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Roles of Gap Junctions and Connexins in Non-Neoplastic Pathological Processes in which Cell Proliferation Is Involved

Maria Lúcia Zaidan Dagli · Francisco Javier Hernandez-Blazquez

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Abstract Cell proliferation is an important process for reproduction, growth and renewal of living cells and occurs in several situations during life. Cell proliferation is present in all the steps of carcinogenesis, initiation, promotion and progression. Gap junctions are the only specialization of cell membranes that allows communication between adjacent cells. They are known to contribute to tissue homeostasis and are composed of transmembrane proteins called "connexins." These junctions are also known to be involved in cell proliferation control. The roles of gap junctions and connexins in cell proliferation are complex and still under investigation. Since pioneer studies by Loewenstein, it is known that neoplastic cells lack communicating junctions. They do not communicate with their neighbors or with non-neoplastic cells from the surrounding area. There are many studies and review articles dedicated to neoplastic tissues. The aim of this review is to present evidence on the roles of gap junctions and connexins in non-neoplastic processes in which cell proliferation is involved.

Keywords Gap junction · Connexin · Regeneration · Wound healing · Angiogenesis

M. L. Zaidan Dagli (✉)
Laboratory of Experimental Oncology, Department of Pathology, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508-900 São Paulo, Brazil
e-mail: mlzdagli@usp.br

F. J. Hernandez-Blazquez
Laboratory of Immunohistochemistry, Department of Surgery, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508-900 São Paulo, Brazil

Introduction

Cell proliferation is an important process for reproduction, growth and renewal of living cells and occurs in several situations during life. In embryonic and fetal development, well-controlled cell proliferation and differentiation are key processes. In vertebrate adult life, cells can be classified as stable, labile or permanent depending on how often they enter the cell cycle. Cell proliferation is important for the renewal of both stable and labile cells. For example, hepatocytes keep the ability to proliferate; however, they do so at such a low level that they are classified as stable cells. On the other hand, some bone marrow cells and epithelial cells from surface recovering tissues are almost permanently in the cell cycle, and therefore, the renewal of these tissues is very fast. That is why these cells are called "labile." Cell death by apoptosis also occurs in these tissues, warranting the tissue homeostasis. Cells which never proliferate are known as "permanent" cells, and examples are neurons and heart striated muscle cells (Kumar, Abbas & Fausto, 2004).

There are many situations in living cells and tissues which involve increased cell proliferation as a response to increased cell death, tissue removal or even the release of growth factors or excessive hormonal stimulation. These are considered pathological, but still reversible, situations. Most of them involve stimulation by a hormone or a growth factor and include liver regeneration, kidney compensatory hyperplasia, epithelial growth during skin wound healing and angiogenesis.

Cell proliferation is present in all the steps of carcinogenesis, initiation, promotion and progression. However, it is only during the preneoplastic stages that increased cell proliferation is considered reversible.

Gap junctions are the only specialization of cell membranes that allow communication between adjacent cells. They are known to contribute to tissue homeostasis and are composed of transmembrane proteins called "connexins." Gap junctions and connexins are reportedly involved in tissue homeostasis due to the exchange of molecules smaller than 1 kDa. These junctions are also known to be involved in cell proliferation control. The roles of gap junctions and connexins in cell proliferation are complex and still under investigation.

Since pioneer studies by Loewenstein, it is known that neoplastic cells lack communicating junctions (Loewenstein 1966; Loewenstein & Kanno 1966). They do not communicate with their neighbors or with non-neoplastic cells from the surrounding area. There are many studies and review articles dedicated to neoplastic tissues. The aim of this review was to present evidence on the roles of gap junctions and connexins in non-neoplastic processes in which cell proliferation is involved. We focused mostly on *in vivo* experiments, using laboratory animal or human samples, since interactions between cells of a given tissue and between different tissues in a given organ are important to keep homeostasis.

Preneoplastic or Hyperplastic Lesions during Carcinogenesis

Several studies have been conducted in order to evaluate the role of gap junctions and connexins during carcinogenesis. Most of those showed that at progression phase the generated cancers presented reduced expression of connexins and, therefore, lower communication capacity. There are examples of this in studies on hepatocarcinogenesis (Sakamoto et al., 1992) and skin carcinogenesis (Budunova, Carbajal & Slaga, 1995; Sawey et al., 1996). This review includes also endometrium, prostate and mammary glands.

Liver

Beer et al. (1988) studied hepatocarcinogenesis in Harlan Sprague-Dawley rats. The model consisted of a 70% partial hepatectomy and the administration of diethylnitrosamine 18 h later. After 1 week, animals were fed a diet containing phenobarbital. Groups of rats were killed 6 and 15 months later. The hyperplastic, preneoplastic focal lesions showed a decrease in gap junction immunoreactivity, while decreased expression of gap junction mRNA was observed in all liver tumors. Krutoviskikh, Oyamada & Yamasaki (1991) studied chemical carcinogenesis in Fischer-344 rats and showed that glutathione *S*-transferase, placental form (GSTP), -positive focal lesions showed markedly lower gap junctional intercellular communication (GJIC) and a significantly lower number of Cx32-positive spots in comparison to surrounding hepatocytes. Hepatocellular carcinomas had significantly lower reduced communicational capacity accompanied by a large decrease in Cx32 expression.

Sakamoto et al. (1992) showed a reduction in the number of Cx32-positive gap junctions in foci and decreased expression of Cx32 in hyperplastic liver nodules, whereas increased expression of Cx26 was seen in some of those preneoplastic lesions. They concluded that Cx26 and -43 seem to be differentially regulated during carcinogenesis.

Neveu et al. (1994) also showed that preneoplastic nodules expressed reduced Cx32 and increased Cx26 during liver hepatocarcinogenesis. The authors concluded that alterations in the expression of connexins represent common modifications during hepatocarcinogenesis. It is apparent that preneoplastic and neoplastic rat hepatocytes, however, fail to use a common mechanism to modify connexin expression. Tsuda et al. (1995) studied the hepatocarcinogenesis induced by the nongenotoxic compound clofibrate. The preneoplastic lesions in this model lacked expression of gamma-glutamyl-transpeptidase (GGT) or GSTP and presented fewer spots of Cx32 in the membranes. The same authors observed that GSTP-positive foci in the same livers, which they considered spontaneous lesions, did not show any decrease in Cx32 spots in the membranes. In this interesting study, the authors conclude that the decrease of Cx32 expression is apparently related to preneoplastic lesions during hepatocarcinogenesis, irrespective of the enzyme phenotype of neoplastic focal lesions and the carcinogens used for their induction.

The era of genetically engineered mice started in the 1990s with the Cx43 knockout mice (Reaume et al., 1995), followed by the Cx32 knockout mice (Temme et al., 1997). Connexin-deficient mice present lower expression of connexins. As expected, they display a lower capacity to communicate and exhibit a higher susceptibility to carcinogenesis. Cx32 knockout mice present higher susceptibility to spontaneous or chemically induced liver cancer (Temme et al., 1997), and Cx43 knockout mice exhibit a higher susceptibility to urethane-induced lung carcinogenesis (Avanzo et al., 2004). These mouse models are now being used to investigate the role of gap junctions and connexins in other pathological processes.

Moennikes et al. (2000) studied the role of Cx32 in phenobarbital carcinogenesis. It is known that phenobarbital is a promotional carcinogen and blocks GJIC *in vitro*. It has been suggested that this effect is relevant for clonal expansion of neoplastic cells *in vitro*. When phenobarbital was administered to Cx32 knockout mice, these presented fewer preneoplastic lesions compared to wild-type mice.

The authors state that even more pronounced differences were observed with respect to tumor response. These results suggest that functional Cx32 protein is required for tumor promotion by phenobarbital.

Livers from Cx32 knockout mice were analyzed during carcinogenesis (Evert et al. 2002). Loss of Cx32 did not alter the morphology of liver tissue; however, preneoplastic lesions obtained by injection of diethylnitrosamine were larger and occupied a higher volume fraction compared to wild-type mice. Cx32 deficiency did not interfere in the spontaneous development of preneoplastic lesions.

Transgenic rats were created which express a dominant-negative mutant of Cx32 (Hokaiwado et al., 2005). Animals were separated according to high-expressing and low-expressing transgenes, depending on the number of transgenes. Both rat strains present lower communication capacity. However, only rats which express higher levels of the mutant gene present a significant inhibition of GJIC capacity and increase in GST-positive liver foci after injection of diethylnitrosamine. The authors concluded that only high levels of transgenes present an enhancing effect on liver carcinogenesis.

Skin

Contrary to hepatocarcinogenesis, during skin carcinogenesis, Budunova et al. (1995) found that skin hyperplasia induced by one topical application of 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was accompanied by hyperexpression of both Cx26 and Cx43 and decreased expression of Cx31.1. Sawey et al. (1996) verified that expression of Cx26 was greatly elevated in nonpapillomatous hyperplasias of mouse skin. In the same model, Cx43 and Cx26 were extinguished in squamous cell carcinomas. The authors concluded that the perturbations in the expression of Cx43 and Cx26 may be associated with the malignant conversion of mouse epidermal cells.

Endometrium

A study conducted with human endometrial samples (Saito et al., 2001) showed that Cx26 and Cx32 were aberrantly expressed in all samples, varying from weak or negative expression to diffuse expression in cytoplasm. In endometrial carcinoma samples, these patterns were also differentially expressed.

Prostate

Human prostatic samples of normal, hyperplastic (benign prostate hyperplasia, BPH) and neoplastic glands were evaluated with respect to gap junction proteins, the connexins (Habermann et al., 2001b). It has been verified that in BPH specimens there was a marked increase in incidence and intensity of Cx43 and Cx32 immunostaining within epithelial cells, while Cx43 and Cx32 expression was reduced or absent in prostate cancer specimens.

Habermann et al. (2001a) studied the expression of Cx32, Cx43 and e-cadherin in developing, adult and aged rat prostate gland exposed to estrogens in early life. Estrogens are known to interrupt prostate development in the neonatal period and to cause hyperplasia and dysplasia in aging animals. Estrogen-treated rats presented a marked decrease in Cx32 staining and increased proportion of Cx43-expressing cells.

Mammary Glands

Two studies investigated gap junction protein expression in mammary carcinogenesis steps (normal, hyperplastic, benign and malignant neoplasms). One of them was performed on human breast tumor samples and the other on canine breast tumor samples. Gap junction protein (Cx26 and Cx43) expression was investigated by immunohistochemistry in normal and neoplastic (benign and malignant) mammary glands (Jamieson et al., 1998). The most interesting finding is that Cx26 was upregulated in the carcinoma cells of most samples, and the staining was usually cytoplasmic and heterogeneous. The authors discuss that the finding of higher expression of connexins, localized in the cytoplasm, is not necessarily inconsistent with a tumor-suppressor role for GJIC.

Torres et al. (2005) studied the expression of Cx26 and Cx43 in canine hyperplastic and neoplastic mammary glands. They verified that normal, hyperplastic and benign mammary gland specimens showed a punctate pattern of Cx26 and Cx43 staining and intercellular staining of E-cadherin. However, malignant neoplasm presented either fewer gap junction spots on cell membranes or increased cytoplasmic immunostaining. In malignant neoplasm, expression of E-cadherin was also reduced. It is important to note that cell proliferation, quantified by proliferating cell nuclear antigen (PCNA) immunostaining of the nuclei, increased from normal to neoplastic mammary glands.

Inflammation

Inflammation is defined as the complex response of the vascularized connective tissue to exogenous or endogenous injury. The reaction is driven to eliminate both the initial cause of injury and the necrotic cells and tissues arising as a consequence of such injury. Inflammation is also intimately interwoven with tissue repair and wound healing. Inflammation is grouped into two basic forms, acute and chronic. Although acute inflammation is of relatively short

duration, chronic inflammation lasts from days to years and is manifested histologically by the influx of lymphocytes and macrophages and by tissue destruction and repair.

The role of gap junctional communication in tissue inflammation and repair has been reviewed by Chanson et al. (2005). According to those descriptions, gap junctions and connexins do participate in the inflammatory response. Therefore, only recent findings will be described here.

Schistosoma mansoni infection and the development of a granuloma have been studied by Oloris et al. (2007) in Cx43 heterozygous or wild-type knockout mice. Granuloma cells express Cx43, as revealed by real-time polymerase chain reaction in isolated granulomas and by immunohistochemistry. Cx43 expression was reduced in $Cx43^{+/-}$ mice, as expected. No differences in the mean area of the granulomas or number of cells per granuloma were observed between mice of different genotypes. However, granuloma cells from $Cx43^{+/-}$ mice displayed reduced PCNA labeling index at 8 and 12 weeks postinfection. Moreover, unexpectedly, $Cx43^{+/-}$ granulomas presented higher fibrosis, as revealed by Sirius red staining and morphometric analysis. Our results indicate that the deletion of one allele of the Cx43 gene, and possibly the diminished GJIC capacity, may impair the interactions among granuloma cells, reduce granuloma cell proliferation and increase the collagen content, therefore modifying *S. mansoni* granuloma reaction in mice.

Tissue Repair

Repair of cells and tissues occurs after injury or tissue loss. Two processes are involved in repair: *regeneration* (or compensatory hyperplasia) is defined as the repair of injured tissue by parenchymal cells of the same type. It occurs after tissue removal or injury and when the cells are still able to proliferate and create a new tissue of the same type. However, severe or persistent tissue injury and inflammation with damage both to parenchymal cells and to the stromal framework leads to a situation where repair cannot be accomplished by parenchymal regeneration alone. Under these conditions, *repair by connective tissue* follows, and nonregenerated parenchymal cells start to be replaced by proliferating fibroblasts and vascular endothelial cells, resulting in a permanent scar. Therefore, angiogenesis, fibrosis and scar remodeling are part of the process.

The process of wound healing is therefore complex and systematic. After an initial injury and the induction of an inflammatory reaction, parenchymal cells proliferate and regenerate; connective tissue proliferates, migrates to the injury site and synthesizes extracellular marix proteins; and the connective tissue is remodeled to achieve wound strength. What is the participation of gap junctions and connexins in these processes?

Regeneration and Compensatory Hyperplasia

Liver

Many studies of regeneration have been performed in the liver, as this organ can easily regenerate after partial hepatectomy. Liver connexins, as far as we know, were the first to be described and identified; therefore, more methods of investigation were available for this organ and connexins 26 and 32. Traub, Druge & Willecke (1983) investigated, in rats, the modifications of gap junction proteins during liver regeneration after partial hepatectomy or bile duct ligation. Quantitative immunoblots were used. The loss and reappearance of Cx26 roughly paralleled the loss and reappearance of the gap junction plaques analyzed by freeze-fracture. At 28–35 h after partial hepatectomy, there was a decrease in Cx26 expression at the level of 15%, while <1% of the gap junction plaques were observed. The authors conclude that the decrease and increase of the total amount of Cx26 may be due to degradation of resynthesis of this protein during liver regeneration. Sugiyama & Ohta (1990) studied the disappearance and reappearance of rat liver gap junctions after partial hepatectomy, and their location in the liver acinar zones was analyzed by immunohistochemistry and morphometry. They verified that 20 h after partial hepatectomy, there was a rapid decrease in the gap junction population in the periportal area of the liver acinus. The disappearance of gap junction proteins progressed toward the central vein afterward. At 48 h after surgery, the density of gap junctions reached the lowest level and started to reappear at 72 and 96 h. In another study, Miyashita et al. (1991) verified that a decrease in Cx 32 was also observed in rat liver after a single administration of dimethylnitrosamine. This decrease failed at <10% of the normal value 24 h after injection of 25 mg/kg and returned to normal level 240 h later. Kren et al. (1993) further studied the role of gap junctions and connexins during rat liver regeneration. The authors verified that the level of expression of Cx26, Cx32 and Cx43 genes changed minimally until 12 h after partial hepatectomy; and the corresponding connexin levels showed a marked decrease only at 18 h after partial hepatectomy. There was a further increase in the Cx32 and Cx26 genes between 24 and 42 h and another decrease at 48 h post-partial hepatectomy. The return to base levels occurred only at 72–84 h after partial hepatectomy, and they were maintained thereafter.

Yamasaki et al. (1993) historically reviewed the role of connexins and gap junctions during multistage carcinogenesis and stated that the relationship between GJIC and cell proliferation is not well known. However, results from

various experiments suggest that that there is a close relationship between inhibition of GJIC and stimulation of cell proliferation. Different stimuli may affect cell proliferation and GJIC differentially and by different mechanisms. Ikejima et al. (1995) studied the effects of hepatocyte growth factor (HGF) on cultured rat hepatocytes. They verified that HGF reduces GJIC and that treatment with transforming growth factor beta 1 (TGF-β1) maintained intercellular communication in the presence of HGF. The authors conclude that regulation of intercellular communication by HGF and TGF- β1 may play an important role during liver regeneration.

In an interesting technical work, Fujimoto et al. (1997) examined the dynamics of connexins, E-cadherin and α-catenin during gap junction disassembly and assembly in hepatocytes during regeneration. The authors used immunofluorescence microcopy and immunogold-electron microscopy with sodium dodecyl sulfate-digested freeze replicas. They verified that during the disappearance of gap junctions these are broken up into smaller aggregates, and then the connexins may be removed from the cell membrane; some of the connexons or connexins remain dispersed in the plane of the membrane as pure, morphologically indistinguishable intramembrane proteins. In addition, connexin immunoreactivity was observed along tight junctional strands, suggesting that gap junctions may also form along the tight junctions.

The effect of loss of gap junctional proteins Cx32 and Cx26 during liver regeneration and whether this loss could affect liver regeneration after two-thirds partial heptectomy was investigated (Temme et al., 2000). Cx32 knockout mice were used. These mice do not express Cx32 and present a reduction in Cx26 expression. The authors verified that the ratio of liver to body weight in regenerating livers was not affected by loss of the Cx32 gene. The peak of DNA synthesis occurred at the same time, i.e., 36–96 h after partial hepatectomy in Cx32-deficient and in wild-type mice. However, during this time only about half of the hepatocyte nuclei incorporated bromodeoxyuridine (BrdU) compared to wild-type mice. At 1–2 weeks after full recovery of liver mass, a higher level of BrdU was incorporated into hepatocytes of Cx32-deficient mice. These results suggest that the extent of synchronous initiation and termination of DNA synthesis in regenerating livers was altered in Cx32-deficient mice.

Dagli et al. (2004) tested the effect, in liver, of one of the mutations found in Charcot-Marie-Tooth patients. A transgenic mouse expressing the V139M mutation, linked to the albumin promoter, was created. This mutant connexin is known to be dominant-negative, and transgenic mice present lower communication capacity between liver cells. Interestingly, when submitted to partial hepatectomy, it was verified that there was a retardation of 24 h in the peak of S-phase cells. These mice also showed a higher susceptibility to hepatocarcinogenesis induced by diethylnitrosamine. Omori et al. (2001) compared these results with the ones obtained by Temme et al. (1997) investigating Cx32 knockout mice. The results were quite similar and indicate that impairment of communication capacity by a dominant-negative strategy or knocking out the Cx32 gene delays liver regenerative capacity. In fact, liver regeneration is an orchestrated phenomenon that initiates soon after the ablation of either 30% or 70% of the liver tissue and culminates with the restoration of the initial liver weight. The process is fast such that within 7 days the initial liver mass is restored. Gap junction communication capacity appeared to be very important in this process, perhaps allowing the spread of cell proliferation signals for the cells to enter the cell cycle.

Yamamoto et al. (2005) investigated the role of stress-responsive p38 mitogen-activated protein (MAP) kinase signaling in the function of gap and tight junctions during regeneration of rat hepatocytes. After 70% partial hepatectomy, p38 MAP kinase is activated and downregulation of Cx32 and upregulation of claudin-1 were observed. However, when p38 MAP kinase was inhibited, downregulation of Cx32 was also inhibited and upregulation of claudin-1 was enhanced. The increase in hepatocyte proliferation was not affected by treatment with MAP kinase inhibitor. These results suggest that p38 MAP kinase signaling partially controls the dynamics of formation of gap and tight junctions.

The role of Cx43 on bile duct proliferation was investigated in mice using the bile duct ligation model. Cx43 heterozygous knockout or wild-type mice were used (Teixeira et al., 2007). Bile ducts were ligated in animals of different genotypes; and 3, 7 and 14 days later, the livers were analyzed for connexin expression and for BrdU positivity. The authors verified that no differences were obtained in the quantification of newly formed intrahepatic bile ducts; however, morphometric quantification of liver components revealed that knockout mice showed fewer blood vessels in their livers.

Gastric mucosa

The role of gap junction in gastric mucosa during the regenerative process of ethanol-induced gastric mucosal injury was also studied (Endo et al., 1995). The number of immunoreactive spots for gap junctions was markedly decreased 1 h after ethanol treatment. The time course of the reappearance of gap junctions closely paralleled the appearance of BrdU-positive cells. This result indicates that morphological repair is different from the recovery of cell maturity and cell proliferation in the regenerative gastric mucosa.

Kidney

The mechanism of compensatory renal growth is not yet well understood. However, participation of Cx43 in this process was recently demonstrated by Li et al. (2002). The right urethras of 5-week-old mice were unilaterally obstructed, and the animals were killed at varying intervals. The control group underwent a sham operation. Freeze-fractured kidney tissue samples were studied using electron microscopy. Numbers of PCNA-positive cells and Cx43 proteins were determined by immunohistochemistry and Western blotting, respectively. PCNA-positive cells in the renal tubules increased on days 1 and 2 after surgery that obstructed the urethra and decreased to normal levels by day 14. The number of gap junctions significantly decreased on days 1 and 2 and then gradually increased to normal levels 3–14 days after surgery. The amount of Cx43 protein in the renal tubules decreased until day 2 and recovered to the same level as that of the control by day 14 after surgery. Significantly, a hyperphosphorylated band of Cx43 in the control kidney was not detected in the operated kidney. These data suggest that the GJIC of renal tubular cells during compensatory renal growth after unilateral urethral obstruction could be temporarily reduced concomitant with a decrease of the expression of a phosphorylated Cx43 protein in renal cortical tubular cells after unilateral urethral obstruction of the contralateral kidney.

Silva et al. (2006) studied the role of Cx43 in the remanescent kidney from Balb/c mice after unilateral nephrectomy. Groups of mice were killed at 24, 48 and 72 h and at 7 and 30 days. An increase in kidney weight was observed at days 7 and 30 postnephrectomy. Total count of BrdU-positive epithelial cells increased 24 h postnephrectomy, with a peak of proliferation 48 h postnephrectomy. In the analyses of volume fraction, no important differences were observed in the volume represented by different histomorphological structures. Relating to the quantity and intensity and location of Cx43 immunostaining, no differences were observed in the nephrectomized groups. Western blot revealed that during the first 24 and 48 h after nephrectomy P0 (unphosphorylated) and P1 (phosphorylated) Cx43 disappeared and the products of Cx43 degradation were reduced. After 72 h, the P0 and P1 states were detected and the amount of degradation increased. At 7 and 30 days after nephrectomy, a higher intensity of P0 and P1 states and a lower P2 (hyperphosphorylated) band were observed. The authors conclude that Cx43 phosphorylation results in the retention of Cx43 in cytoplasm, without accentuated degradation, eventually modulating mitotic activity in renal cells.

Heart

Cardiac muscle does not proliferate in normal conditions. Under chronic overloading, myocardial fibers adapt suffering hypertrophy, which is defined as the increase in fiber volume due to the multiplication of cytoplasmic organelles.

Formigli et al. (2003) investigated expression of Cx43 during the myocardial adaptation to acute and chronic volume overloading. Using the pig left ventricle, an aortocaval fistula was created. After 48 h, the tissue developed a hypertrophic response that was associated with early dynamic changes and upregulation in Cx43 expression. However, from 168 h to 3 months, a reduction in the myocardial expression of Cx43 was observed.

Peripheral nervous system and central nervous system

Cx32 is present in Schwann cells and in oligodendrocytes in the peripheral and central nervous systems, respectively. Scherer et al. (1995) examined Cx32 expression in normal and pathological situations. In the peripheral nervous system, Cx32 expression changes in parallel with that of other myelin-related genes during development, Wallerian degeneration and axonal regeneration. In the central nervous system, Cx32 protein and mRNA increase during development in parallel with other myelin genes. The authors showed that Cx32 plays an important role in the biology of myelin-forming cells.

The changes in expression of Cx32, Cx43 and Cx46 during peripheral nerve injury were investigated by Chandross et al. (1996). The authors verified that by 3 days after crush injury, Cx46 mRNA rapidly increased in the degenerating regions. In parallel, Cx43 mRNA also increased in endoneural fibroblasts in the crush and distal regions by 3 days, coincident with macrophage infiltration. By day 12, Cx43 decreased to normal levels. These results show that both Cx43 and Cx46 are important for injury and regenerative response of peripheral nerves.

The alterations in Cx43 during axon regeneration after motor axon injury were studied by Aldskogius & Kozlova (1998). Astrocytes and microglia are involved in this process. The authors verified that there is upregulation of Cx43 expression in astrocytes within 1 h after injury and within 24 h the astrocytes upregulate glial fibrillary acidic protein.

Dezawa et al. (1998) verified the possibility that atypical gap junctions develop between heterologous tissues, such as regenerating nerve axons and Schwann cells, during peripheral nerve regeneration in adult rats. After a complete transection and subsequent regeneration in the rat sciatic nerve distal segment, small gap junction-like structures were observed between the regenerating axons and adjoining Schwann cells. Immunoelectron microcopy detected Cx32, and biocytin, a small-molecular weight dye,

was transported from regenerating axons into Schwann cells. These finding suggest that regenerating axons communicate directly with Schwann cells through small gap junctions. This may play a role in the mechanism of regeneration after nerve transection.

Nagaoka et al. (1999) investigated the expression of gap junction protein connexin in normal and crush-injured rat sciatic nerves. Using confocal laser scanning microscopy, Cx26 was seen in the perineurium, Cx43 was present in the perineurium and epineurium and Cx32 was present in the paranodal regions of the nodes of Ranvier. After crush injury, expression of Cx32 was transiently lost but recovered, whereas Cx43 expression appeared in the endoneurium. No alterations were observed in Cx43 during nerve regeneration, but Cx26-positive spots decreased.

Cx32 is expressed in the pronodes and Schmidt-Lanterman incisures of myelinating Schwann cells, in which it is believed to form a reflexive pathway between the abaxonal and adaxonal cytoplasmic domains. Charcot-Marie-Tooth is a neurodegenerative disease in which many mutations in Cx32 genes are found. Patients that bear the mutation Val1881Ala have a severe peripheral neuropathy. Abrams et al. (2003) investigated the behavior of Schwann cells that express this mutant. In a xenograft system in nude mice, they verified that the ability to regenerate myelinated fibers is impaired. They suggest that loss of function of Cx32 may impact on the function of precursors of myelinating Schwann cells before the formation of the hypothesized reflexive pathway. On the other hand, the Glu102Gly (E102G) mutation leads to a milder phenotype and early regeneration of myelinated fibers is not impaired.

Bone marrow hematopoietic cells

Mouse and human bone marrow cells were studied by Krenacs & Rosendaal (1998) in order to verify the cell type association of the connexin types in bone marrow under different physiological and pathological conditions and to test the pathway of communication in bone marrow cultures. In mouse and human bone marrow, Cx43 is the main connexin, Cx37 was found only in the arteriolar endothelium and no expression of Cx32 or Cx26 was detected. These cells do communicate in culture. After treatment with 5-fluorouracil, mouse regenerating bone marrow stromal processes expressed high Cx43 levels; when in culture, there was communication between stromal cells and between stromal cells and hematopoietic cells. In a few human leukemia samples (acute lymphoblastic leukemia, acute myeloid leukemia and myelodysplastic syndrome), Cx43 expression was found to increase fourfold more. The authors conclude that direct cell-to-cell communication may be involved in hematopoiesis in cases of demand of progenitor cells and in regeneration. However, gap junctions may not play as important a role in resting adult hematopoiesis and in leukemias.

Lymphoid and myeloid development was studied in the bone marrow of mice in which Cx43 was heterologously deleted (Montecino-Rodrigues, Leathers & Dorshkind, 2000). Cx43 deletion ($Cx43^{-/-}$) compromises the terminal stages of primary T and B lymphopoiesis; Cx43 knockout mice had a reduced frequency of $Cd4^{+}$ cells, T cell receptor-expressing thymocytes and surface immunoglobulin M^{+} cells compared to wild-type mice. $Cx43^{+/-}$ mice also showed defects in B- and T-cell development, similar to those observed in Cx43 knockout mice. Regeneration of lymphoid and myeloid cells after cytoablative treatment was severely impaired in these mice. These data suggest that loss of the Cx43 allele can affect blood cell formation.

Presley et al. (2005) used hematopoietic stem cells in culture to study the role of Cx43 on proliferation and differentiation *in vivo*. Cx43 Mx-Cre mice were created, generating an inducible Cx43 knockout mouse. Interferon-inducer poly(i)-poly was used to generate control ($Cx43^{+/+}$) and Cx43-deficient ($Cx43^{-/-}$) mice. When mice from both genotypes were treated with 5-fluorouracil, it was verified that Cx43-deficient mice presented severely impaired hematopoiesis recovery, as demonstrated by absence of recovery of blood cell counts, including neutropenia, anemia and thrombocytopenia, and a five- to eightfold decrease of cellularity and hematopoietic progenitor content. These data suggest that hematopoietic regeneration after cycle-specific chemotherapy is blocked in Cx43-deficient mice and that Cx43 expression seems to be crucial in the development of an efficient response to hematopoietic stress.

Endothelial cells

Yeh et al. (2000) investigated the role of Cx37, Cx40 and Cx43 in endothelial cells from rat carotid artery after denudation injury. They verified, *in situ*, that after injury the regenerating endothelium initially expressed small, sparse gap junctions, the number of which progressively increased to values equivalent to those of controls. Cx40 spots returned to normal levels by 28 days after injury; however, Cx37 and -43 exceeded values in uninjured artery. These results suggest that different intercellular communication requirements are necessary during the various phase of the healing process.

Angiogenesis

Angiogenesis is involved in several physiological and pathological processes, being an essential event during reproduction, development and wound repair. Control of angiogenesis may be desirable in diseases that are driven by persistent unregulated angiogenesis, such as arthritis,

diabetic retinopathy, tumor growth and cancer metastasis (Folkman & Shing, 1992). Therefore, it is highly important to understand the events that can influence endothelial cell growth to form new blood vessels.

Endothelial growth is the central event during formation of new blood vessels (Folkman & Shing, 1992). One of the factors that can influence cell proliferation is the ability to communicate with adjacent cells (Cheng, Smith & Charnock-Jones, 2003). Although particularly complex (Vestweber, 2000), understanding molecular pathways of crosstalk between endothelial cells is crucial for the comprehension of angiogenesis dynamics.

Endothelial cells express Cx43, Cx40 or Cx37. Errede et al. (2002) verified that there is differential expression of Cx43 in fetal, adult and tumor-associated human brain endothelial cells. Cx43 is highly expressed in the cortical plate and astrocytoma blood vessels, while it is virtually absent in the cerebral cortex microvessels. There is also high expression of Cx43 in the developing brain. The authors conclude that endothelial Cx43 expression is developmentally regulated in the normal human brain and that it may be controlled by the microenvironment in both normal and tumor-related conditions.

Ashton et al. (1999) studied the *in vitro* endothelial cell migration, the vascular tube formation and the influence of thromboxane A_2 on the process. They detected that GJIC increases in migrating human endothelial cells. However, thromboxane A_2, a biologically active eicosanoid released by platelets, monocytes and damaged vessel wall, inhibited endothelial cell migration, intercellular communication capacity as well as vascular tube formation. Moreover, it was verified by immunohistochemistry that thromboxane A_2 may impair functional coupling by altering the cellular distribution of gap junctions, leading to increased Cx43 internalization.

Suarez & Ballmer-Hofer (2001) detected that vascular endothelial growth factor (VEGF) stimulates angiogenesis by directly acting on endothelial cells and maximally blocked GJIC capacity 15 min after administration. The cells restored their communication capacity 1–2 h after treatment. The authors suggest that this effect may be due to the phosphorylation state of Cx43. Thuringer (2004) verified that this VEGF-induced disruption of GJIC is relayed by an autocrine communication via secretion of adenosine triphosphate to preserve intercellular calcium signaling in the capillary endothelium.

Nitric oxide is a potent modulator of gap junctional coupling in endothelial cells; it enhances the *de novo* formation of endothelial gap junctions by increasing the incorporation of Cx40 into the plasma membrane (Hoffmann et al., 2003).

The expression of Cx43 in intimal vascular hyperplasia, a vascular remodeling process that occurs after a vascular injury, was investigated by Deglise et al. (2005). This process may cause stenosis and may occur during venous bypass grafts. In this process, there is involvement of proliferation, dedifferentiation and migration of medial smooth muscle cells toward the subintimal space. A time-course analysis of saphenous veins in culture revealed that there was progressive upregulation of Cx43 during the intimal hyperplasia, while Cx40 expression remained unchanged during the process.

Acute vascular injury induced by percutanous coronary interventions is associated with increased Cx43 expression in neointimal muscle cells, and reducing the expression of gap junction connexin (Cx43) inhibits the progression of atherosclerosis. Chadjichristos et al. (2006) showed that reducing Cx43 limits neointima formation after acute vascular injury by decreasing the inflammatory response and reducing smooth muscle cell migration and proliferation. The authors suggest that decreasing Cx43 expression may offer a novel therapeutic strategy for reducing restenosis after coronary interventions.

Using Cx43 heterozygous knockout mice (Reaume et al., 1995), Walker et al. (2005) verified that Cx43 deficiency causes dysregulation of coronary vasculogenesis, which may be one of the causes of newborn deaths of Cx43 homozygous knockouts. In knockout animals, there was a marked reduction in the branching complexity of the coronary artery and a profusion of large coronary plexuses. There was also elevated expression of VEGF and abnormal epicardial cell morphology. Cx43 is therefore important for coronary vasculogenesis and vascular remodeling.

Rodrigues et al. (2006) used heterozygous knockout mice for Cx43 and tested *in vivo* the role of Cx43 in the formation of new blood vessel sprouts. The corneal model of angiogenesis (Sunderkotter et al., 1991; Cao & Cao, 1999), which represents one of the best *in vivo* models to study angiogenesis, was applied in $Cx43^{+/-}$ and $Cx43^{+/+}$ male or female mice. As the cornea is devoid of preexisting vascularization, any vessels found there after cauterization or stimulation by angiogenic factors are newly formed blood vessels (Auerbach et al., 2003). Two parameters were quantified by image analysis at 2 or 6 days after cauterization: newly formed blood vessel density and length. At days 2 and 6 after corneal cauterization, $Cx43^{+/-}$ mice showed smaller density of newly formed blood vessels compared to $Cx43^{+/+}$ mice. Therefore, deletion of one *Gja1* allele (Cx43 gene) may have impaired cell-cell communication, diminishing angiogenesis in the mouse cornea.

Wound Healing and Fibroplasia

Wound healing is a process in which cell proliferation occurs to restore the normal tissue architecture with connective tissue.

Skin

The expression of connexins during epidermal wounding was investigated by Goliger & Paul (1995). The authors detected that Cx26 was upregulated in epidermal cells close to the wound but downregulated in cells at the wound edge. On the contrary, Cx31.1 and Cx43 were downregulated in cells at both epidermal localizations. Interestingly, increased expression of Cx26 was still evident in the hyperproliferative epidermis of 6-day-old wounds. Coutinho et al. (2003) studied the dynamics of gap junction proteins Cx26, 30 31.1 and 43 in murine epidermis and dermis during wound healing. They verified that connexin expression is extremely plastic between 6 h and 12 days postwounding. Interestingly, the immediate response (6 h) is downregulation of all connexins in the epidermis, but thereafter the expression profile of each connexin changes dramatically. The authors conclude that the changing patterns of connexin expression are key events in the wound-healing process.

Qiu et al. (2003) studied the effect of targeting Cx43 in the skin wound-healing process. Incisional and excisional skin lesions were provoked in rats and followed by topical application of a gel containing Cx43 antisense. Cx43 knockdown resulted in reduced inflammation and a dramatic increase in the rate of wound closure. In addition, a significant reduction in the deposition of granulation tissue was obtained, with a smaller, less distorted scar. Cx43 antisense treatment, therefore, was considered a potential therapeutic strategy to improve the wound-healing process.

The expression of Cx26, Cx30 and Cx43 was investigated in human cutaneous wound healing and nonhealing wounds and in *ex vivo* accelerated wound healing after transplantation of keratinocytes (Brandner et al., 2004). The authors showed a loss of Cx43 staining at the wound margins during initial wound healing and after the transplantation of keratinocytes. In contrast, Cx43 remains present at the margins of most nonhealing wounds. The authors also showed a subsequent induction of Cx26 and Cx30 near the wound margins in spontaneous wound healing and even earlier, after the transplantation of keratinocytes. Cx26 and Cx30 were present at the wound margins of most nonhealing wounds. The authors showed that Cx43 downregulation is an important event in human wound healing. GJIC may contribute to the acceleration of wound healing after the transplantation of keratinocytes.

According to Richards et al. (2004), phosphoserine 368 expression levels were increased after wounding of human skin but not at 6 or 72 h. Evidence indicates that protein kinase C-dependent phosphorylation of Cx43 at S368 creates dynamic communication compartments that can temporally and spatially regulate wound healing.

Coutinho et al. (2005) studied the effect of Cx43 antisense application in the early stages of partial-thickness cutaneous burn wound healing. Antisense reduced the spread of tissue damage and neutrophil infiltration around the wound following injury. Epithelial cell proliferation was increased, and therefore the rate of wound closure was accelerated, compared to controls. Resultant scarring was smaller, with less granulation tissue, and more dermal appendages were seen in comparison to controls. These results confirm that Cx43 antisense treatment speeds partial-thickness burn healing and reduces scarring.

Mori et al. (2006) studied the role of acute downregulation of Cx43 with antisense oligonucleotides at wound sites in the wound-healing process. Both *in vivo* and *in vitro* studies were conducted. Treated wounds exhibited accelerated skin healing with significant increases in keratinocyte and fibroblast proliferation and migration. In addition, *in vitro* knocking down of Cx43 is associated with increased mRNA for TGF-ß1 and collagen α1 and general collagen content at the wound site. Granulation tissue, angiogenesis, myofibroblast differentiation and wound contraction occurred faster, concomitantly with a reduction in leukocyte infiltration. These data may suggest a therapeutic effect of using Cx43 antisense in skin wound healing.

Cornea

Expression of Cx26, Cx32, Cx43 and Cx50 was studied in rabbit corneal epithelium during wound healing (Matic et al., 1997). After a 3 mm-wide lesion, corneas were allowed to heal *in vivo* for up to 45 h. The authors verified that after wounding the migrating epithelial monolayer lacked Cx43 and Cx50; this pattern appeared as early as 6 h after wounding and lasted for 24 h. However, staining of the epithelial membrane was increased in the transition zone between monolayered and multilayered epithelium. The authors concluded that corneal epithelial healing involves biphasic changes in the expression of connexins and cell-to-cell communication. Ratkay-Traub et al. (2001) studied the gap junction proteins Cx43 and Cx26 in the healing process of rabbit corneas after keratectomy with excimer laser. During regeneration, both connexins were expressed throughout the corneal epithelium including the migrating cells. They also showed transient upregulation 24 h after wounding in the form of overlapping relocation of the upper cell layers, when the cells were highly proliferating. Cao et al. (2002) studied, for the first time, the differentially expressed genes in the healing of mouse corneas 18–22 h after transepithelial excimer laser ablation. cDNA microarrays on mouse nylon arrays showed that 1,176 genes were upregulated and 27 were downregulated. The authors detected that Cx31, ZO1 and occluding, among others, were downregulated during cornea healing.

Tongue

The changes in Cx26 and Cx43 were studied in hamster tongue during wound healing (Saitoh et al., 1997). Shortly after the injury, expression of Cx26 and Cx43 decreased at wound edges; but by 1–3 days after injury, the expression of both proteins increased and both connexins colocalized at the same spots in the epithelium near the wound edges. Therefore, quantitative changes were associated with differentiation, migration and proliferation of keratinocytes.

Cardiac muscle

Kostin et al. (2002) tested the hypothesis that structural remodeling of cellular connections, alterations in the expression of connexins and an increase in fibrosis represent anatomic substrates of atrial fibrillation in human patients. The authors detected that Cx40 had marked heterogeneous distribution and Cx43 was markedly decreased. These changes, together with the augmentation of fibrosis, may underlie localized conduction abnormalities and contribute to initiation and self-perpetuation of reentry pathways and atrial fibrillation.

Liver fibrosis and cirrhosis

Liver fibrosis occurs as a consequence of repeated injury to liver cells, causing cell death. Liver cells can regenerate; however, after repeated injury, there is activation of collagen-producing cell types, which starts the fibrosis process. Cirrhosis is an irreversible process, which is characterized by intense fibrosis linking portal spaces and/or hepatic terminal venules and regenerating hepatocyte nodules that confer a nodular and hard appearance for the livers.

Fat-storing cells, previously denominated "Ito cells" or perisinusoidal cells, play an important role in collagen deposition in normal and cirrhotic livers.

Greenwel et al. (1993) studied liver fat-storing cell clones obtained from CCL4-induced cirrhotic rat livers with regard to proliferation, expression of extracellular matrix components, interleukin 6 and connexin 43. The authors detected that fat-storing cell clones are heterogeneous with regard to proliferation index, expression of procollagens, interleukin 6 and TGF-ß mRNAs. They also verified that the clones are coupled through functional gap junction but heterogeneous with regard to the expression of Cx43. Yamaoka et al. (2000) studied the expression of Cx32 in human chronic liver diseases, including chronic viral hepatitis and liver cirrhosis. They verified that the number of gap junction plaques was significantly smaller than in normal liver and that the number of gap junction plaques tended to be lower in liver cirrhosis than in chronic viral hepatitis. These results reinforce a possible role of decrease in cell-to-cell communication capacity during chronic liver diseases, which can possibly lead to liver cancer. Nakashima et al. (2004) investigated the expression of Cx32 in chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. They observed that Cx32 expression decreased gradually as the disease progressed to cirrhosis and hepatocellular carcinoma. Interestingly, Cx32 was detected in the cytoplasm of neoplastic hepatocytes in hepatocellular carcinoma, and it was also seen, although less frequently, in liver specimens showing hepatitis and cirrhosis. These findings suggest that the changes in both the amount and the subcellular localization of Cx32 may be implicated in human hepatocarcinogenesis.

Concluding Remarks

The pioneer studies by Loewenstein in the 1960s proposed to the scientific community important questions about the role of communication capacity on cell proliferation. Loewenstein's group verified that cancer cells did not communicate with other cells, while normal cells did communicate rather freely with other cells (Loewenstein & Kanno, 1966, 1967). However, when Loewenstein & Penn (1967) examined the intercellular communication in (non-neoplastic) regenerating rat liver and urodele skin, they verified that the communication capacity was as good as the respective normal intact state. They also discussed why cell proliferation ceased when the tissue was restored, by the process called "contact inhibition." The authors proposed that "normal tissue growth and differentiation depend on the flow of materials from one cell interior to another through the junctional cell surfaces."

Loewenstein's concept was examined in depth by Trosko & Ruch (1998), who proposed that negative or positive signals could pass through gap junctions from one cell to another and control cell growth. They also stated that in cells lacking gap junctions growth would not be suppressed by neighboring negative signals or would be stimulated by accumulation of positive signals, and this could lead to dysregulated growth.

The role of connexin genes in growth control in several situations was reviewed by Yamasaki et al. (1999). In this article, the authors stated that connexin expression per se, rather than GJIC level, is more closely related to growth control, suggesting that connexins may have a GJIC-independent function.

From the review presented here, it can be verified that GJIC capacity and the proteins which form these junctions, the connexins, are indeed associated with growth control in non-neoplastic pathological processes. In general, in most situations and in several tissue types, there is a direct

correlation between GJIC and connexin expression and an inverse correlation between GJIC capacity and cell growth. One of the most interesting examples is skin wound repair, in which Cx43 antisense applied in skin during wound repair could accelerate the process. This is a proposed therapeutic method that still needs to be tested in humans and other animal species.

Exceptions to the rule "diminished GJIC capacity and increased cell proliferation" were found in connexin knockout mice. Liver regeneration was delayed in Cx32 knockout mice (Temme et al., 1997) and in Cx32 transgenic mice expressing a dominant-negative Cx32 mutant (Omori et al., 2001; Dagli et al, 2004). Angiogenesis was dysregulated in Cx43 knockout embryos and fetuses (Walker et al. 2005) and diminished in mice in which Cx43 was heterologously deleted (Rodrigues et al, 2006). *Schistosoma* sp. granuloma cells in infected mice also presented lower cell proliferation (Oloris et al., 2007). These differences may be related to the cell communicating status when the proliferation stimulus occurs. Poorly communicating cells tend to present disturbed growth behavior. Another important statement is that although genetically modified animals are considered excellent tools for research, the exact extent of the genetic modifications performed are not known, so the animals may show unexpected responses. These may be due to the complex relationships in the living body, including the release of growth hormones or growth factors or other reactions still to be investigated.

Up to now, and according to the evolution of the scientific knowledge, it is possible to affirm that GJIC and connexins do interfere with cell proliferation in most physiological and pathological situations, and the mechanisms are yet to be discovered.

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