Connexin 37 Mutations in Rat Hepatic Angiosarcomas Induced by Vinyl Chloride

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Abstract

Connexin genes have been shown to restore normal cell growth when transfected into certain tumorigenic cells and thus are considered to form a family of tumor suppressor genes. In this study, we have analyzed mutations of the connexin 37 (Cx37) gene in rat hepatic angiosarcomas induced by vinyl chloride. A total of 25 rat liver tumors (22 hepatic angiosarcomas and 3 hepatocellular carcinomas) were analyzed by PCR-single-strand conformation polymorphism analysis and DNA sequencing. Four mutations were detected in three tumors: (a) one GGGGIY to GAGGIU mutation at codon 168; and (b) three silent mutations, CGA**® to GCC**® at codon 166. In addition, we found that codon 88 is polymorphic (GAG**® to GAA**®). Cx37 proteins are detectable in endothelial cells of normal liver by immunohistochemical analysis, but none of the angiosarcomas showed Cx37-positive spots. These results suggest that Cx37-mediated gap junctional intercellular communication may be disturbed in most of these angiosarcomas, but mutation of the Cx37 gene is rare.

Introduction

The major role of GJIC 1 is considered to be the maintenance of homeostasis in multicellular organisms. Gap junctions mediate the transfer of substances of molecular weight less than 1000 from one cell into adjacent cells (1). It is believed that through GJIC, the necessary level of second messengers that are important for growth control is maintained among cells in a given tissue (2, 3). Aberrant growth control is an essential feature of cancer cells. Therefore, it has long been considered that altered GJIC might play an essential role in carcinogenesis (3, 4).

GJIC is mediated by a group of related proteins called Cxs, containing conserved transmembrane and extracellular domains but unique cytoplasmic regions that may confer Cx-specific physiological properties. There are several lines of evidence that suggest that disruption of intercellular communication facilitates the clonal growth of potential cancer cells and that it can work as a tumor-suppressive element. For example, transfection of various Cx genes into GJIC-deficient cancer cells restores normal cell growth (5, 6). Because most tumor suppressor genes have been found to be mutated in tumors, our laboratory has studied the occurrence of Cx gene mutations in various tumors. We found no Cx32 gene mutations in primary gastric cancers (7) and hepatocellular cancers, but we found a base-substitution mutation of the Cx32 gene in a rat liver tumor induced by a nitrosamine (8).

By peptide analysis of a tumor-associated antigen, Mandelboim et al. (9) found that Lewis lung carcinoma-specific peptide is derived from a mutated Cx37 gene. Because Cx37 is expressed in the lung and mammary gland, we examined human lung and breast cancer samples for the presence of Cx37 mutation; although we found a polymorphism, no somatic mutations of the Cx37 gene were found (10).

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1 To whom requests for reprints should be addressed.
2 The abbreviations used are: GJIC, gap junctional intercellular communication; SSCP, single-strand conformation polymorphism; Cx, connexin.

Because Cx37 is highly expressed in endothelial cells (11), we decided to examine angiosarcomas.

Vinyl chloride monomer has been shown to be a multipotent carcinogen in rodents, being capable of causing a variety of tumors and inducing angiosarcoma of the liver (12). Human hepatic angiosarcomas are also strongly associated with occupational exposure to vinyl chloride (12). A multitude of studies have established the metabolic pathways and mutagenic properties of vinyl chloride and have allowed the identification of vinyl chloride-DNA adducts (13–16). In this study, we have examined mutation of Cx37 genes in 25 hepatic tumors induced in rats by vinyl chloride and found four mutations by PCR-SSCP and DNA sequencing.

Materials and Methods

Samples. Twenty-five DNA samples extracted from rat hepatic tumors induced with vinyl chloride were analyzed in this study. Eleven of the tumor samples were obtained at IARC, and 14 samples were provided by Dr. C. Maltoni (Institute of Oncology, Bologna, Italy). For tumor induction, as described previously (13), female Sprague Dawley rats with their pups (both sexes) were exposed for 8 h/day, 6 days/week to 500 ppm of vinyl chloride from day 3 through day 28 postpartum and after weaning were exposed for an additional 2 weeks (total exposure, 33 days). Twenty-two tumors were hepatic angiosarcomas, and three tumors were hepatocellular cancers. DNA from the tails of 40 Sprague Dawley (OFA) rats obtained from Ifba-Credo (L'Arbresle, France) was extracted by a phenol-chloroform procedure and analyzed for comparison with DNA from tumor cells.

SSCP Analysis of Cx37 Genes. For analysis of the Cx37 gene (as shown in Fig. 1), six pairs of primers were synthesized to produce overlapping fragments covering the whole coding part of the gene (17). Pairs of primers used were (5 to 3' ):
A: GAGGAGGACCGCCGGAACAT and CGGTGAGCGCTGCTGCGCT; B: GACGATCTGGATTTCCAGTCTTAC and CCCTACATGTAAAGGTTCT; C: GAGGGAGACCTTGCGCCACT and CCAGGCCTAGAGCGACCCACT; D: GCAGGGTCTCTCTCTAGGCCG and TTACTCTCCGTCGACAGAG; E: ACTGGAGCTGTGACGT CCTC CTGCGTACCTTTCACTC; and F: TTTTCCTCTCTTCTGCCTACTT.

For SSCP analysis, 2.5 μl of PCR products were mixed with the same volume of sample buffer (95% formamide, 20 mM EDTA, and 0.05% bromophenol blue). The samples were denatured for 4 min at 94°C and applied to a 5% polyacrylamide gel (acrylamide:bisacylamide, 99:1) with or without 5% glycerol. The gel was prepared with buffer containing 89 mM Tris-borate, 89 mM boric acid, and 0.16 mM EDTA. After electrophoresis, the gels were dried and exposed to X-ray film (Biomax; Eastman Kodak).

Sequencing of Mutated DNA. The aberrant bands in PCR-SSCP were cut from the gels, and DNA was eluted by boiling in water for 1 h. The eluted DNA was submitted to PCR again and sequenced by the dideoxynucleotide chain termination method using DNA Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, OH) and primers end-labeled with [γ-32P]ATP.

Immunohistochemistry. Frozen sections of 13 angiosarcoma tissue samples, 3 of which had a Cx37 gene mutation, were fixed with ethanol and immunostained using anti-Cx37 antibody provided by Dr. Daniel Gros (Université de la Méditerranée, Laboratoire de Génétique et Physiologie du Développement, Marseille, France). As a positive control, a frozen section of normal rat liver was used. For negative control, nonimmunized rat immunoglobulin was used as the first antibody. After immu-
nostaining with diaminobenzidine, silver intensification of the nickel-diaminobenzimidate was performed according to Merchenthaler et al. (18).

Results

PCR-SSCP Analysis. A total of 25 DNA samples from vinyl chloride-induced hepatic tumors were analyzed by SSCP. In four samples, aberrant bands were detected in the primer A region (see Figs. 1 and 2), and in three samples, aberrant bands were detected in the primer C region (Fig. 3). When 40 DNA samples from normal rats were similarly analyzed, the same aberrant band was detected in the primer A region in six cases but was not detected in the primer C region. In one (No. 2 in Table 1) of the three samples with an aberrant band in the primer C region, we were able to recover DNA from nontumor tissue for SSCP analysis and found no aberrant band. From these results, we could tentatively conclude that these tumor samples contain mutations in the primer C region and that the other aberrant bands found in the primer A region in four samples are probably a polymorphism.

Sequencing of the Aberrant Bands. To characterize the putative mutation and the polymorphic aberrant bands seen in the SSCP analysis, the aberrant bands and corresponding normal bands were cut from the SSCP gels, and the DNA samples extracted were subjected to DNA sequencing. The results are summarized in Table 1. As expected, the sequence of aberrant bands in the primer C region found in three tumor samples contained a base substitution (Fig. 4), namely a CGA to CGC mutation at codon 166. Furthermore, one sample contained an additional mutation, GGG to GAG at codon 168. Whereas the mutation at codon 168 altered the coding amino acid from glycine to glutaminic acid, the mutation at codon 166 did not change the amino acid, arginine. Therefore, the latter mutation was silent, but as described above, this base change is not polymorphic. A possible genetic polymorphism identified by SSCP analysis of tumor samples and normal rat samples was also sequenced. This was found to be a GAG/GAA polymorphism at codon 88, giving no change in the amino acid glutamine (data not shown). All mutations and polymorphisms were verified by carrying out further independent PCR of the original DNA samples.

Immunohistochemistry. By immunohistochemical analysis of normal rat liver, positive expression of Cx37 was detected as spots in endothelial cells of the central vein but was not detected in hepatocytes or epithelial cells of the bile duct (Fig. 5a). To compare protein expression of the Cx37 gene, either mutated or not mutated, we performed immunohistochemical analysis on 13 hemangiosarcoma samples, 3 of which had a Cx37 gene mutation. No expression was detected in the 3 samples that had Cx37 gene mutation nor in the other 10 samples, which did not have any Cx37 gene mutation (Fig. 5b). These results suggest that Cx37 protein expression is down-regulated and/or aberrantly localized in hepatic angiosarcomas.

Discussion

The results presented here indicate that Cx37 mutations can be found in rat hepatic angiosarcomas induced by vinyl chloride and that a genetic polymorphism of the Cx37 gene is also present among Sprague Dawley rats. Only one mutation found in the tumors was a base-substitution change resulting in a coding amino acid change. This mutation is located in the third transmembrane region. The Cx37 coded by such a mutation may have the potential to down-regulate wild-type Cx37 in a dominant-negative fashion because it is likely that mutant Cx37 and wild-type Cx37 would produce a heteromeric connexon that would not be functional. We have recently shown that several point mutations in the transmembrane as well as the extracellular domains of Cx32 caused a dominant-negative down-regulation of GJIC (19). Our more recent preliminary results suggest that Cx32 mutants even down-regulate Cx26 mediated GJIC, suggesting that Cx26/mutant Cx32 heteromeric connexons were formed. In fact, the occurrence of Cx26/Cx32 heteromeric connexons in vitro (20) as well as in vivo (21) has been reported. These results imply that mutation of a Cx gene at a certain domain could be a powerful mechanism for disruption of GJIC.

Three tumors had the same silent mutation at codon 166, which is also located in the third transmembrane domain. Because this base substitution was not found in 40 randomly chosen Sprague Dawley rats, we believe that it was a somatic mutation. It is puzzling why this silent mutation should be present in 3 of 25 tumors analyzed in this study; if the mutation is biologically silent and gives no selective growth advantage, such a mutation should be found more rarely. It is conceivable that the silent mutation codon would compete for tRNA and thus modify the rate of protein synthesis, but we are unaware of
any precedent for such an effect. However, in the three mutated samples, one sample was from a hepatocellular carcinoma; therefore, there is still a possibility that the silent mutation is a polymorphism.

In this study we also found a genetic polymorphism in the rat Cx37 gene that does not change the coding amino acid. The common codon was GAG, and the rare allele GAA was found in 4 of 25 tumor samples (16%) and in 6 of 40 normal individual rats (15%). These results suggest that there is no apparent relationship between these polymorphic alleles of the Cx37 gene and susceptibility to vinyl chloride-induced hepatic angiosarcomas. Nevertheless, this is the first report of a polymorphism of the Cx37 gene in the rat. We have previously shown that a genetic polymorphism that codes for different amino acids (valine and isoleucine) exists at codon 130 of the Cx37 gene in humans (10).

Vinyl chloride is a DNA-damaging carcinogen that induces liver angiosarcomas in humans and animals (15). It seems likely that mutations of tumor suppressor genes may be induced by the carcinogen and that such mutations contribute to the development of hepatic angiosarcoma. Hollstein et al. (22) and Trivers et al. (23) have examined the p53 tumor suppressor gene in six vinyl chloride-associated human liver angiosarcomas and found three A:T to T:A transversions. Soini et al. (24) and Andersson et al. (25) examined the p53 gene in 30 hepatic angiosarcomas not associated with vinyl chloride exposure and found 2 G:C to A:T transitions. Froment et al. (13) examined ras proto-oncogenes in seven rat liver tumors induced by vinyl chloride, whereas in two hepatocellular carcinomas, a transversion was found in Ha-ras. In five liver angiosarcomas, bp substitutions were found in N-ras A (in two samples), in N-ras B (four samples), and in N-ras C (three samples). The mutation found in a rat hepatic angiosarcoma in our study was GGG<sup>168</sup> to GAG<sup>168</sup> at codon 168. The G:C to A:T mutation was also observed in the Ki-ras gene of a vinyl chloride-associated human hepatic angiosarcoma, which is consistent with the known mutation pattern induced by vinyl chloride in bacteria (15, 26).

Although Cx37 mutation seems to occur only rarely in vinyl chloride-induced hepatic carcinogenesis, we have observed that Cx37 protein expression is decreased in all 13 hepatic angiosarcomas examined. Our results suggest that Cx37-mediated GJIC is disrupted by mechanisms other than Cx37 gene mutation. Our hope of further analyzing Cx37 protein by Western blot was hampered by the lack of sufficient frozen samples. In a similar study with nitrosamine-induced rat liver tumors, we also found that Cx32 protein expression was aberrant in many tumors, but only one of them contained a Cx32 gene mutation. Although we can tentatively conclude that Cx gene mutations are rarely involved in carcinogenesis, additional studies are warranted because mutations of only 2 of 13 Cx genes, Cx32 and Cx37, have thus far been studied.

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Fig. 5. Immunohistochemistry of Cx37 in normal liver and hemangiosarcoma. In the normal sample (a), small spots were detected in endothelial cells of the central vein but were not detected in hepatocytes or in bile duct epithelial cells. In hemangiosarcoma (b), no expression of Cx37 was detected.

References

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