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Changes in Gap Junction Protein (Connexin 32) Gene Expression During Rat Liver Carcinogenesis

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A rat liver gap junction (GJ) cDNA probe that detects mRNA encoding the 32 Kd GJ-protein (connexin 32) was employed to study GJ-protein gene expression in rat liver tumors induced by a single exposure to diethylnitrosamine (DEN) followed by exposure to 2-acetylaminofluorene (AAF)/CCl_4/AAF or induced by systemic administration of N-ethyl-N-hydroxyethylnitrosamine (EHEN). All carcinomas generated by these carcinogens showed markedly reduced levels of GJ-protein mRNA. This may indicate that GJ-protein levels and gap-junctional intercellular communication (GJIC) capacity are also severely compromised. Moreover, all hyperplastic nodules also showed a reduced level of GJ-protein mRNA. Taken together with our earlier finding that the liver tumor promoter phenobarbital inhibits GJ-protein gene expression, these results suggest that deranged GJIC is a relatively early event in liver multistage carcinogenesis. A range of other cDNA probes was also used to characterize gene expression in the DEN-induced tumors. Induction of expression was seen for glutathione S-transferase (placental form) (GST-P), γ-glutamyltranspeptidase (GGT), and c-raf but not for c-Ha-ras or c-myc.

Key words: diethylnitrosamine, GJ-protein mRNA, cDNA probes, carcinomas, carcinogens

From the time of discovery of the gap junction (GJ) as a channel providing direct cell-cell exchange of ions and low MW molecules, it has become clear that this intercellular communication (GJIC) system is intimately involved in the basic processes of tissue differentiation and homeostasis [1,2]. This has been due in part to the observations of decreased GJ number or function in various cancers [2–4]. More recently, reports that certain tumor promoters inhibit GJIC or reduce GJ number have led to the hypothesis that tumor *development* may occur if there is disturbance of GJIC

Abbreviations used: AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine; EHEN, N-ethyl-N-hydroxyethylnitrosamine; GGT, γ-glutamyltranspeptidase; GJ, gap junction; GJIC, gap-junctional intercellular communication; GST-P, glutathione S-transferase (placental form).

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between a carcinogen-altered cell and surrounding normal cells [5–7]. For example, the rat liver tumor promoter phenobarbital inhibits GJIC in rodent liver cells in vitro [8,9] and reduces the number of GJs in rat liver after systemic treatment [10]. In a recent in vivo study, we determined that systemic administration of phenobarbital to rats results in specific reduction of GJ-protein mRNA amount in the liver [11]. Thus, at the molecular level, this may indicate one point of control of GJIC that is susceptible to tumor promoter action. We wished to extend these studies and to examine whether reduced GJ-protein gene expression is also a permanent feature of carcinogen-induced rat liver tumors. Such a feature may indicate heritable impairment of GJIC, thus providing an escape of tumor cells from GJIC-mediated homeostatic control exerted by normal tissue.

MATERIALS AND METHODS

Adult male Fischer-344 rats were used in these studies. For obtaining liver neoplasms, two different carcinogenesis protocols were employed: 1) one dose of 200 mg/kg diethylnitrosamine (DEN) was administered i.p. followed 2 wk later by five daily gavages of 10 mg/kg 2-acetylaminofluorene (AAF) in corn oil, one gavage of 2 ml CCl_4/kg (50% mixture in corn oil), then four daily gavages of 5 mg/kg AAF; resulting tumors were removed after 9 months; and 2) 0.1% N-ethyl-N-hydroxy-ethylnitrosamine (EHEN) was provided in the drinking water for various times (4, 12, 20 wk) and then removed (for 16, 8, 0 wk, respectively) before collection of liver tumors. EHEN-induced tumors were stored as frozen blocks which were used for histology and RNA extraction. All other samples were divided for formalin fixation and liquid nitrogen freezing with −70°C storage. Total RNA was extracted from tissues, and northern blot analysis was conducted by using [α-^{32}P]labeled cDNA probes [11]. The origins of the probes were as follows: GJ-protein cDNA, Dr. D. Paul, Harvard Medical School, Boston [12]; GST-P cDNA, Dr. M. Muramatsu, University of Tokyo Faculty of Medicine [13]; GGT cDNA, Dr G. Guellaen, INSERM Unit 99, Créteil, France [14]; c-raf cDNA, c-Ha-ras cDNA, and c-myc cDNA were purchased from Oncoprobe, Gaithersburg, Maryland.

RESULTS AND DISCUSSION

Figure 1 is a composite analysis of expression of various genes in DEN-induced rat liver tumors (lanes 5–16) and in untreated liver (lane 1), DEN-initiated liver (lane 2), and in cells surrounding tumors (lanes 3, 4). GJ-protein gene expression was markedly reduced in all tumors and in fact was barely detectable in some of the large hyperplastic nodules and in the hepatocellular carcinoma. That this reduced expression was characteristic of nodules indicates an effect occurring in early, precarcinomatous stages of carcinogenesis. This effect was not due to any prolonged action of DEN itself on gene expression since no expression decrease was observed in DEN-initiated liver (lane 2). Also, the reduced GJ-protein gene expression appeared to be confined to the tumor tissue since normal expression levels were seen in liver tissue adjacent to tumors (lanes 3, 4).

In another recent study, reduced GJ-protein gene expression in rat liver tumors was also observed [15] although this result may have been complicated by the use of continuous systemic phenobarbital to promote tumor formation, since phenobarbital itself decreases GJ-protein gene expression [11]. However, recent immunocytochemistry studies showing reduced GJ-protein levels in preneoplastic liver foci and in tumors

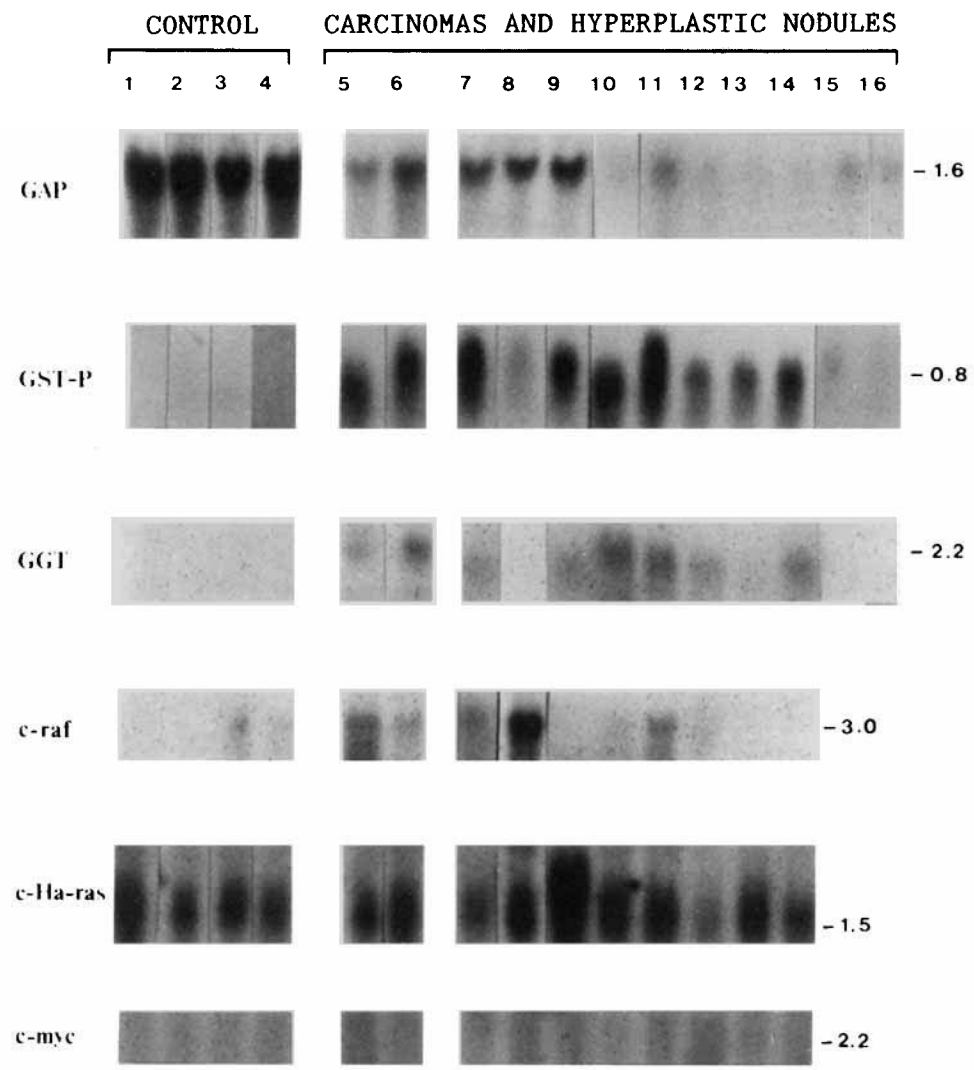

Fig. 1. Northern blot analysis of transcripts of genes encoding GJ-protein (GAP), GST-P, GGT, c-raf, c-Ha-ras, and c-myc in rat liver controls and in DEN-induced rat liver tumors. Total RNA was extracted by the guanidium/cesium chloride centrifugation method [27] and northern blot analysis was conducted on 10 μg RNA samples by using [α-^{32}P]labeled cDNA probes [11]. Numbers at the right refer to the size (Kb) of each transcript. **Lane 1:** Liver of untreated rat. **Lane 2:** Normal-looking liver of DEN-treated rat at 3 months after ip-DEN. **Lanes 3** and **4:** Normal-looking tissue surrounding large hyperplastic nodules (lanes 8, 15). **Lane 5:** From a pool of six hyperplastic nodules of diam. 3–6 mm. **Lane 6:** From a pool of three hyperplastic nodules of diam. 8–11 mm. **Lanes 7** and **8:** Hyperplastic nodules of diam. 1.5–2.0 cm. **Lane 9:** Adenocarcinoma. **Lane 10:** Hepatocellular carcinoma. **Lane 11:** Hyperplastic nodule – 2 cm diam. **Lane 12:** Hyperplastic nodule with some features of carcinoma. **Lanes 13–16:** Hyperplastic nodules of diam. 1.5–2.0 cm.

[15,16] suggest that GJIC within these neoplasms may indeed be impaired. Such impairment therefore may result from disturbances at the protein level and at the gene expression level (this study). Figure 2 illustrates that decreased GJ-protein gene expression is observed also in rat liver tumors generated by a different protocol, i.e., systemic exposure to the carcinogenic nitrosamine EHEN.

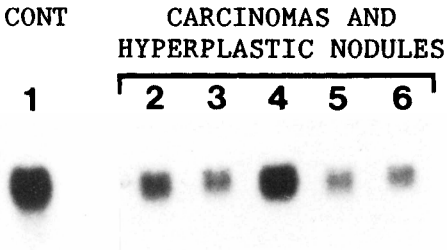

Fig. 2. Northern blot analysis of transcripts of the GJ-protein gene in normal liver and liver tumors obtained after exposure to EHEN. Total RNA was isolated by using a single-step thiocyanate-phenol-chloroform extraction method [28] and northern blot analysis was conducted on 10 μg RNA samples by using the [α-^{32}P]labeled GAP-cDNA probe [11]. **Lane 1:** Liver of untreated rat. **Lane 2:** Hyperplastic nodule remaining after 4 wk EHEN exposure followed by 16 wk without EHEN. **Lanes 3** and **4:** Hepatocellular carcinomas remaining after 12 wk EHEN exposure followed by 8 wk without EHEN. **Lanes 5** and **6:** Hepatocellular carcinomas remaining after 20 wk EHEN exposure.

We examined also in this study the expression of other genes in the DEN-induced tumors (Fig. 1). In most tumors, but not in control samples, expression of GST-P and GGT was seen. The induction of expression of these genes and the synthesis of their proteins are strongly associated with carcinogen-induced neoplasia in liver [17]. Of the various known proto-oncogenes, we studied three in this report which were previously reported to be expressed during liver carcinogenesis, viz., c-raf, c-Ha-ras, and c-myc. c-raf expression occurred at varying degrees in half of the tumors and some expression was noted in one sample of neighbouring tissue. Previously, enhanced c-raf expression has been reported in phenobarbital-treated rat liver [15], in DEN + phenobarbital-induced rat liver tumors [15], in ENU-induced mouse lung tumors, and in 60% of human lung carcinoma cell lines [18]. For c-Ha-ras expression, there was no consistent change amongst the tumors with the adenocarcinoma being the only tumor to express the gene above control levels. However, reports exist of elevated-c-Ha-ras expression in Morris hepatoma [19] and carcinogen-induced hepatocellular carcinoma [20–22], though interestingly, no enhanced expression was found in GGT-positive cells isolated from preneoplastic foci [23]. Finally, for c-myc, we observed no expression in all of the samples. This is in contrast to the numerous reports of elevated c-myc expression in Morris hepatomas [19] and rat liver tumors generated by various carcinogens [20,22,24]. The reason for our result is not yet clear.

The principal finding of the present report is that GJ-protein gene expression is markedly reduced in rat liver tumors, and this phenomenon is common for protocols employing two nitrosamine carcinogens, viz., DEN and EHEN. This finding, together with our previous report of phenobarbital's specific suppression of GJ-protein gene expression in rat liver, indicates that reduced levels of GJ-protein mRNA are a feature associated with both tumor promotion and the tumorigenic phenotype in this system. Such reduction may result in lowered levels of GJ-protein and subsequent GJIC capacity. It is interesting then to speculate on the significance of the heterogeneity of GJ-protein gene expression in the rat liver tumors, i.e., complete or near-complete absence of expression was not observed in all tumors (Fig. 1). This is possibly due to contamination of normal cells. Alternatively, it may be that the tumors expressing the gene, albeit at lower than normal levels, are maintaining some GJIC with surrounding

normal cells and are thus prone to be influenced to undergo remodelling and subsequent regression. Indeed, not all hyperplastic nodules progress to carcinoma.

Recently, we have extended our studies to include human hepatocellular carcinomas. To date, two tumors have been examined and in these no change in GJ-protein gene expression was seen (unpublished data). However, other preliminary experiments using an SV-40 large T-transformed human hepatocyte cell line have shown that while these cells displayed levels of GJ-protein mRNA similar to the normal parent cell strain, their GJIC capacity was extremely poor in comparison to the parent cells (S. Swierenga, unpublished observations). Clearly then, it is difficult to interpret the human carcinoma data and further work is required, particularly at the GJ-protein level. However, even if no alteration was to be found at this level, caution should be engendered since 1) this would not indicate the functional state of the gap junctions, and 2) even a full complement of functioning gap junctions (i.e., normal homologous GJIC) within the tumor does not inform us of the existence of selective heterologous communication, i.e., lack of GJIC between tumor cells and normal cells. Such selective communication, which we have observed in vitro [25,26], would, as with reduced homologous GJIC within a tumor cell compartment, provide a means for transformed cells to evade suppressive influences of surrounding normal cells.

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REFERENCES

1. Pitts JD: J Cell Sci [Suppl] 4:239–266, 1986.
2. Loewenstein WR: Biochim Biophys Acta 605:33–91, 1979.
3. Weinstein RS, Merk FB, Alroy J: Adv Cancer Res 23:23–89, 1976.
4. Tachikawa T, Yamamura T, Yoshiki S: Virchows Arch [B] 47:1–15, 1984.
5. Murray AW, Fitzgerald DJ: Biochem Biophys Res Commun 91:395–401, 1979.
6. Yotti LP, Chang CC, Trosko JE: Science 206:1089–1091, 1979.
7. Yamasaki H: In Slaga TJ, Butterworth B (eds): "Nongenotoxic Mechanisms in Carcinogenesis." Cold Spring Harbor, NY: Cold Springs Harbor Laboratory, 1987, pp 297–309.
8. Williams GM: Ann NY Acad Sci 349:273–282, 1980.
9. Ruch RJ, Klaunig JE: Cancer Res 48:2519–2523, 1988.
10. Sugie S, Mori H, Takahashi M: Carcinogenesis 8:45–51, 1987.
11. Mesnil M, Fitzgerald DJ, Yamasaki H: Mol Carcinog 1:79–81, 1988.
12. Paul DL: J Cell Biol 103:123–134, 1986.
13. Suguoka Y, Kano T, Okuda A, Sakai M, Kitagawa T, Muramatsu M: Nucleic Acids Res 13: 6049–6057, 1985.
14. Laperche Y, Bulle F, Aissani T, Chobert ML, Aggerbeck M, Hanoune J, Guellaen G: Proc Natl Acad Sci USA 83:937–941, 1986.

15. Beer DG, Neveu MJ, Paul DL, Rapp UR, Pitot HC: Cancer Res 48:1610–1617, 1988.
16. Janssen-Timmen U, Traub O, Dermietzel R, Rabes HM, Willecke K: Carcinogenesis 7:1475–1482, 1986.
17. Pitot HC, Sirica AE: Biochim Biophys Acta 605:191–212, 1980.
18. Rapp UR, Cleveland JL, Bonner TI, Storm SM: In Reddy EP, Skalka AM, Curran T (eds): "The Oncogene Handbook." Amsterdam: Elsevier, 1988, p 237.
19. Cote GJ, Chiu JF: Biochem Biophys Res Commun 143:624–629, 1987.
20. Makino R, Hayashi K, Sato S, Sugimura T: Biochem Biophys Res Commun 119:1096–1102, 1984.
21. Corcos D, Defer N, Raymondjean M, Paris B, Corral M, Tichonicky L, Kruh J: Biochem Biophys Res Commun 122:259–264, 1984.
22. Tashiro F, Morimura S, Hayashi K, Makino R, Kawamura H, Horikoshi N, Nemoto K, Ohtsubo K, Sugimura T, Ueno Y: Biochem Biophys Res Commun 138:858–864, 1986.
23. Beer D, Schwarz M, Sawada N, Pitot HC: Cancer Res 46:2435–2441, 1986.
24. Yaswen P, Goyette M, Shank PR, Fausto N: Mol Cell Biol 5:780–786, 1985.
25. Yamasaki H, Fitzgerald DJ: In Langenbach R, Barrett JC, Elmore E (eds): "Tumor Promoters—Biological Approaches for Mechanistic Studies and Assay Systems." NY: Raven Press, 1988, pp 131–147.
26. Mesnil M, Yamasaki H: Carcinogenesis 9:1499–1502, 1988.
27. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ: Biochemistry 18:5294–5299, 1979.
28. Chomezynski P, Sacchi N: Anal Biochem 162:156–159, 1987.