Carcinogenesis vol.14 no.10 pp.2127–2129, 1993

Intercellular communication in primary cultures of putative preneoplastic and 'normal' hepatocytes

E.Leibold and L.R.Schwarz[1]

GSF—Institut für Toxikologie, Ingolstädter Landstrasse 1, D-85758 Oberschleißheim, Germany

[1]To whom correspondence should be addressed

Gap junctional intercellular communication (IC) was studied in γ-glutamyltranspeptidase (γ-GT)-positive and -negative hepatocytes isolated from carcinogen-treated rats. Putative preneoplastic γ-GT-positive hepatocytes were visualized in monolayer cultures by indirect immunofluorescence using anti-γ-GT-antibodies. IC was evaluated by studying dye coupling of the cells. γ-GT-positive hepatocytes showed a significantly lower dye coupling than did γ-GT-negative liver cells. Spread of the dye Lucifer Yellow CH to neighboring cells was decreased further by the tumor-promoting chemical phenobarbital in both cell types *in vitro*. Also treatment *in vivo* with the barbiturate significantly reduced dye coupling of hepatocytes. The findings suggest that as a result of their decreased ability to communicate, preneoplastic hepatocytes may escape from growth control and differentiation signals given out by surrounding 'normal' cells.

Introduction

Signalling through gap junctions is considered to play a pivotal role in the physiological control of growth, tissue homeostasis and differentiation (1). Accordingly, several authors have proposed that dysfunction of gap junctional intercellular communication (IC*) is involved in carcinogenesis (cf. 2–4). This hypothesis is supported by several lines of evidence: (i) many tumor cells express a heritable phenotype of reduced IC (3,5); (ii) several oncogene products, such as those from *src* and *ras*, have been shown to be associated with a decrease in IC (6,7); (iii) many tumor-promoting chemicals inhibit gap junctional IC (cf. 4).

It has been shown recently that both primary hepatic tumors (8–11) and preneoplastic liver foci (9,11,12), i.e. pre-stages of hepatic tumor development, exhibit a decreased expression of gap junctional protein or mRNA. In their studies, Beer *et al.* (9) and Neveu *et al.* (12) demonstrated a decreased immunoreactivity for the major hepatic gap junction connexin (Cx 32) of the rat in some preneoplastic lesions. Furthermore, Neveu *et al.* (12) showed that treatment of the animals with the hepatic tumor promotor phenobarbital (PB) results in a marked increase in the number of putative preneoplastic liver cell foci deficient in Cx 32. Krutovskikh *et al.* (11) reported a lower IC and a significantly decreased number of immunoreactive Cx 32 spots in altered hepatic foci expressing the placental isoenzyme of glutathione S-transferase (GST-P).

The present investigation was aimed at studying IC between γ-GT-positive and -negative hepatocytes using dye coupling, and the effect of the hepatic tumor promoter PB under the defined conditions of cell culture. Viable putative preneoplastic γ-GT-positive hepatocytes were detected by indirect immunofluorescence using anti-γ-GT antibodies.

*Abbreviations: IC, intercellular communication; Cx 32, connexin 32; PB, phenobarbital; GST-P, placental glutathione-S-transferase; 2-AAF, 2-acetylaminofluorene; γ-GT, γ-glutamyltranspeptidase.

Materials and methods

Male Wistar rats from the GSF breeding colonies (100 g body wt) were given diethylnitrosamine (100 mg/kg body wt, i.p. in 0.9% NaCl) followed 2 weeks later by 0.02% 2-acetylaminofluorene (2-AAF) for 2 weeks administered in the basal diet. In the middle of the 2-AAF treatment the rats were anesthetized with ether and subjected to a two-thirds hepatectomy. After the 2-AAF treatment the animals received 0.05% PB in the drinking water for 1 month. During this period the rats were fed the semi-synthetic diet 1534 (Altromin, Lage, FRG), which efficiently prevents the appearance of the 'non-specific' γ-glutamyltranspeptidase (γ-GT)-positive hepatocytes around the portal venules (13) which are not considered to represent preneoplastic hepatocytes (14). Similarly, Glauert *et al.* (1986) reported that feeding a purified diet prevents the periportal increase in γ-GT activity (15). Histochemical demonstration of γ-GT in cryostat sections of livers after the carcinogen treatment showed multiple γ-GT-positive foci. On average there were 30.5 ± 4.2 γ-GT-positive foci per cm^2, occupying 22 ± 4.6% of the total section area. γ-GT enzyme activities in cell suspensions isolated from untreated and carcinogen-treated rats amounted to 0.11 ± 0.02 and 0.6 ± 0.13 U/mg protein (mean ± SEM, $n = 4$) respectively.

Hepatocytes were isolated by collagenase (Boehringer, Mannheim, Germany) perfusion as described recently (16). Viability of the cells was determined routinely by staining with trypan blue. Hepatocyte preparations were used only when >80% of the cells excluded the dye.

For the investigation of IC, 1×10^6 viable hepatocytes were plated on 60 mm plastic culture dishes coated with rat tail collagen. The cells were suspended in 2 ml Leibovitz L-15 medium supplemented with 21.4 mM $NaHCO_3$, 20 mM HEPES, 0.1 mM dexamethasone, 10 mU/ml insulin, 100 U/ml penicillin and 100 mg/ml streptomycin. After 1.5 h of attachment, cultures were washed once with HEPES-buffered PBS and re-fed with 2 ml fresh medium. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

γ-GT-positive hepatocytes were demonstrated using biotinylated polyclonal anti-γ-GT antibodies (17). Three hours after plating, hepatocytes were incubated with culture medium containing the antibody at a dilution of 1/400. After 1 h, the cultures were washed twice with HEPES-buffered PBS and 2 ml of fresh medium containing 5% of phycoerythrin-conjugated streptavidin solution (Becton Dickinson, Heidelberg, Germany) were added to the cultures. Incubation with the streptavidin conjugate was performed for 20 min, after which hepatocytes were washed again and re-fed with culture medium (Figure 1). The process of immunostaining did not affect dye coupling between cultured hepatocytes and no staining of cells could be observed in hepatocyte cultures from untreated animals.

IC between hepatocytes was detected by microinjection of Lucifer Yellow CH (Fluka Feinchemikalien, Neu-Ulm, Germany). Injection needles with a guaranteed diameter of 0.3–0.5 mm were filled with 5% (w/v) Lucifer Yellow CH in 0.1 M LiCl (Femtotips™, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Hepatocyte cultures were observed after 5–8 h under a Zeiss Axiovert 10 epifluorescence microscope (Zeiss, Oberkochen, Germany) and 20–30 cells/dish were injected with the fluorescent dye solution using an automatic pneumatic micromanipulator and injector system (Eppendorf, Micromanipulator Model 5170, Microinjector Model 5242). Dye solution was injected into cells at a pressure of 30–50 hPa for 0.7 s. Five minutes after dye injection, hepatocytes in direct contact with 'dye-donor' hepatocytes were evaluated for evidence of dye coupling. The percentage of dye-coupled neighboring cells was determined for each of the experimental conditions.

Results

Hepatocytes were isolated from carcinogen-treated animals and cultured at subconfluent density on collagen-coated dishes. After 5 h, hepatocytes form clusters of cuboid cells whose plasma membranes are in close contact.

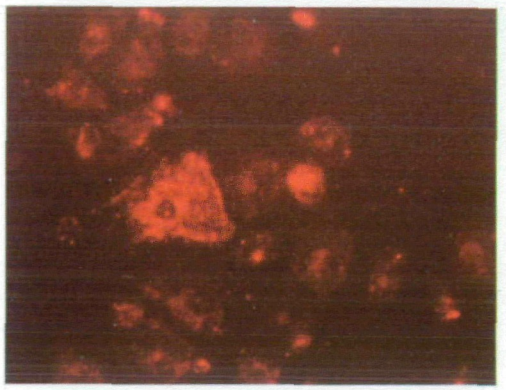

Fig. 1. Immunostaining of cultured primary hepatocytes isolated from a carcinogen-treated rat with antibodies directed against γ-GT. γ-GT-positive cells show a bright phycoerythrin fluorescence. Magnification ×1000.

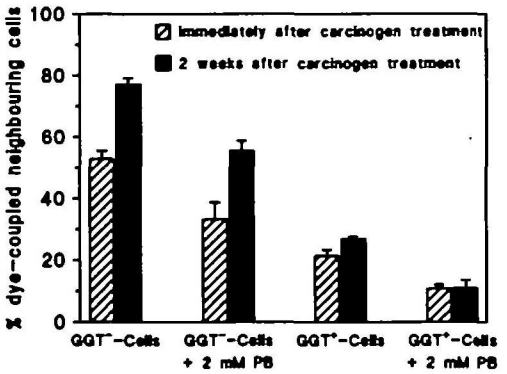

Fig. 2. Intercellular communication in cultured γ-GT-positive and γ-GT-negative hepatocytes. Cells were cultivated for 5 h in the absence or presence of 2 mM PB. Results are the mean ± SEM of four different experiments, in each experiment 20–30 cells were injected with Lucifer Yellow CH. All differences shown in the figure (γ-GT-negative versus γ-GT-positive cells; cells isolated immediately after the carcinogen treatment versus cells isolated 2 weeks after the carcinogen treatment; absence of PB versus presence of PB during cell culture) are significant with $P < 0.009$.

IC of γ-GT-positive and -negative hepatocytes was evaluated by microinjection of Lucifer Yellow CH and observing inhibition of spread of dye into adjacent cells.

Dye coupling by γ-GT-negative dye donors from carcinogen-treated animals was ~50% (Figure 2, first hatched column). In contrast dye coupling between hepatocytes obtained from untreated animals was ~86.8 ± 1.1 % (mean ± SE of 19 independent experiments). This relatively low capacity of 'normal' cells from treated animals to communicate was mainly due to the treatment of the animals with PB. When cells were isolated after discontinuing the PB treatment for 2 weeks, the percentage of dye-coupled neighboring cells increased significantly (Figure 2; first filled column). Accordingly, hepatocytes isolated from rats that were treated with PB only (100 mg PB/kg body wt, i.p., and subsequently from day 2 0.1% PB in the drinking water for 5 days or 9 weeks) showed a reduced dye coupling of 69.3 ± 1.7 or 59.3 ± 1.8 (mean ± SE of five independent experiments) respectively. Dye coupling of γ-GT-positive dye donors was significantly lower than that of γ-GT-negative cells from carcinogen-treated rats and amounted to only 22% (Figure 2; third hatched column) when cells were isolated immediately after the PB treatment. Discontinuing the PB treatment of the rats prior to the isolation of hepatocytes had only a minor effect on dye coupling of γ-GT-positive donor cells (Figure 2, third filled column).

Addition of PB to cell cultures for ~5 h further lowered the extent of IC in hepatocytes isolated from carcinogen-treated rats. Dye coupling was decreased by ~20% and 10–15% in γ-GT-positive and -negative hepatocytes respectively (Figure 2; second and fourth hatched and filled column). Interestingly, PB lowered IC irrespective of whether the cells were isolated immediately, or 2 weeks after, PB promotion.

Discussion

During the past years several attempts have been made to study the biochemical characteristics of γ-GT-positive putative preneoplastic hepatocytes under defined *in vitro* conditions. In several studies, γ-GT-positive hepatocytes were isolated by binding to anti-γ-GT-antibodies and subsequently analyzed for various biochemical functions (17–20). In other studies, mitogenic stimulation of γ-GT-positive hepatocytes was assessed in mixed cultures of γ-GT-positive and -negative liver cells by combining enzyme histochemistry and autoradiography (13,21). Since the enzyme histochemical demonstration of γ-GT is performed on fixed/dead cells this procedure cannot be used for live cells. We therefore developed another methodology to visualize viable γ-GT-positive hepatocytes. Using biotinylated anti-γ-GT antibodies and phycoerythrin-conjugated streptavidin, it was possible to stain viable γ-GT-positive liver cells by indirect immunofluorescence.

γ-GT-positive hepatocytes showed a marked decrease in IC compared to γ-GT-negative hepatocytes from the same animals. This result is in good agreement with findings of others showing a reduced expression of gap junction protein in putative preneoplastic foci in liver slices of rats (9,12). More recently Krutovskikh *et al.* (11) demonstrated that not only was the expression of Cx 32 significantly decreased in liver cell foci expressing the placental isoenzyme of glutathione S-transferase, but that dye coupling was also severely compromised in these putative preneoplastic cells. Our results with γ-GT-positive cells indicate that this might be a general property of cells in preneoplastic foci. As a result of their low capacity to communicate, preneoplastic liver cells may escape from the growth control and differentiation signals sent out by surrounding normal cells (3,4).

The hepatic tumor promoter PB caused a marked decrease in IC in both γ-GT-positive and γ-GT-negative hepatocytes. There are two different mechanisms by which the barbiturate PB might affect IC. On the one hand, treatment of rats with PB has been shown to decrease mRNA levels of gap junction proteins (22), on the other hand it may affect the physiological control of gap junctional permeability (23). The reduced dye transfer in hepatocytes isolated immediately after subchronic treatment of rats with PB is likely to be due to decreased expression of gap junction protein (12,22). It is unlikely, however, that interference with gene expression is the mechanism underlying the decrease of IC following the addition of PB to hepatocyte cultures. This 'acute' effect of the barbiturate might be attributable to changes in the permeability of gap junctions. It has been shown that reduced dye coupling occurs within 2–4 h after exposure of cultured hepatocytes to PB, and that the effect of PB is rapidly reversible following removal of the barbiturate (24; E.Leibold and L.R.Schwarz, unpublished results). Accordingly, hepatocytes isolated immediately after the PB treatment may recover from the 'acute' effects of PB during culturing of the cells. This may

explain why exposure of cultured liver cells to PB *in vitro* affects not only dye coupling between hepatocytes that were isolated 2 weeks after treating the animals with the barbiturate but also immediately after tumor promotion with PB.

In summary, the present study clearly shows that IC is severely compromised in γ-GT-positive hepatocytes; the decreased IC may favor the maintenance of the preneoplastic phenotype and the progression of the cells towards more malignant phenotypes. Identification of viable γ-GT-positive hepatocytes by staining will allow further study of the function of preneoplastic hepatocytes under defined cell culture conditions.

References

1. Loewenstein,W. (1988) The cell-to-cell channel gap junction. *Cell*, **48**, 725–726.
2. Trosko,J.E., Chang,C.C. and Madhukav,B.V. (1990) Modulation of intercellular communication during radiation and chemical carcinogenesis. *Radiat. Res.*, **121**, 241–251.
3. Yamasaki,H. (1990) Gap junctional intercellular communication and carcinogenesis. *Carcinogenesis*, **11**, 1051–1058.
4. Klaunig,J.E. and Ruch,R.J. (1990) Biology of disease. Role of inhibition of intercellular communication in carcinogenesis. *Lab. Invest.*, 62, 135–146.
5. Nicolson,G., Dulski,K.M. and Trosko,J.E. (1988) Loss of intercellular junctional communication correlation with the metastatic potential in mammary adenocarcinoma cells. *Proc. Natl. Acad. Sci. USA*, **85**, 473–476.
6. Azarnia,R. and Loewenstein,W.R. (1984) Intercellular communication and the control of growth: alteration of junctional permeability of the src gene. A study with temperature-sensitive mutant Rous Sarcoma virus. *J. Membr. Biol.*, **82**, 191–205.
7. de Feijter,A.W., Trosko,J.E., Krizman,D.B., Lebovitz,R.M. and Lieberman,M.W. (1992) Correlation of increased levels of Ha-*ras* T24 protein with extent of loss of gap junction function in rat liver epithelial cell. *Mol. Carcinogenesis*, **5**, 205–212.
8. Janssen-Timmen,U., Traub,O., Dermietzel,R., Rabes,H.M. and Willecke,K. (1986) Reduced number of gap junctions in rat hepatocarcinomas detected by monoclonal antibody. *Carcinogenesis*, **7**, 1475–1482.
9. Beer,D.G., Neveu,M.J., Paul,D.L., Rapp,U.R. and Pitot,H.C. (1988) Expression of the c-*raf* protooncogene, γ-glutamyltranspeptidase, and gap junction protein in rat liver neoplasms. *Cancer Res.*, **48**, 1610–1617.
10. Fitzgerald,D.J., Mesnil,M., Oyamada,M., Tsuda,H., Ito,N. and Yamasaki,H. (1989) Changes in gap junction protein (Connexin 32) gene expression during rat liver carcinogenesis. *J. Cell. Biochem.*, **41**, 97–102.
11. Krutovskikh,V.A., Oyamada,M. and Yamasaki,H. (1991) Sequential changes of gap-junctional intercellular communications during multistage rat liver carcinogenesis: direct measurement of communication *in vivo*. *Carcinogenesis*, **12**, 1701–1706.
12. Neveu,M.J., Hully,J.R., Paul,D.L. and Pitot,H.C. (1990) Reversible alteration in the expression of the gap junctional protein connexin 32 during tumor promotion in rat liver and its role during cell proliferation. *Cancer Commun.*, **2**, 21–31.
13. Neumann,I., Thierau,D., Andrae,U., Greim,H. and Schwarz,L.R. (1992) Cyproterone acetate induces DNA damage in cultured rat hepatocytes and preferentially stimulates DNA-synthesis in γ-glutamyltranspeptidase-positive cells. *Carcinogenesis*, **13**, 373–378.
14. Fischer,S., Lilienblum,W., Ullrich,D. and Bock,K.W. (1986) Immunohistochemical differentiation of γ-glutamyltranspeptidase in focal lesions and in zone I of rat liver after treatment with chemical carcinogens. *Carcinogenesis*, **7**, 1405–1450.
15. Glauert,H.P., Schwarz,M. and Pitot,H.C. (1986) The phenotypic stability of altered hepatic foci: effect of the short-term withdrawal of phenobarbital and of the long-term feeding of purified diets after the withdrawal of phenobarbital. *Carcinogenesis*, **7**, 117–121.
16. Watkins,J.B.,III, Thierau,D. and Schwarz,L.R. (1992) Biotransformation in carcinogen-induced diploid and polyploid hepatocytes separated by centrifugal elutriation. *Cancer Res.*, **52**, 1149–1154.
17. Lebsanft,J., Luippold,G. and Schwarz,L.R. (1985) Activities of drug metabolizing enzymes in isolated γ-glutamyltranspeptidase-positive preneoplastic hepatocytes from carcinogen treated rat. *Cancer Lett.*, **29**, 29–36.
18. Hanigan,M.H. and Pitot,H.C. (1982) Isolation of γ-glutamyltranspeptidase positive hepatocytes during the early stages of hepatocarcinogenesis in the rat. *Carcinogenesis*, **3**, 1349–1354.
19. Lebsanft,J., Luippold,G. and Schwarz,L.R. (1986) Uptake of taurocholate by isolated γ-glutamyltranspeptidase-positive, putatively preneoplastic hepatocytes from 2-acetylaminofluorene treated rat. *Carcinogenesis*, **7**, 1925–1926.
20. Sargent,L., Xu,Y., Sattler,G.L., Meisner,L. and Pitot,H.C. (1989) Ploidy and karyotype of hepatocytes isolated from enzyme-altered foci in two different protocols of multistage hepatocarcinogenesis in the rat. *Carcinogenesis*, **10**, 387–391.
21. Xu,Y.-H., Sattler,G.L. and Pitot,H.C. (1988) A method for the comparative study of replicative DNA synthesis in GGT-positive and GGT-negative hepatocytes in primary cultures isolated from carcinogen-treated rats. *In Vitro Cell. Dev. Biol.*, **24**, 995–1000.
22. Mesnil,M., Fitzgerald,D.J. and Yamasaki,H. (1988) Phenobarbital specifically reduces gap junction protein mRNA level in rat liver. *Mol. Carcinogenesis*, **1**, 79–81.
23. Ruch,R.J. and Klaunig,J.E. (1988) Kinetics of phenobarbital inhibition of intercellular communication in mouse hepatocytes. *Cancer Res.*, **48**, 2519–2523.
24. Klaunig,J.E., Ruch,R.J. and Weghorst,C.M. (1990) Comparative effects of phenobarbital, DDT, and lindane on mouse hepatocyte gap junction intercellular communication. *Toxicol. Appl. Pharmacol.*, **102**, 553–563.

Received on March 18, 1993; revised on June 24, 1993; accepted on July 1, 1993