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MEETING REPORT

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Gap junctions in health and disease

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Abstract An international symposium was held on gap junctions in health and disease in Regensburg, Germany, gathering together a panel of international scientists who discussed normal functions of gap junctions and their contribution to a variety of human diseases. The emphasis was on strategies and models for a better understanding of gap junction-mediated cell-to-cell communication in a variety of tissues, including null mutations of gap junction genes in recombinant transgenic mice. The topics varied from the normal function of cardiac gap junctions and its contribution to cardiac dysfunction up to the recently discovered point mutations of a gap junction gene encoding the gap junction protein connexin32 in Charcot-Marie-Tooth syndrome of the X1 type. A perspective of the future development of gap junction research and its contribution to unravelling pathophysiological mechanisms of human diseases was given by M.V.L. Bennett.

Key words Gap junctions · Genes · Connexin · Cell-to-cell communication

Introduction

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Gap junctions constitute intercellular channels that allow the direct transport of ions and metabolites, including second-messenger molecules, between adjacent cells without leakage into the extracellular space. This form of short-range signal transfer is collectively termed "direct cell-to-cell communication". It exists in all parenchymal organs and between stromal cells with the exception of mature skeletal muscles, spermatocytes and circulating blood cells. Gap-junction channels are formed by connexin proteins, which are encoded by a family of at least 13 (soon to be 14) genes. Connexin genes have a common structure, with each gene exhibiting a single intron that separates a small noncoding upstream exon from a larger exon that contains the entire coding sequence. In the human and mouse genomes, these single-copy genes map to at least four chromosomes, including chromosome X. Structurally, the gap-junction channel is thought to consist of two identical hemichannels (or connexons), each of which is formed of six subunits (Fig. 1).

Importantly, the biophysical properties of gap-junction channels depend on the type of connexins that form the channel. Recent evidence suggests that functional criteria such as voltage dependence, pH sensitivity, degree of phosphorylation, and conductance are determined by the connexon types that combine with each other to form a functional and competent gap-junction channel. Since the expression of gap-junction channels varies widely in dif-

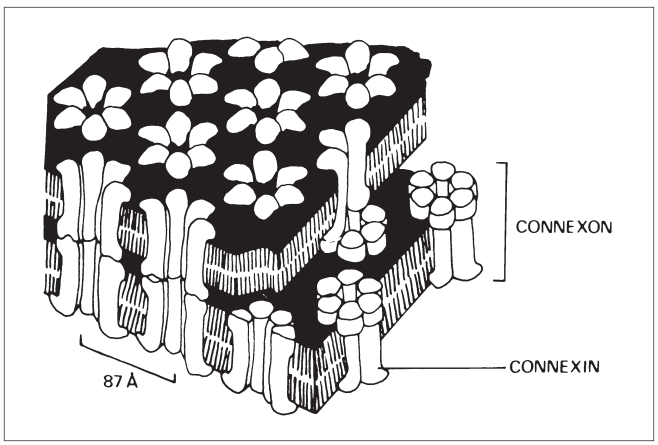

Fig. 1 Model of a gap junction with hemi-channels (connexon) and hexameric oligomerized subunits (connexin)

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ferent tissues, we have to envisage considerable differences in the functional properties of gap junction-coupled compartments within the various organ systems. By learning the molecular make-up of gap junctions we will gain a better knowledge on their normal function and hopefully of the various disease states in which this structure is involved. The contributors to this meeting report will pass on information about the state of the art in gap junction research and discuss the implications that alterations in gap junction expression, distribution and function may have for the normal function of tissues.

Gap junctions in health and disease: a perspective

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Holding an "International Symposium on Gap Junctions in Health and Disease" implies that there are diseases in which gap junctions play an important part. In the study of normal function, that is of health, one can describe what an organelle such as a gap junction does, and often the function is demonstrated by removing the structure or blocking its action, or more recently by overexpressing it. Then malfunctions associated with loss or gain of normal function become candidates for a disease process, as well as delineating the action in health. Gap junctions have been around for almost 40 years, but only recently has it become possible to have a symposium that includes presentations on real diseases in which alterations in gap junctions are demonstrated.

Historically, gap junctions were recognized as a substrate of electrical transmission between cells, especially cardiac muscle and certain neurons [1]. Speed of transmission and reciprocity of action were plausible explanations of their occurrence in most instances. Suggested diseases or disease processes included recurrent propagation in heart muscle and associated excessive positive feedback in epilepsy. Several years later, gap junctions were found to mediate the exchange of small molecules, and the roster of permeant substances now includes nucleotides, second messengers and molecules of intermediary metabolism, but not proteins and nucleic acids. Moreover, there appear to be differences in the permeability of different types of gap junction. Ca^{2+} should be included as a permeant second messenger, although there was a group that held that Ca^{2+} closed gap junctions and ignored evidence indicating that it would cross the junctions at low concentrations.

Gap junctions were thought to mediate "metabolic cooperation" in which cells that were metabolically capable of synthesizing some essential intermediate could supply it via gap junctions to other cells that were incapable of making it [3]. This process was readily demonstrated in tissue culture and proposed as an explanation of how X-linked diseases could be asymptomatic in females heterozygous for a loss of function mutation on the X chromosome. Although X-inactivation or lyonization would result in half the cells lacking the function, gap junctional communication with cells in which the normal X chromosome was active was thought to rescue the incompetent cells. A problem not considered, at least not by this author, was the grain of the mosaic of lyonization, which can be much too coarse to allow diffusional communication between competent and incompetent cells.

A question from this early period was why there were gap junctions between seemingly identical cells that did not generate action potentials. The uninteresting possibility was that gap junctions just helped out by allowing cells doing well at some time to help other cells that were doing not quite so well. Whether it was a matter of closeness to nutrient sources or randomness in gene expression, innervation, or something else, the spatial and temporal cooperation permitted might make a system operate better. The operation was not obviously much better, but even a small improvement provides powerful selection over evolutionary time frames. When it became apparent that the proteins forming gap junctions, or connexins, were encoded by a gene family now of 12 (soon to be 14) members [8], it seemed likely that if loss of a connexin were not an embryonic lethal, there would be genetic diseases associated with mutations in these genes. Genetic diseases are an excellent expression of Murphy's law ("Anything that can go wrong will [go wrong]").

Cloning of connexins has led to dramatic advances, including in situ hybridization to determine which connexin RNAs are expressed where. Antibodies can be made to specific (or common) sequences permitting determination where the proteins are expressed, and some of these antibodies allow identification at electron microscopic resolution. Patch clamping permits recording from small cells, and tissue culture makes many more cell types accessible. New blocking methods of increased specificity, targeted gene knockout by homologous recombination and transgenic mice provide the means for determining what loss or gain of function can do. The modern cell biology and biophysics of gap junctions make the analysis of the healthy function much more direct and demonstrate or suggest where they are relevant to disease. Some of the early questions about the roles of gap junctions have been clarified; for some there is still no satisfactory answer.

Cardiac gap junctions: discontinuous conduction in normal and diseased heart

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Impulse conduction in the heart is microscopically discontinuous, in that action potentials are regenerated in each cell and pass from one cell to the next with measurable delays. This discontinuity is due to each cardiac myocyte's being connected to other cells through gap junction channels, and results in anisotropic conduction attributable to nonuniform cellular geometry and to nonuniform gap junction distribution between the myocytes [15]. Recent studies of human gap junction channel diseases and animal model systems resulting in connexin overexpression or loss of function suggest that alterations in the expression and arrangement of gap junctional channels within the heart may have a strong influence on the propensity for cardiac arrhythmogenesis by modifying the extent of discontinuous propagation, and that disrupted expression may also result in abnormal cardiac development.

Gap junction channel proteins (connexins) are encoded by a gene family with more than a dozen members in rodents; three connexins are expressed in cardiac myocytes (Cx40, Cx43, Cx45). Cardiac dysfunctions appear to be mediated by several types of connexin disturbances. In transgenic animals, over- or underexpression of Cx43, the major gap junction protein in the cardiovascular system, leads to embryonic laterality deficits. In mice in which Cx43 has been deleted through homologous recombination, the right ventricle is hypertrophied to such an extent that outflow is impeded, and animals die at birth owing to pulmonary artery stenosis [13]. Cardiac myocytes cultured from these animals display much lower junctional conductance than those from wild-type littermates, virtually no spread of intracellularly injected Lucifer Yellow, less regular and less coordinated spontaneous beating, and retarded spread of Ca^{2+} waves from one cell to the next under Ca^{2+} overload conditions. Control mouse cardiac myocytes exhibit channels corresponding in unitary conductance to those of Cx43 and Cx40, whereas in Cx43 KO animals only channels corresponding to Cx40 are detected. Laterality defects may also occur in humans with Cx43 mutations. Though still somewhat controversial, it has been claimed that a subset of heterovisceral atriotaxia patients (the phenotype apparently characterized by concomitant polysplenia or asplenia) display coding region mutations within the phosphorylatable serine residues in the cytoplasmic tail of the molecule [4]. Biophysical studies on cells transfected with human Cx43 mutated in this region, however, show only modest electrophysiological differences, and even severely truncated Cx43 can still form functional channels.

Following myocardial infarction in both patients and animal models, gap junction expression is reduced, exaggerating the anisotropic nature of ventricular conduction [14]. Because lateral gap junctions are less abundant than longitudinal ones, and because of the collagen deposition in infarcted areas, this remodeling of connections can result in enhanced discontinuity in impulse conduction. Resultant slowing of conduction and the appearance of longer conduction pathways leads to the appearance of re-entrant circuits in animal models. A particularly striking example of gap junction rearrangement is seen in cardiac myocytes in tissue culture infected with *Trypanosoma cruzei* [6], the causative protozoan parasite in Chagasic cardiomyopathy. In these cells, Cx43 staining at cellular boundaries virtually disappears, although abundance of both Cx43 and its mRNA is not affected. In this case, infection produces changes in conduction that are even more extreme than in the Cx43 KO model, and the cause of the Cx43 disappearance appears to be high-affinity interaction between the parasite and the gap junction protein.

In summary, in a variety of examples cardiac conduction disturbances can be regarded as gap junction-related diseases. It follows that it is worth exploring the possibility that therapeutic measures might be developed in which cardiac gap junetions or their genes are targeted. Although such studies are only just beginning, spinoffs for further gap junctions research might include the development of gap junction-specific pharmacological and genetic tools.

Insulin secretion and gap junctions

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Insulin secretion results from the activity of about 2×10^9 β-cells (in humans), which are clustered in pancreatic islets. Coordination of these cells is achieved via their interaction with neurotransmitters and numerous signal molecules carried by blood and also with neighbouring cells. The latter interactions are thought to be of prime importance, since insulin secretion is markedly al-

tered after disruption of cell-to-cell contacts and is rapidly corrected after cell reaggregation. Since β-cells are functionally connected by gap junctional channels, we have assessed whether the direct intercellular exchanges of cytoplasmic ions and small molecules that these channels permit (an event also referred to as junctional coupling) participate in the synthesis, storage and release of insulin.

We have found that single β-cells (which cannot form gap junctions) show alterations in both basal and stimulated release of insulin, in protein biosynthesis, and in the expression of the insulin gene. Restoration of β-cell-to-cell contacts is paralleled by an improvement of these defects, and in particular by a rapid increase in insulin release. This change, however, is prevented in the presence of either alkanols, which block gap junction channels, or of antisense oligonucleotides designed to interact specifically with the mRNA coding for the gap junction protein Cx43. We have further observed that sustained stimulation of insulin release is associated with an increase in β-cell coupling and in the expression of gap junctions and Cx43, both in vivo and in vitro. Other experiments have revealed that conditions inhibiting insulin release decrease or abolish β-cell-to-cell coupling. In vivo, however, these conditions result in hyperglycaemia and increased coupling, suggesting that the level of circulating glucose and the ability of β-cells to recognize the sugar properly may influence junctional channels independently of each other. In addition, the acute pharmacological blockade of junctional channels markedly increases the basal release of insulin and abolishes that stimulated by glucose in both isolated islets of Langerhans and intact pancreas. These alterations are not paralleled by changes in the second messengers known to control insulin secretion, do not affect single β-cells, and are fully reversible after washout of the uncoupling drugs. We have observed that tumour insulin-producing cells, which have retained at least some of the glucose sensitivity that characterizes normal β-cells, are interconnected by gap junctions made of Cx43. In contrast, several cell lines that feature abnormally low insulin production and marked glucose insensitivity do not express connexins or gap junctions and are uncoupled. In at least some of these lines, the stable transfection of the gene coding for Cx43, the only gap junction protein found in β-cells, induces the expression of functional gap junction channels and improves both the biosynthetic and secretory defects of the cells. In vivo, Cx43-transfected cells also grow at a lower rate and secrete more insulin than wild-type, noncommunicating cells.

Compared with other forms of cell-to-cell communication, gap junctional coupling provides a unique mechanism for direct equilibration of ionic and molecular gradients between nearby cells. Whenever the resulting concentration reaches a threshold level for activation of an effector mechanism, functioning will be simultaneously modified in all coupled cells, hence ensuring a larger recruitment of cells functioning in a coordinated way. This mechanism is particularly advantageous in the case of β-cells, which, taken individually, differ substantially in their ability to biosynthesize and release insulin [9]. The molecular mechanism whereby junctional coupling balances these disparities, may involve changes in free intracellular Ca^{2+}, in the electrophysiological characteristics of β-cells, or in the spreading of current-carrying ions across large cell populations.

It is worth stressing that the increased basal insulin release and loss of β-cell responsiveness that are observed during acute uncoupling and with single β-cells are reminiscent of the defects that characterize residual β-cell function in type II (non-insulin-dependent) diabetes and in animal models of this disease. Also, β-cells increase the expression of the gap junction protein Cx43 and coupling after in vivo treatments with glibenclamide, a sulphonylurea that promotes insulin release from the glucose-insensitive β-cells of type II diabetics.

Taken together, these observations indicate that insulin secretion results from the activity of numerous and functionally heterogeneous β-cells, whose integration depends in an obligatory manner on proper communication. Full control of this intercellular communication is ascribed to multiple mechanisms, including cell-to-cell signaling by nutrients, intrinsic and extrinsic neural inputs, local and circulating hormones, and as direct interactions between adjacent cells. While the precise contribution of these mechanisms and their hierarchic organization is not yet understood, compelling evidence indicates that junctional coupling is operative under conditions which abolish most other forms of cell-to-cell communication. Hence gap junctional coupling appears to play a fundamental, hitherto disregarded role in the regulation of both the moment-to-moment release of insulin and its longer term β-cell specific expression. Most likely, gap junctions have become an obligatory feature of β-cells, and of all other endocrine cell types, since they provide a direct pathway for compensating their individual, intrinsic metabolic and effector differences. Future studies should investigate the physiological relevance of the gap junction control in vivo (this is now under testing using transgenic animal approaches), and assess whether alterations in connexin expression or coupling function significantly contribute to the pathogenesis of the β-cell defects which are observed in type II diabetics. [Supported by grants from the Swiss National Science Foundation (32-34086.95), the Juvenile Diabetes Foundation International (195077), and the European Union (SC1*-CT92-0833).]

Nerve growth factor, gap junctions and nerve regeneration

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Peripheral neuropathies are among the most common and incapacitating neurological disorders. Although palliative treatments are available for management of this group of disorders, therapeutic approaches in the past have been limited. One new promising approach involves the therapeutic uses of neurotrophic factors/cytokines. Since neuropathy can result from dysfunction of either Schwann cells (demyelinating neuropathies) or neurons themselves (axonopathies), different classes of cytokines acting on glia or neurons will probably be useful for the treatment of different types of neuropathy.

The recent recognition that mutation of connexin32 underlies one form of Charcot-Marie-Tooth neuropathy highlights the importance of gap junction proteins for Schwann cells and peripheral nerve function [2]. The first part of this presentation discusses the effects of several inflammatory cytokines/growth factors including transforming growth factor beta (TGFB), tumor necrosis factor alpha (TNF alpha), new differentiation factor beta (NDFB), and glial growth factor (GGF) on the phenotype, function, and expression of gap junctions by cultured Schwann cells. These observations are correlated with changes in connexin expression that occur after nerve injury [5]. The second part of the presentation discusses ongoing clinical trials of nerve growth factor (NGF) in peripheral neuropathy. The preclinical studies leading to these trials, as well as unexpected biological actions of NGF observed in both animals and humans, are discussed. Findings of the phase 1A and 1B trials are presented, along with an update on progress with the phase II trial of NGF in diabetic small fiber neuropathy. Finally, the status of clinical trials of several other growth factors in peripheral neuropathy is discussed briefly.

The role of gap junctions and capacitive coupling in neural synchrony

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There are many putative mechanisms for neural synchrony in seizures. Our group has collected evidence indicating a role for gap junctional electronic coupling in the low-calcium model of seizure activity in the hippocampal slice [11]. Whole-cell electrophysiological recordings were used. When the extracellular calcium concentration was rapidly lowered by perfusing with no added calcium, synchronous seizure-like activity was observed, as noted by others. In this situation, increased dye coupling between CA1 neurons was seen. Also, spikelets (or fast prepotentials) were seen; many investigators consider that these reflect electrotonic gap junction-mediated coupling, especially in dye-coupled neurons. The spikelets occurred in the same pattern as spike bursts. When integrated, spikelets took on the same appearance as spikes. Conversely, when spikes were differentiated, they resembled spikelets. It is hypothesized that spikelets represent a form of capacitative coupling across the closely apposed pre- and postsynaptic membranes around gap junctions that are usually seen in clumps on the neuronal surface membrane. Biophysical modelling of gap junctional apposition between two theoretical neurons supports the concept of capacitive coupling. Evidence for rapid modulation of gap junctional coupling was obtained. Intracellular acidification or drugs known to block electrotonic coupling caused a marked decrease in or abolition of neuronal dye coupling, synchronous seizure-like activity in the slice, and spikelet activity. The last two effects were measured within minutes of the onset of treatment. However, intracellular alkalosis caused an increase in seizure-like activity and in spikelets. It is hypothesized that gap junctional mechanisms, including capacitative coupling, can be rapidly modifiable and play an important part in epileptogenesis. (Supported by the MRC.)

X-linked Charcot-Marie-Tooth disease and connexin32

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Mutations in connexin32 (Cx32) cause the X-linked form of Charcot-Marie-Tooth disease (CMTX), which is one of the inherited forms of demyelinating neuropathy. Cx32 is expressed by myelinating Schwann cells in peripheral nerve and is localized principally to the Schmidt–Lanterman incisures and the paranodal regions. The expression of Cx32, like that of other myelin-related genes, is highly regulated by axon-Schwann cell interactions. The levels of connexin32 mRNA and protein increase during development and fall after axotomy, but return to normal if axons regenerate and are remyelinated. To determine whether there are functional gap junctions in the incisures of myelinating Schwann cells, we have injected carboxyfluorescein (molecular mass 376 Da) and 3,000 Da rhodamine-conjugate dextran into single living teased nerve fibers of rodent sciatic nerve. Our preliminary results indicate that carboxyflourescein, but

not 3,000 Da rhodamine-conjugated dextran, can pass through incisures, suggesting that there are functional gap junctions between adjacent layers of myelinating Schwann cells that allow small molecules and ions to diffuse radially across the myelin sheath. To determine whether mutant Cx32 proteins are synthesized and properly localized in mammalian cells, constructs expressing various Cx32 mutations were introduced into PC12J cells by permanent transfection. Some Cx32 mutants (e.g., Argl5 → Gln) were synthesized and properly targeted, others (e.g., Glu186 → Lys, Glu208 → Lys) were synthesized but accumulate in the cytoplasm, while at least one (175 frameshift) was not detectably expressed. Thus, there are multiple effects of Cx32 mutations on the synthesis and localization of Cx32, potentially causing more than a simple loss of function.

Gap junctions and tumorigenesis

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Gap junctional intercellular communication (GJIC) has been implicated in the control of cell growth. This is based on the observations that many tumours and tumour cell lines exhibit reduced or altered GJIC [17]. In addition, GJIC is decreased by some oncogenes and by various carcinogens. Since there is substantial evidence implicating loss of GJIC in transformation, a variety of approaches have been used to upregulate intercellular communication to restore growth control. For example, many reports describe effects of retinoids on increasing GJIC and decreasing cell growth and transformation [16]. With the recent cloning of many of the connexin (Cx) cDNAs, a direct approach to increasing connexin expression and subsequent GJIC has allowed a more thorough examination of the role of intercellular communication on tumour suppression. While functional analyses have been carried out either by injection of specific connexin RNAs into *Xenopus* oocytes or by transfection into a variety of cell lines, because of the variety of interests of investigators only some of the studies transfecting connexin cDNAs into mammalian cells have reported on effects on cell growth.

The introduction and overexpression of connexin cDNAs in tumour cells by transfection has shown that the presence of functional gap junctions can suppress growth and/or tumorigenicity of some types of transformed cells (summarized in Table 1). Transfection and expression of Cx43, Cx32, Cx26 and Cx40 in various cells resulted in increased communication. However, only some connexins caused a slower growth in vitro and in vivo that correlated with the level of expression. The unique presence of certain connexins in different cell types suggests functional cell specificity for connexins. This concept is supported by the finding that chemically transformed mouse fibroblasts, rat glioma cells, and human rhabdomyosarcoma cells, which are deficient in

Table 1 Transfection of connexin genes

Cell line	Transfected connexin	Growth in vitro	Growth in vivo	Reference[d]
SkHep hepatoma	Cx32	↔	↓	Eghbali et al. 1990,1991
WB-F344, liver epithelial cells	Cx43	↓[b]	nd	Esinduy et al. 1995
Rat-1 fibroblasts	Cx43as[a]	↓[c]	nd	Goldberg et al. 1994
Transformed 10T1/2	Cx43	↓	↓	Mehta et al. 1991
MCA-10	Cx43	↓	↓	Rose et al. 1993
HeLa	Cx26	↓	↓	Mesnil et al. 1995
	Cx40	↔	↔	
	Cx43	↔	↔	
C6 glioma	Cx43	↓	↓	Zhu et al. 1991 Naus et al. 1992
	Cx32	↔	↓	Bond et al. 1994
Rhabdomyosarcoma	Cx43	↓	nd	Lin et al. 1995
Transformed kidney epithelial cells	Cx43	↓	nd	Chen et al. 1995

[a] Transfected with antisense Cx43 cDNA
[b] Suppressed growth of cocultured transformed cells
[c] Lost ability to suppress growth of cocultured transformed cells
[d] References listed in Table 1 are cited in order of citation: Eghbali et al. (1990) Proc Natl Acad Sci USA 87:1328–1331; Eghbali et al. (1991) Proc Natl Acad Sci USA 88: 10701–10705; Esinduy et al. (1995) Carcinogenesis 16:915–921; Goldberg and Bertram (1994) In Vivo 8:745–754; Mehfa et al. (1991) J Membr Biol 124:207–225; Rose et al. (1993) Carinogenesis 14:1073–1075; Mesnil et al. (1995) Cancer Res 55:629–639; Zhu et al (1992) Proc Natl Acad Sci USA. 89:10218–10221; Naus et al. (1992) Cell Mol. Neurobiol. 12:163–175; Bond et al. (1994) Cell Growth Differ 5:179–186; Lin et al. (1995) Sci China B 38:305–312; Chen et al. (1995) Cell Growth Differ 6:681–690

Cx43 expression compared with their nontransformed counterparts, exhibit reduced growth and tumorigenicity following transfection of Cx43 cDNA [18]. A similar observation was seen following transfection of human hepatoma cells with Cx32 [7], which is normally present in hepatocytes and following Cx26 introduction into HeLa cells which were derived from cervical cells which normally express Cx 26 in vivo. From these observations, it is clear that the establishment of GJIC in tumour cells does not always lead to effects on cell growth. There appears to be selective effects on cell growth mediated by specific connexins.

We are currently pursuing the mechanism(s) by which GJIC might alter cell growth. One approach has been to identify changes in gene expression associated with GJIC. We have shown that glioma cells transfected with Cx43 secrete growth-inhibitory factor(s), which are currently being characterized. In addition, expression of insulin-like growth factor and associated binding proteins is altered in these transfected cells. A second approach has been to isolate and characterize transjunctional molecules which may be involved in growth control.

Given the accumulation of evidence supporting a role for GJIC in growth control and differentiation, one might expect to see evidence of transformation in vivo in transgenic mice with null mutations of connexin genes. Targetted Cx43 gene knockout through homologous recombination resulted in lethality at birth, which was due to cardiac malformations. Preliminary studies have also been carried out with Cx26 and Cx32. Unfortunately, Cx26 knockout mice die at 10–11 days in utero. In contrast, connexin32 knockout mice are viable. These transgenic mice provide interesting models in which to test the transformability of cells in tissues with reduced or absent GJIC.

Characterization of connexin32- and connexin26-deficient mice

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The murine connexin gene family consists of at least 13 members, which are cell type specifically expressed, with overlapping specificity, and which code for the subunit proteins of gap junctions. Hepatocytes coexpress connexin (Cx) 32 and Cx26. In addition, Cx32 is expressed in Schwann cells, oligodendrocytes, and other cell types, whereas Cx26 is found in brain, placenta, intestine, and uterus. In order to study the function of these genes in mice, we have inactivated the Cx32 and Cx26 genes by gene targeting in cultured embryonic stem cells and have generated homozygous mutant animals.

Surprisingly, Cx32-deficient mice are viable and fertile [10]. In contrast to human patients suffering from Charcot-Marie-Tooth (X) disease, which is due to a mutation in the human Cx32 gene, Cx32-deficient mice show normal nerve conductance at 4 months of age. It is possible, however, that these mice develop neuropathological symptoms at an older age. Lucifer yellow coupling in cultured embryonic hepatocytes from Cx32 deficient mice is reduced to about one third of the value observed in wild type hepatocytes. Electrical stimulation of sympathetic nerves in isolated Cx32-deficient liver released only one fourth of the amount of glucose mobilized in wild-type liver. We conclude that Cx32-containing gap junctions between hepatocytes mediate intercellular propagation of the signal received from sympathetic nerve endings via release of noradrenaline.

Mice deficient in Cx26 die in utero between 10.5 and 12.5 d.p.c. They lack expression of Cx26 in the labyrinth region of the placenta between syncytiotrophoblasts I and II, which functions in nutrient transport from maternal to embryonic blood vessels. We speculate that the intercellular transport of nutrients through Cx26 gap junction channels in the labyrinth region is inhibited in Cx26-deficient mice. This may cause embryonic lethality.

Concluding remarks

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The idea of reentrant action potential propagation in cardiac muscle probably antedates the demonstration that the heart was not a true syncytium. Because cardiac fibers are core conductors with the gap junctions between cells as part of the pathway of axial current, conduction velocity can be regulated by the junctional conductance. Spray reported decreases in junctional conductance in cardiocytes infected with the intracellular parasite causing Chagas' disease. Cardiac arrhythmias are one of the major causes of death in chronic Chagas' disease, and it is tempting to suppose that islands of infected tissue might divide conducting tissue into pathways that permitted reentrant propagation. He also pointed out that halothane, which lost favor as a general anesthetic because of its propensity to produce cardiac arrhythmias, is an excellent blocker of gap junctions at anesthetic doses. The issue of connexin regulation is raised in respect to heart. Cx43 is the principal connexin expressed in the ventricular muscle (others are found in the conducting system). Not surprisingly, a Cx43 KO mouse is lethal; it dies at birth. Surprisingly, however, it dies not from failure of the heart to contract – the other connexins keep the action potentials going – but from a malformation in the right outflow tract that obstructs blood flow to the lungs. It seems that Cx43 is essential for embryonic development of the heart. In possible confirmation, several mutations of Cx43 are found in humans with cardiac and visceral developmental abnor-

malities. These mutations are in putative phosphorylation sites, which may control expression or conductance, but for developmental regulation rather than transmission of action potentials.

An aspect of metabolic cooperation was raised by Meda, who presented data indicating that coupling between pancreatic beta cells increases the amount of insulin that they secrete. Nominally, all beta cells are alike, and coupling would not be expected to do much for them; if cells have a secretory cycle, however, they may get out of phase and thus be different much of the time. For the increase in secretion, one can give an analogy in terms of the psychological phenomenon of "social facilitation." An isolated chick will eat a certain amount, but chicks in a group will eat more per chick; to anthropomorphize, chicks, seeing their companions eat, become hungry. Meda's data suggest that communication among secreting beta cells leads them to secrete more than they otherwise would. The question with any form of nonelectrical communication between cells is the identity of the message. Actually, communication between beta cells may be electrical. There is evidence that secretion is triggered by a regenerative Ca^{2+} action potential, and spontaneous firing in one cell may recruit others to the secretory process. Although Meda's hemolytic plaque assay for insulin secretion is a thing of beauty, Ca^{2+} imaging applied to cells in isolation and in groups may give a clearer picture of the secretory events.

Gap junctions were first identified at electrical synapses, and I view them as perfectly good synaptic structures, since they are anatomically specialized sites of transmission between neurons. Although there remains in the literature the concept that electrical synapses are somehow primitive and more characteristic of lower species, there is little foundation for this view, which is in any case irrelevant, since mammals, including humans, have them in the central nervous system, including the neocortex. Arguments can be put forward concerning what electrical synapses can do, and they can do most if not all of the things that chemical synapses do. They can be regulated on a fast scale by a number of mechanisms including phosphorylation, transjunctional voltage and changes in cellular pH and upon a Ca^{2+} short time scale. They are modifiable on a somewhat slower time scale by changes in transcription and translation and in formation and degradation. The question now is not what they can do, but what do they do in fact do.

Since epilepsy is a phenomenon of excessive electrical activity, excessive electrical transmission has long been a candidate cause. There is little evidence to support such an etiology, and the number of different possible "lesions" in excitability mechanisms that could cause epilepsy is large, including block of inhibition, loss of Na^+ inactivation, loss of K^+ activation, and increase in the number of electrical synapses. Of particular interest in Carlen's presentation was that ephaptic, (nonsynaptic, electrical) mechanisms appear to be important in a least some epileptiform activity. The timecourse of transmitted potentials suggests capacitative coupling, which could only arise from large areas of apposition between cells. One would consider these areas electrical synapses, if one were to decide that they were specialized. In any case, they mediate interactions that the nervous system "lives with," even if there are occasional difficulties. With respect to epilepsy one should also consider the possibility of spatial buffering. Gap junctions between astrocytes are expected to facilitate redistribution of extracellular K^+ accumulated during neuronal activity; as one astrocyte is depolarized by locally increased external K^+, it will depolarize more distant cells and drive K^+ out of them; and if uptake of KCl is a mechanism of removal of extracellular K^+, then coupling between cells will, by metabolic cooperation, increase the effectiveness of removal of excess K^+ at localized sites, if not a uniform rise in K^+. Again, as a possibility rather than a fact, decreases in glial coupling may increase the susceptibility to epileptiform discharges. However, a change observed in glial coupling in an epileptic animal may represent a maladaptive or an adaptive response to previous seizures rather than a cause of current seizures.

An early suggestion was that loss of gap junctional communication led to malignancy. In the 1966 paper by Potter et al. [12] in which the idea first appears, it was also demonstrated that some transformed cells were still coupled, and subsequently from the same laboratory there came extensive evidence that malignant cells in vitro and in vivo could be coupled, even if at a reduced level. As for many bad hypotheses, it was very difficult to prove or disprove that reduced coupling or permeation causes cancer. With the discovery of oncogenes, it became even clearer that connexin genes were not oncogenes, although they might function as suppressors by allowing an inhibitory regulator to diffuse in from adjacent cells or a growth stimulator to leak out to adjacent, nonmalignant cells. Thus, in multistage carcinogenesis, gap junctional communication could provide a modest brake. Gap junctions are not affected by initiation, but may slow promotion and progression. Furthermore, malignant cells have many changes in their surface molecules, and loss of gap junctions may be an epiphenomenon. Conversely, metastasis may require the malignant cell to be able to form gap junctions to cross the endothelium and invade a new site. Transfection of malignant cells with connexin genes has allowed the demonstration that gap junction expression can slow growth in culture and in vivo. Naus presented some of these data obtained with a malignant glial cell line: poorly coupled cells grew more rapidly; well coupled cells grew more slowly. A further fascinating result was that growth of poorly coupled cells was reduced by conditioned medium from the same cells in which coupling had been increased by transfection with the connexin cDNA. Less well-coupled cells grew faster, but medium from Cx43 transfected cells reduced this growth. One could now suppose that the growth regulation is mediated by an extracellular signal that is secreted more effectively by better coupled cells. Again, we have to ask what the

message is, and Naus is progressing towards its characterization.

The gap junction field finally does have a genetic disease caused by mutations in a connexin gene; the pathology was unexpected and is entertaining as well as interesting in what it does and does not show. In X-linked Charcot-Marie-Tooth disease, or CMTX, the gene for connexin32 (Cx32) is mutated. Only after linkage analysis demonstrated the involvement of Cx32 was it shown that this connexin is expressed in myelin. The gap junctions are apparently reflexive (between different parts of the same cell) and are presumed to improve interchange of small molecules between the periaxonal cytoplasm and the outermost part of the Schwann cell. Light microscopic immunocytochemistry shows Cx32 at perinodes and Schmitt–Lanterman incisures, but their arrangement has not been clearly resolved in thin section or freeze-fracture electron microscopy. Scherer showed dye coupling between inner and outer parts of the Schwann cell that was sensitive to gap junction blockers, but surprisingly failed to show cytoplasmic continuity between periaxonal and perinuclear space with labeled dextran. Further fine structure studies are required. The question remains as to what is communicated from outside to inside or vice versa. And it is unclear why loss of this communication, which has no apparent effect on myelin formation during growth and development, leads eventually to demyelination and degeneration of nerve fibers. In studies described by Kessler it was shown that in Wallerian degeneration, proliferating Schwann cells cease expressing Cx32; instead they express Cx46 mRNA and in culture are coupled by junctions whose properties are more characteristic of another connexin than either Cx32 or Cx46. There is no obvious relation of this switch in connexin expression to CMTX. Kessler also presented data showing that nerve growth factor can protect against peripheral neuropathy caused by microtubule-blocking agents. The potential for growth factors as a symptomatic treatment for CMTX must be considered. The long latency of disease onset and greater susceptibility of long axons suggest the possibility of involvement of axonal transport, particularly of the slow variety in which transport to the extremities could take years.

A striking feature of CMTX is that the only obvious pathology is in peripheral nerve. Yet Cx32 is expressed in liver, pancreas, kidney, central myelin and some electrical synapses. In terms of survival advantage, Cx32 is most important in peripheral nerve. Its expression in the other tissues does not appear to provide much additional selection advantage, since it can be so well done without. If there were an advantage, its independent selection would have to involve promoters that led to restricted expression in these tissues without affecting expression in peripheral nerve. This line of argument is independent of the question of whether another connexin compensates for the absence of Cx32. The possibility of dominant negative forms of Cx32 that block gap junction formation by other, coexpressed connexins, is moot in the absence of symptomatology in the tissues in question.

An animal model of CMTX may shed some light on these questions. Willecke described a mouse in which the Cx32 gene was disrupted (“knocked out”) by homologous recombination. As in humans, the mice are quite viable and reach young adulthood without obvious neurological problems. (Willecke did say that others have seen evidence of neuropathy in later life.) As predicted, there is a reduction in gap junctions in liver, but unexpectedly the expression of Cx26, which is normally coexpressed in liver with Cx32, is also decreased. (A response of liver to partial hepatectomy is loss of gap junctions by down-regulation of both Cx26 and Cx32 before cell division leads to compensatory hypertrophy; a liver lacking Cx32 may shut down its Cx26 expression by a similar mechanism.) The major physiological difference observed was that glucose mobilization in response to sympathetic nerve stimulation is reduced, while the response to glucagon or norepinephrine is unaffected. The inference is that acini are innervated at one end and that neurally released norepinephrine acts only on innervated cells in the Cx32 knockout animal, but that second messengers diffuse to involve additional cells in the wild type with normal communication between hepatocytes. In addition, the Cx32 knockout animals have a somewhat lower body mass and a slightly modified diurnal rhythm in glycogen content of the liver.

So where is the field after all this time, and where is it going? We now know most if not all of the connexin genes and for the most part where they are expressed. However, connexin distribution is not yet fully characterized, particularly in the nervous system, and the example of CMTX, Schwann cells and Cx32 suggests caution. We know much more about the biophysics of gap junctions, and more detailed descriptions of connexin-specific permeation, gating and regulation are being developed. As mechanisms of secretion and of regulation of the cell cycle become clearer (and the permeability of different gap junctions is better characterized), the role of gap junctions in these processes will be determined. Application of new molecular reagents and techniques for identifying and perturbing connexins will clarify their functions in the nervous system. Transgenic and knockout mice will provide further indications of where we can expect human disease, although the homologous lesions do not always have the same effects in the two species. In any case, manipulations of gene expression will allow examination of pathogenesis and reveal possibilities for therapeutic intervention.

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