹ A more direct role for TNF- α in mediating down-regulation of Cx43 in the heart after administration of bacterial endotoxin has been demonstrated by analysis of the response of the Cx43 promoter to this cytokine.⁷ However, injection of endotoxin into the kidneys or lungs resulted in increased expression of Cx43 at these sites.¹² Although these data appear to be contradictory, it is possible that the influx of inflammatory cells could contribute to this response, since Cx43 has been detected in activated leukocytes.¹³ However, whether gap junctions form in macrophages remains controversial, and our own experiments failed to detect the presence of functional gap junctions or hemichannels in these cells.¹⁴

More recently, we have examined the expression of Cx43 in reactive astrocytes associated with MS lesions. These lesions are known to contain high levels of IL-1 β , a cytokine that down-regulates gap junction connectivity, as well as Cx43 mRNA and protein in human astrocytes *in vitro*.⁸ By immunohistochemistry, the data show a marked loss of Cx43 immunoreactivity within the center of active and chronic-active MS lesions, with normal expression of Cx43 immunoreactivity in the adjacent normal-appearing white matter (G. R. John, manuscript in preparation). Taken together, these data support the conclusion that gap junction expression may indeed be altered at sites of inflammation *in vivo* and could thus contribute to the pathogenic state.

Implications for Cell-Cell Communication in Inflamed and Injured Tissues

Gap junctions are considered to form the molecular link for coordinated long-distance signaling among individual members of various syncytia. The most widely studied of these coordinated responses is the propagation of cal-

Accepted for publication March 12, 2001.

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cium waves after stimulation of any one cell within the network. Gap junction-mediated intercellular communication can potentially be life-saving or catastrophic for survival of cell populations challenged with injury or cell death to their members. The potential benefit of intercellular coupling is illustrated by the phenomenon of metabolic cooperation, in which it has been demonstrated that cocultures of cells, each deficient in thymidine or purine nucleotide synthesis, resulted in cell populations with growth rates equivalent to those of wild-type cells.¹⁵ The rescue of cell populations that would otherwise be destined to die has been termed the "kiss of life" or the "Good Samaritan effect." In inflammatory conditions, the closure of gap junction channels could have several distinct advantages for the host tissue. It could serve to restrict the passage of activatory molecules to neighboring cells, thereby containing the spread of the inflammatory response, as suggested by Chanson et al.¹ Closing of gap junction connections could also restrict the spread of apoptotic signals to adjacent cells within the network; consistent with this are studies that have shown a reduction of infarct size in both the heart and the brain when coupling of cells is reduced.^{16,17}

Alternatively, intercellular communication may be a bad thing, if highly toxic molecules produced in one cell are small enough to penetrate the channels from one cell to another. Such an effect was originally shown for transfer of toxic nucleotides by Fugimoto et al¹⁸ and termed the "kiss of death"; more recently, the same phenomenon has been termed the "bystander effect" and used to increase the extent of tumor cell killing with gancyclovir.^{19–21} Moreover, loss of the ability to communicate with adjacent cells could also prove detrimental to the normal coordinated functioning of affected tissues, as well as significantly impede the formation of an organized response to insult. This could be particularly important in the induction of an appropriate protective response against noxious substances and/or infectious agents. Recent studies of calcium wave formation, however, have suggested that cells that function as a coordinated syncytium use two distinct pathways to mediate this response: an intercellular and an extracellular pathway, with gap junctions composing the intercellular pathway and signaling via P2 receptors the extracellular pathway.²² Extracellular nucleotides function as ligands for P2 receptors, which fall into two major classes: metabotropic P2Y receptors and ionotropic P2X receptors. Activation of metabotropic P2Y receptors leading to the release of Ca²⁺ from intracellular stores is generally considered to be the extracellular mechanism involved in the generation of calcium waves in non-excitable cells.

A particularly striking observation that has emanated from studies of cells in which gap junction connectivity is lost either by cytokine-induced down-regulation⁸ or through gene-targeting techniques²³ is that this can be shown to result in the enhancement of signaling via the extracellular pathway. Furthermore, in both the Cx43 knockout mouse and in IL-1-treated human astrocytes, this enhanced P2Y receptor signaling was associated with a functional switch in the P2Y nucleotide receptor subtype from one dominated by a response to ATP

(P2Y₁-like) to one in which ATP and UTP were equipotent (P2Y₂-like).^{8,23} This functional switch in P2Y receptor expression was further supported by reverse transcription-polymerase chain reaction evidence of enhanced P2Y₂ expression in the IL-1 β -treated cells.⁸ These data show that communication within the astrocytic syncytium is sustained by a finely tuned interaction between gap junction-dependent and -independent mechanisms, such that a reduction of gap junction-mediated intercellular communication in Cx43 knockout mice is compensated for by an increased autocrine communication. Such an interplay between gap junctions and paracrine/autocrine signaling provides a high degree of plasticity for intercellular communication between astrocytes. Whether a similar maintenance of syncytial activity will be found in other cell systems, as well as for responses other than calcium wave formation, will be an interesting question to address.

What is the possible relevance of an interplay between gap junctions and P2 receptor signaling for inflammatory gene expression in the inflamed airway epithelium? Firstly, we have been able to show that in astrocytes signaling via P2 receptors intersects with components of the cytokine signaling cascade, modulating the nature of the inflammatory genes activated in these cells by cytokines such as IL-1 β and TNF- α .^{24,25} These data fit well with accumulating evidence that in cells of both myeloid and non-myeloid origin, agonists or antagonists of P2Y and P2X receptors modulate the inflammatory cascade. So, for example, it has been shown that in human macrophages, ATP provides a second stimulus required for the processing and secretion of lipopolysaccharide-induced IL-1.^{26,27} In these studies, the activation of the P2X₇ receptor has been implicated, which results in the formation of a large transmembrane pore that allows the bidirectional passage of molecules up to 900 Da.²⁸ Additional data supporting a role for this receptor in lipopolysaccharide-activated cytokine production have recently been generated in mice in which the P2X₇ gene has been inactivated.²⁹ ATP has also been found to modulate the generation of reactive oxygen intermediates^{30,31} and to regulate lipopolysaccharide-induced nitric oxide synthase II and TNF expression both *in vivo* and *in vitro*^{30–35} in macrophages, as well as IL-1-induced cytokine and chemokine expression in astrocytes.^{24,25} The different specificities for endogenous agonists displayed by P2 receptors suggests that the composition and concentration of nucleotide triphosphates, diphosphates, and monophosphates, as well as nucleosides, in the extracellular space provides the cell with important information concerning changes in the extracellular environment and, in the case of ciliated epithelium, may help coordinate cell activity.³⁶ Furthermore, changes in the expression of these receptors may alter sensitivity to these events. High levels of extracellular nucleotides are released from sources such as platelets, activated leukocytes, and damaged or dying cells, in a number of injurious conditions. This has led to the hypothesis that autocrine/paracrine activation of P2 receptors permits different cell types to communicate with each other and with the extracellular environment through the release

and sensing of nucleotides such as ATP and to use this information to fine-tune the response to the extent and nature of the injury.^{24,25}

Secondly, in recent years it has been proposed that extracellular nucleotide activation of P2 receptors may be useful in the symptomatic therapy of CF. In particular, UTP acting on a P2Y₂ receptor has been shown to activate alternative Cl⁻ conductances primarily via the phospholipase C/inositol 1,4,5-triphosphate/intracellular Ca²⁺ signaling pathway, leading to restoration of salt and water secretion via the activation of a [Ca²⁺]_i-mediated anion conductance.^{37,38} Additional studies now support the conclusion that activation of P2Y₂ receptors may also restore a portion of electrogenic bicarbonate secretion in CF airways expressing the [Ca²⁺]_i-mediated anion conductance pathway, which may help to normalize trans-luminal pH across CF epithelia.³⁹ Support for the concept that the P2Y₂ receptor is the dominant P2Y purinoceptor that regulates airway epithelial ion transport has been provided by studies in mice with targeted gene deletions of the P2Y₂ receptor.⁴⁰

Thus, if indeed the expression of Cx43 and the P2Y₂ receptor show a similar inverse relationship in other cell types, then the failure to down-regulate gap junctions in response to TNF- α in airway epithelial cells expressing the mutant form of the CFTR gene would block the shift from P2Y₁ to P2Y₂ expression, rendering the cells less sensitive to the potentially beneficial effects of P2Y₂ receptor agonists. The mechanism by which Cx43 and P2 receptor expression is linked is unknown. One possible way this might occur would involve the different selective diffusion of signaling molecules through gap junction channels formed of different connexins. Such a mechanism recently was proposed for osteoblastic cell lines in which transcriptional activities of osteoblast-specific promoters were modulated in opposite directions by overexpressing either Cx43 or Cx45.⁴¹ Alternatively, the recent recognition of protein-protein interactions involving connexins allows the possibility that expression of Cx43 might recruit a specific type of P2 receptor to the membrane preferentially (as occurs via the direct binding of ZO-1^{42,43}) or might selectively affect P2 receptor gene expression directly or via a binding protein.

Connexin-Protein Interactions: The Nexus

Intercellular communication can be regarded as a collective process involving gap junctions, membrane receptors, and other membrane and cytosolic proteins whose activities are coordinated. As for other specialized membrane domains, such as caveolae and synapses, the integral membrane components of gap junctions appear to be linked into a macromolecular complex, the Nexus.⁴⁴ Because cytoplasmic domains differ greatly among the 16 known connexins, it seems likely that Nexus components may also vary, and binding affinities within the Nexus containing an individual connexin may be altered by such factors as cytosolic pH, phosphorylation, and binding of other components. For the Cx43 Nexus, binding sites include src homology (SH) and PSD, disks large, zonula

occludens (PDZ) binding domains on the Cx43 molecule, and most probably other domains as well.

Connexin43 (Cx43) is a tetraspan membrane protein with cytoplasmic amino and carboxyl termini (NT and CT) and a cytoplasmic loop (CL) connecting the second and third transmembrane domains.⁴⁵ Protein-protein interactions between CT and CL are hypothesized to be responsible for channel closure by acidification,⁴⁶ and the ~16-kd carboxyl terminus contains multiple phosphorylation sites, WSH2/SH3 binding sites, and a single distal PDZ recognition motif. Interactions of Cx43 with proteins containing PDZ, SH2, and SH3 domains is thus hypothesized to serve as a scaffold on which to assemble components of the intercellular signaling pathway into the multiprotein Nexus complex, which couples their activity to downstream signaling molecules.

PDZ domains are approximately 90 amino acid modules, which mediate protein-protein interactions by binding to the last 3 or 4 amino acids of the C-termini of their target proteins. The specificities of PDZ domain-containing proteins are quite diverse, with the minimal recognition motif being a hydrophobic residue at the carboxyl terminus.⁴⁷ In the case of Cx43, the second PDZ domain of the tight junction-associated protein zonula occludens (ZO)-1 has been shown to interact with the most distal four amino acids (DLEI^{42,43}), and such interaction has been implicated in targeting Cx43 to cell-cell interfaces.⁴³

The effectors possessing association motifs, known as src homology 2 (SH2) and src homology 3 (SH3) domains, are protein modules of about 100 and 50 amino acids, respectively.^{48,49} SH2 domains bind to proteins containing phosphotyrosine regions and thus regulate signal transduction events involving tyrosine phosphorylation.⁵⁰⁻⁵² The SH3 domains, which bind to proline-rich regions, are supposed to regulate signal transduction involved in cytoskeletal organization and cell morphology.^{48,49} In the case of Cx43, the proline-rich region of the carboxyl terminus comprises amino acids 273-285, and a phosphorylated tyrosine at position 265 has been shown to interact with the SH2/SH3 domain-containing proteins v-src (pp60) and c-src.⁵³⁻⁵⁵ Phosphorylation of Cx43 CT by v- and c-src may be involved in decreased gap junctional conductance^{43,54-58}; c-src binding to Cx43 has been shown to result in loss of interaction with ZO-1.⁵⁵

Modulation of Coupling Strength

One of the most fundamental questions raised by the report of Chanson et al¹ concerns the mechanism by which Lucifer Yellow dye spread is reduced in non-CF cells treated with TNF- α . Reduction in intercellular communication was detected within 2 minutes after TNF- α addition and reached a plateau at 20 minutes, coinciding with the plateau of induction of NF- κ B translocation to the nucleus. The rapidity of the uncoupling would appear to constrain the probable mechanisms to those modulating activity of channels already present in the junctional membrane, rather than acting on connexin synthesis or degradation, which is consistent with unpublished results cited in Chanson et al¹ that Cx43 expression is not com-

promised by treatment with TNF- α for as long as 90 minutes. Plausible candidates for transduction of the Lucifer Yellow uncoupling would include direct closure of the gap junction channels by cytoplasmic factors such as low pH or lipophilic products of phospholipase activation. It is also possible that kinase activation by TNF- α triggers a transduction cascade that closes the intercellular channels either as a direct result of conformational change in Cx43 or as a consequence of altering the affinities of Cx43 to its binding partners within the Nexus.

Although the concept that ion channels (such as connexins, P2Y receptors, and CFTR) may interact with each other as well as with other proteins (such as ZO-1 and other PDZ and/or SH domain proteins) is relatively new, the implications of this concept for intercellular signaling under physiological and pathological conditions are profound. As efforts to understand the interactions highlighted by Chanson et al¹ proceed, we anticipate that the links between cytokines and the channels that mediate intercellular signaling (especially gap junctions and P2 receptors) will become clearer and will point the way toward therapeutic intervention for the regulation of inflammation in a number of different cell types and tissue pathologies.

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