The Gap Junction Protein Connexin32 Is a Mouse Lung Tumor Suppressor

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Abstract

Although loss of connexin expression and/or gap junction intercellular communication correlates with decreased growth control and increased neoplastic potential, there is limited evidence directly linking gap junction intercellular communication function with tumor suppression in situ. Here, we show for the first time that a gap junction protein, connexin32 (Cx32), acts as a lung tumor suppressor in a mouse model. Cx32-deficient nontumorous lung tissue exhibited an increased proliferative index (P < 0.001), and, after exposure to the carcinogen diethylnitrosamine, Cx32-deficient mice exhibited a highly statistically significant (P < 0.001) increase in bronchioloalveolar lung tumor incidence (28 of 45, 62%) and a 45% increase in average multiplicity compared with wild-type mice (7 of 29, 24%). Tumors from Cx32-deficient mice also showed increased activation of mitogen-activated protein kinase (P < 0.001) compared with wild-type tumors, implicating this signaling pathway in Cx32/gap junction intercellular communication-associated lung tumorigenesis.

Introduction

Gap junction proteins, connexins, mediate gap junction intercellular communication and are a group of at least 20 highly conserved proteins with developmental and tissue-specific expression patterns (1, 2). Gap junction intercellular communication allows for the direct transmission between neighboring cells of ions, small hydrophilic metabolites, and messengers less than 1,000 to 2,000 in size. Connexins and gap junction intercellular communication play an important role in normal development and physiology with a loss of function implicated in various human diseases including nonsyndromic deafness and peripheral nerve disorders (3). Several studies with transgenic mouse strains with germline-inactivated connexin genes (knockouts) have revealed the importance of connexins in normal developmental and physiologic processes (4). Just as connexins have been implicated in the control of normal development and growth, many studies have shown an association between reduced gap junction intercellular communication and decreased growth control/increased tumorigenesis (1, 2, 5). Specifically, inducible expression of exogenous connexin43 in gap junction intercellular communication-incompetent tumor cells can also restore partial growth control in vitro and/or in vivo (6). The best evidence directly linking gap junction intercellular communication and growth control comes from studies with genetically engineered mice deficient in connexin32 (Cx32; ref. 7). These mice exhibit increased susceptibility to chemical (diethylnitrosamine) and radiation-induced liver tumorigenesis, observed as increased tumor incidence and tumor size (8–12).

Cx32 is expressed in liver, lung, kidney, pancreas, and several other tissues. Cx32-deficient mice exhibit abnormal physiology as well as peripheral nerve demyelination reminiscent of human Charcot-Marie Tooth Syndrome (4). Although previous studies have established that connexins are expressed in the pulmonary system, the precise influence of connexins on development and physiology has yet to be determined (13–16). Although several studies have suggested the influence of connexins on lung tumor cell behavior (12, 17, 18), no conclusive studies have linked connexin deficiency with pulmonary oncogenesis in situ. Here, we evaluated the role of Cx32 in mouse lung carcinogenesis with a genetically deficient mouse model in combination with the chemical lung carcinogen diethylnitrosamine and observed increased tumorigenesis strongly implicating Cx32 as a tumor suppressor in mouse lung.

Materials and Methods

Mice and Diethylnitrosamine Treatment. Cx32-deficient heterozygous mice originally created in the laboratory of K. Willecke (Institut für Genetik, Bonn, Germany; Ref. 7) and generously provided by Steven Scherer (School of Medicine, University of Pennsylvania, Philadelphia, PA) in a FVB/N background were crossed with C57-BL6 mice and inbred for eight generations before the study. PCR-based genotyping was conducted as described previously (12). Diethylnitrosamine experiment was as follows: Wild-type mice (29 total, 17 male/12 female) and Cx32-deficient mice (45 total, 23 male/22 female) were injected intraperitoneally at 12 ± 2 days of age with diethylnitrosamine (Sigma, St. Louis, MO) at 0.12 μmol diethylnitrosamine/mg body weight. Spontaneous tumorigenesis experiment was as follows: Wild-type mice (18 total, 8 male/10 female) and Cx32-deficient mice (49 total, 28 male/21 female) were untreated.

Necropsy, Tissue Processing, Histologic, Immunohistochemical Analysis. Mice were injected with bromodeoxyuridine (BrdUrd; Sigma; 20 mg/ml stock in PBS and 1 mg/10 g mouse injected) 1 hour before sacrifice (at 9 months for diethylnitrosamine experiment or 16 to 24 months for spontaneous tumorigenesis experiment). Complete lungs were formalin fixed, embedded in paraffin, and sectioned into 4-μm slices by standard procedures with subsequent deparaffinization, H&E staining and immunohistochemical detection (12). Lung bronchioloalveolar adenoma/carcinoma classification was based on the following: local tissue and blood/lymph vessel invasion, nuclear atypia, anaplastic appearance, increased nuclear to cytoplasmic ratio, and presence of mitotic figures. All tumors counted were at least 250 cells with tumor area quantified with the following formula: area = πr². Statistical analysis of tumor incidence and mitogen-activated protein kinase (MAPK) activation data were done with two-tailed, two-sample proportions test or Student’s t test (BrdUrd experiment only) with significance at P ≤ 0.05. Immunohistochemical analysis was done as described previously (12) with a primary antibody against surfactant-associated protein C (1:50; Santa Cruz Biotechnology Biotech, Santa Cruz, CA), BrdUrd (1:100; DAKO, Carpinteria, CA), or phosphorylated/activated MAPK (Erk1/p44 and Erk2/p42; 1:100; Cell Signaling, Beverly, MA) for 1 hour at room temperature (MAPK overnight at 4 degrees) followed by washes and a 1-hour incubation at room temperature with a biotinylated antigen (for surfactant-associated protein C; 1:700; Vector Laboratories, Burlingame, CA), horseradish peroxidase-conjugated antimouse (for BrdUrd, 1:200; Southern Biotechnology, Birmingham, AL), or biotinylated antirabbit (for MAPK, 1:250; Vector Laboratories) secondary antibody. In cases where not all of the tumors were analyzed from both groups, a random representative selection of tumors was subjected to immunohistochemistry analysis. BrdUrd indices were calculated by counting positively labeled cells/40× objective field (0.23 mm² area) and averaging results within each group.

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Results and Discussion

Connexin32-Deficient Mice Exhibit Increased Susceptibility to Diethylnitrosamine-induced Lung Tumorigenesis. In mouse lung, Cx32 is expressed in multipotent pulmonary alveolar type II cells (13). We evaluated the effect of Cx32-deficiency on lung tumorigenesis initiated with the carcinogen diethylnitrosamine, a tissue-specific carcinogen shown to cause tumors primarily in mouse liver and lung. In liver, we observed increased tumorigenesis in Cx32-deficient mice in close agreement with previously published studies (data not shown; refs. 8–12). In lung, summarized in Table 1, Cx32-deficient mice showed a 2.6-fold increased susceptibility to bronchioalveolar tumor development (28 of 45, 62%) when compared with wild-type mice (7 of 29, 24%; $P < 0.001$). Cx32-deficient mice also exhibited an increase in average tumor number/mouse (multiplicity; 1.6/mouse) compared with wild-type mice (1.1/mouse). When comparing the number of tumor-bearing mice with more than one tumor/mouse, the Cx32-deficient group had a higher, although not statistically significant, percentage of mice with more than one tumor (12 of 28, 43%) compared with wild-type mice (1 of 7, 14%; $P = 0.072$).

Cx32-deficient mice contained 2.4-fold as many carcinomas (13 of 45, 29%) as wild-type mice (1 of 8, 13%), suggestive of an increased conversion to an invasive tumor type in the absence of Cx32. There was no significant difference when comparing male to female mice in incidence or multiplicity. When present, tumors exhibited papillary, solid, and mixed morphologic phenotypes with no apparent bias to genotype. Fig. 1 illustrates the range of tumor morphology, type, and sizes observed in lungs from wild-type (Fig. 1, A–D) and Cx32-deficient (Fig. 1, E–J) mice.

Cx32-Deficient Mice Do Not Demonstrate Increased Spontaneous Lung Tumor Incidence. As mouse strain/genetic backgrounds can alter tumor susceptibility, we evaluated untreated wild-type and Cx32-deficient mice to determine spontaneous lung tumor incidence. We detected no significant genotype-associated inequality in spontaneous bronchioloalveolar tumor incidence [Table 1; wild-type, 3 of 18 (17%); Cx32-deficient, 11 of 49 (22%); $P = 0.624$] or multiplicity in 16 to 24-month-old mice. These results suggest that in lung, similar to the connexin tumor suppressor function observed in chemical/radiation-induced mouse liver (8–12), the role of Cx32 as a tumor suppressor is only evident after exposure to a carcinogen. Thus, the absence of Cx32 alone is not sufficient to induce spontaneous tumors in these mice. Although the frequency of wild-type mice with tumors in the diethylnitrosamine experiment (24%) is similar to the spontaneous wild-type incidence (17%), diethylnitrosamine-treated mice were analyzed much earlier (9 months) than mice in the spontaneous experiment (16 to 24 months); therefore, results are not directly comparable.

Cx32-Deficient Lung Tumors Express the Type II Alveolar Cell Marker Surfactant-associated Protein C. The adult mouse lung is predominantly composed of type I and type II pulmonary alveolar cells (13, 19). Type I cells mediate gas exchange and occupy a majority of surface area in the adult mouse lung. Type II cells produce surfactants and are direct progenitors of type I cells. Cx32 is expressed in type II alveolar cells, which are the predominant alveolar cell type found in the rudimentary mouse pulmonary system (19) at the time of diethylnitrosamine exposure used in this study (Day 12). To determine the origin of these bronchioalveolar tumors, we used immunohistochemistry to examine expression of a type II-specific product, surfactant-associated protein C (Fig. 2, A–D; ref. 19). All of the Cx32-deficient and wild-type bronchioalveolar tumors analyzed showed surfactant-associated protein C expression supporting the type II alveolar cell derivation of the tumors. This is in agreement with extensive evidence, indicating that the majority of mouse lung tumors originate from the proliferation-competent, normally Cx32-expressing, type II alveolar cells (19).

Cx32-Deficient Nontumorous Lung Tissue Demonstrates Increased BrdUrd Incorporation. Before sacrifice, mice were injected with the S-phase marker, nucleoside analog BrdUrd to allow quantification of nontumor and tumor cell proliferation. As expected, tumors from both wild-type (37.4 ± 36.5/0.23 mm² area) and Cx32-deficient (33.8 ± 11.8/0.23 mm² area) mice displayed dramatically increased BrdUrd incorporation compared with normal tissue (Fig. 2, F, I, and J). However, no significant difference in tumor BrdUrd index was observed between genotype groups ($P = 0.68$). In contrast, nontumorous Cx32-deficient lung tissue showed a statistically significant increase in BrdUrd incorporation (4.2 ± 1.2/0.23 mm² area; Fig. 2, E and H) compared with wild-type nontumorous lung tissue (1.8 ± 1.3/0.23 mm² area; $P = 0.0002$; Figs. 2, E and G). Measure-

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Abbreviations: BAV, bronchioalveolar; DEN, diethylnitrosamine; KO, knockout.
Fig. 1. Cx32-deficient mice exhibit increased bronchioloalveolar tumorigenesis. H&E standard histologic staining of wild-type and Cx32-deficient bronchioloalveolar tumors. Tumor areas varied from 20 mm² (smallest) to 650 mm² (largest). A–D, wild-type; E–J, Cx32-deficient. A–C and E–G, adenomas; D and H–J, carcinomas. Large and inset photos are same tumor at different magnification. A–J, bar = 40 μm. Bar in insets (except inset J) = 160 μm; bar in inset J = 280 μm.
Fig. 2. Wild-type and Cx32-deficient bronchioloalveolar tumors show expression of the type II alveolar cell-associated protein marker, surfactant-associated protein C, with Cx32-deficient nontumorous tissue exhibiting increased BrdUrd incorporation. Immunohistochemical detection of wild-type and Cx32-deficient bronchioloalveolar tumors for surfactant-associated protein C (A–D) and BrdUrd incorporation in nontumorous and tumorous lung tissue (E–J). Brown signal is surfactant-associated protein C or BrdUrd reactivity, and blue is hematoxylin nuclear counterstain. A, nonspecific primary antibody control; B, wild-type adenoma; C and D, Cx32-deficient carcinomas. Large and inset photos are same tumor at different magnification. Bar = 50 μm (A–D) and 270 μm (insets). E and F, immunohistochemical detection of incorporated BrdUrd in nontumorous (E) and tumor (F), wild-type (●), and Cx32-deficient (▲) lung tissue. G–J, photographs of quantified BrdUrd areas. G, wild-type nontumorous lung; H, Cx32-deficient nontumorous lung; I, wild-type adenoma; J, Cx32-deficient carcinoma. *, statistical significance of nontumorous wild-type and Cx32-deficient lung BrdUrd incorporation comparison (P = 0.0002). Bars = 40 μm (G–J). (KO, knockout)
ments were taken from nontumorous tissue distal to tumor areas to avoid any local tumor-related effects.

Cx32-Deficient Lung Tumors Exhibit Increased MAPK Activation. As increased activation of MAPK pathways are frequently observed in many tumor types, including lung and liver (12), and have been implicated in gap junction intercellular communication regulation (1, 2, 20), we analyzed diethylnitrosamine-induced lung tumors from wild-type and Cx32-deficient mice immunohistochemically with an antibody reactive only against the phosphorylated/activated form of MAPK (Erk1/p44 and Erk2/p42; Fig. 3). This analysis revealed a

Fig. 3. Cx32-deficient mice exhibit increased MAPK-active bronchioloalveolar tumors. Immunohistochemical detection of activated-MAPK in wild-type and Cx32-deficient bronchioloalveolar tumors. A and B, wild-type; C–F, Cx32-deficient. A–D, MAPK negative; E–F, MAPK positive. A, wild-type adenoma; B, wild-type carcinoma; C, Cx32-deficient adenoma; D–F, Cx32-deficient carcinoma. Brown signal is activated-MAPK reactivity, and blue is hematoxylin nuclear counterstain. Large and inset photos are same tumor at different magnification. Bar = 40 μm (large panels) and 200 μm (insets).
statistically significant increase in the percentage of MAPK-activated lung tumors from Cx32-deficient mice (12 of 45, 27%; Fig. 3, E and F) compared with lung tumors from wild-type mice (0 of 8, 0%; Fig. 3, A and B; P < 0.001). Although activation of MAPK-related pathways is frequently correlated with tumor progression in mouse lung, here it cannot completely explain the increased MAPK-activation in Cx32-deficient lung tumors, because we observed carcinomas in both genotypes that exhibited no detectable MAPK-activation (Fig. 3, B and D). Additionally, Cx32-deficient lung tumors showed no bias toward MAPK-activation in more advanced tumor types, compare adenomas (5 of 12, 42%; MAPK positive) and carcinomas (7 of 12, 58%; MAPK positive).

Conclusion
This study demonstrates that loss of the gap junction protein Cx32 renders mouse lung more susceptible to carcinogen-induced tumorigenesis (diethylnitrosamine). It is interesting that loss of Cx32 does not result in any dramatic abnormalities in pulmonary development, function, or spontaneous tumorigenesis, most probably because of redundancy in function among other connexins expressed. The growth regulatory role for Cx32 is dramatically evident both in tumor incidence as well as multiplicity. An increased carcinoma to adenoma ratio also suggests an acceleration in conversion toward a more aggressive tumor phenotype in the absence of Cx32. Specifically, we have shown that Cx32 is a critical tumor suppressor in mouse lung, in accord with the previously determined function of Cx32 as a mouse liver tumor suppressor (8–12). In both tissues, Cx32-deficiency alone does not predispose mice to increased spontaneous tumorigenesis; however, loss of Cx32 does dramatically increase tumor incidence/multiplicity after exposure to a chemical carcinogen. Thus, gap junction intercellular communication may act primarily at the tumor promotion level and/or may require that additional oncogenic mutations be sustained for cellular transformation. The increased proliferation (BrdUrd incorporation) observed in the nontumorous Cx32-deficient lung tissue may represent one possible mechanism, contributing to the acquisition of these additional mutational events. Additionally, as Cx32-deficient mice exhibit an increase in tumors with active MAPK pathways, loss of Cx32-mediated gap junction intercellular communication may increase the number of initiated cells activating MAPK or in some manner contribute to the positive selection of tumors harboring activated MAPK pathways.

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