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SHORT COMMUNICATION

Phenobarbital selectively promotes initiated cells with reduced TGFβ receptor levels

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Phenobarbital (PB) is a potent tumor promoter in rodent liver. In this study we investigated whether PB selectively promotes a population of initiated cells with reduced levels of transforming growth factor-β (TGFβ) receptors types I, II and III. Liver tumors were induced in male Fischer F344 rats by diethylnitrosamine (DEN). Following induction the animals were divided into PB-treated (DEN/PB) and untreated groups (DEN). After 3 months of treatment half of the PB-treated rats were removed from PB for the final month (DEN/PB/OFF). At 4 months, the livers from rats in the three treatment groups were removed, tumors excised and frozen with matched surrounding normal tissue. The mRNA levels for the TGFβ receptors types I–III were significantly decreased in tumor tissue from DEN/PB rats when compared with surrounding normal liver tissue or tumors from age-matched untreated controls. In tumors from DEN/PB/OFF rats the TGFβ receptor types I–III were also significantly reduced compared with controls and not different to tumors from DEN/PB rats. There was no difference in the mRNA levels for the TGFβ receptors in tumors from rats exposed to DEN alone, when compared with the surrounding normal tissue. These results demonstrate that PB selectively promotes initiated cells with reduced levels of TGFβ types I–III receptors and suggests a mechanistic role for TGFβ in PB-induced liver tumor promotion.

Approximately 60% of the chemicals determined by the National Toxicology Program to be carcinogenic in rats and mice give rise to liver tumors. Some of these carcinogens, however, are either only weakly genotoxic or have been found to cause no detectable genetic damage. Rather, they appear to function as tumor promoting agents. These agents include chemicals to which humans are exposed, e.g. contraceptive steroids (1,2), tamoxifen (3), benzodiazepine compounds (4), dioxin (5), and phenobarbital (PB*) (6). Although there is no definitive mechanism for the carcinogenic activity of these diverse agents, one hypothesis involves reduced reponsiveness to negative growth signals, especially the potent mitoinhibitor transforming growth factor-β (TGFβ) (7).

In mammals TGFβ exists as three highly homologous isoforms, TGFβ1, TGFβ2 and TGFβ3 (8). These structurally and functionally similar isoforms exist as 25 kDa homodimers in their active form. Throughout this paper the TGFβ isoforms are referred to simply as TGFβ. TGFβ is secreted in an inactive latent complex which cannot bind to the TGFβ receptors (8–10). The TGFβ latent complex contains phosphomannosyl residues and consequently can bind to the mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2r) (11). The binding of the TGFβ latent complex to this receptor results in either activation of TGFβ by the proteolytic enzyme plasmin (12,13) or TGFβ degradation in the lysosomes.

Following activation, TGFβ can bind to three distinct receptors, TGFβ I, TGFβ II and TGFβ III (14–16). Types I and II receptors are involved in signaling and have serine/threonine kinase activity. In order to maximally signal, TGFβ must bind to the type II receptor and subsequently form a heteromeric complex with the type I receptor (10). The type III receptor, also called betaglycan, does not play a role in signaling, but binds active TGFβ for presentation to the type I and II receptors (17). Therefore, changes in cellular membrane concentrations of the three TGFβ receptors will alter the ability of cells to respond to TGFβ.

TGFβ has been demonstrated to be a potent mitoinhibitor for most cell types (8,18,19). In fact, picomolar concentrations of TGFβ inhibit hepatocyte growth in culture (20–22), and TGFβ injected into partially hepatectomized rats significantly delays the onset of DNA synthesis (23). TGFβ-induced growth inhibition, however, is not observed in four retinoblastoma cell lines that lack TGFβ receptors (24). It has been suggested that these retinal tumor cells may gain a selective growth advantage by failing to express the TGFβ receptors, allowing them to escape the growth inhibitory action of TGFβ. In addition, growth inhibition by TGFβ was restored in a TGFβ-resistant hepatoma cell line after transfection and expression of the type II TGFβ receptor (25).

During PB-induced liver tumor promotion the intracellular concentration of TGFβ increases significantly in normal, but not initiated, hepatocytes (26). In addition, PB does not alter the expression of TGFβ receptors in normal hepatic tissue. We have previously demonstrated, however, that diethylnitrosamine (DEN)-initiated/PB-promoted tumors have significantly reduced mRNA and membrane protein levels of the TGFβ type I–III receptors when compared with receptor levels in the surrounding normal tissue (27). To further determine the role of the TGFβ receptors in PB-induced tumor promotion we investigated the effect of removing PB from rats who had previously been exposed to it for 3 months.

Male Fischer 344 rats weighing 80–100 g were obtained from Charles River Laboratories (Raleigh, NC). They were fed Purina rodent chow no. 5010 (PMI Feeds, St Louis, MO) and water *ad libitum* and were maintained in a temperature- and humidity-controlled room under a 12 h light/dark cycle. Following 1 week of acclimatization, the rats were treated with DEN (Sigma, St Louis, MO) at 50 p.p.m. in the drinking water for 1 month. The animals were randomly placed in cages (three per cage). One third of the animals were given

*Abbreviations: PB, phenobarbital; TGFβ, transforming growth factor-β; M6P/IGF2r, mannose 6-phosphate/insulin-like growth factor II receptor; DEN, diethylnitrosamine.

regular water, while the remaining animals received 0.1% PB (Mallinckrodt, Paris, KY) in the drinking water for 3 months to produce PB-promoted liver tumors. This treatment was followed by either another month on 0.1% PB in the drinking water or 1 month of PB-free drinking water. The half-life of PB is 11 ± 2 h in rats (28). The exposure regimens resulted in the formation of liver tumors 1–2 cm in diameter in all three treatment groups, but there was a greater number of tumors in the PB-promoted animals in comparison with the non-promoted animals. Untreated rats served as age-matched controls. All animal use was in full compliance with NIH guidelines for humane care and was approved by the Duke University Medical Center Animal Use Committee.

After 4 months of treatment the rats were killed, tumors and normal liver tissue surrounding the tumors were excised and snap frozen in liquid nitrogen for RNA extraction. Total RNA was extracted by the RNAzol method (Tel-Test B, Friendswood, TX), a modification of the single step acid guanidinium thiocyanate/phenol/chloroform method (29). RNase protection assays were performed as previously described (30).

An unpaired *t*-test was used to compare the mRNA levels of the TGFβ receptor types I–III in the surrounding normal liver tissue with receptor levels in the tumors from all three treatment groups. The means of the mRNA levels of the type I–III TGFβ receptors in the surrounding normal tissue in each of the three treatment groups were compared with each other and to receptor levels in the age-matched controls using analysis of variance with a Scheffé *post hoc* test, with $P < 0.05$ taken to indicate a significant difference.

Analysis of our experimental data (Figure 1) demonstrates that the mRNA levels of the TGFβ receptor types I ($P <$

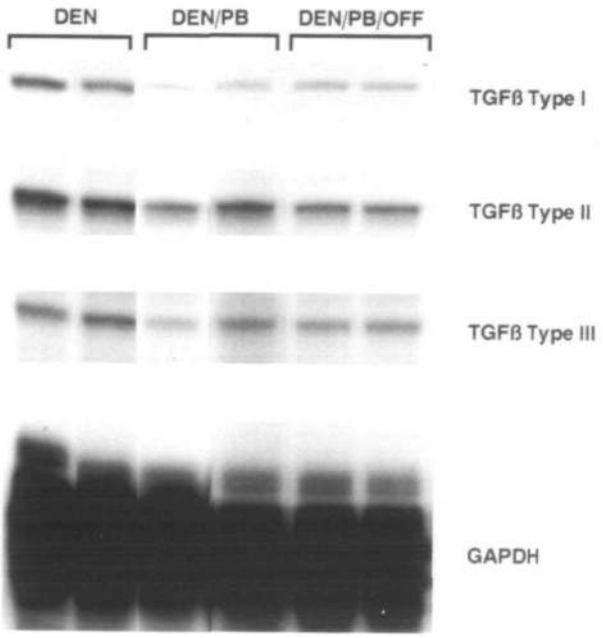

Fig. 1. Representative RNase protection assay data for TGFβ receptor types I–III in liver tumors from rats exposed to DEN alone, DEN/PB or DEN/PB/OFF.

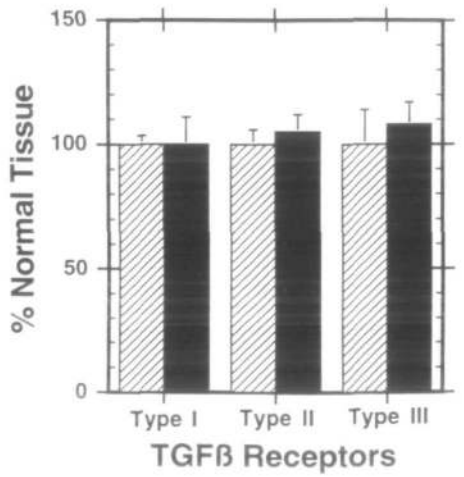

Fig. 2. mRNA levels for TGFβ receptors in tumors (black bars) and the normal liver tissue surrounding the tumors (hatched bars) from DEN animals. Values are relative to those in the livers of untreated age-matched controls. Error bars represent standard error of the means.

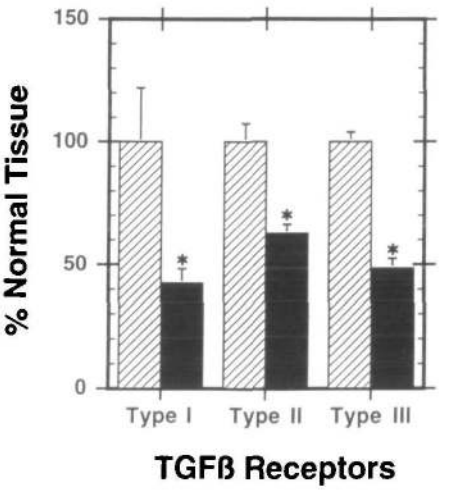

Fig. 3. mRNA levels for TGFβ receptors in tumors (black bars) and the normal liver tissue surrounding the tumors (hatched bars) from DEN/PB animals. Values are relative to those in the livers of untreated age-matched controls. Error bars represent standard error of the means and the asterisks denote values that are significantly different from controls.

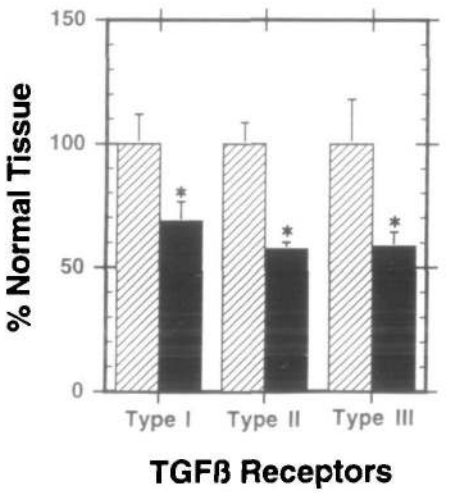

Fig. 4. mRNA levels for TGFβ receptors in tumors (black bars) and the normal liver tissue surrounding the tumors (hatched bars) from DEN/PB/OFF animals. Values are relative to those in the livers of untreated age-matched controls. Error bars represent standard error of the means and the asterisks denote values that are significantly different from controls.

0.01), II ($P < 0.0001$) and III ($P < 0.0001$) were significantly decreased in DEN/PB tumors when compared with the normal liver tissue surrounding the tumors and untreated age-matched control liver tissues, but remain unaltered ($P > 0.1$) in non-promoted tumors (Figures 2 and 3). The mRNA levels of the TGFβ receptors types I ($P < 0.02$), II ($P < 0.0001$) and III ($P < 0.0001$) remained significantly decreased in rat liver tumors following removal of PB from the diet (DEN/PB/OFF; Figure 4). The mRNA levels of the three TGFβ receptors in the normal liver tissue surrounding DEN/PB tumors and DEN/PB/OFF tumors were also not significantly altered when compared with each other or to the receptor levels in the untreated age-matched controls, suggesting that the decrease in TGFβ receptors results from decreased mRNA expression in the tumor tissue, rather than increased mRNA in the surrounding normal tissue.

We have previously shown that PB significantly increases the concentration of TGFβ in normal hepatocytes (31,32), the long-term effect of which is to inhibit hepatocyte proliferation (20,21). We have postulated that long-term PB treatment creates a mitoinhibitory environment in the liver due to elevated levels of TGFβ. An effect of this mitoinhibitory environment is to select for the growth of tumors exhibiting a phenotype characterized by a reduction in responsiveness to TGFβ. It was recently demonstrated that in rats initiated with DEN and promoted by PB tumors have reduced levels of expression of TGFβ receptors both at the mRNA and protein levels (27). These data suggest that PB is either selecting for the growth of a population of initiated cells with reduced expression of TGFβ receptor types I–III or PB itself is down-regulating the receptor levels. The data reported here support our previous observations (27) and demonstrate that with DEN/PB/OFF rats, where removal of PB eliminates the stimulus for the mitoinhibitory environment, the steady-state mRNA levels remained reduced for the TGFβ types I–III receptors. These data provide evidence that PB is selecting for the growth of a population of initiated cells exhibiting an inherent reduction in the expression of TGFβ receptor types I–III.

A model for tumor selection by PB is supported by elegant experiments which demonstrated that long-term exposure of cultured rat liver epithelial cells (WB cells) to increasing concentrations of TGFβ promotes spontaneous neoplastic transformation of a population of liver cells that express increased levels of proto-oncogenes (33). In this experimental model chronic exposure to elevated levels of TGFβ produces a mitoinhibitory environment that selects for the growth of TGFβ-resistant transformed liver cells, analogous to the promotional stage of carcinogenesis.

Hepatocytes have been shown to be less sensitive to the mitoinhibitory effects of TGFβ during remodelling after 2/3 partial hepatectomy. This reduced sensitivity correlates with a 50% decrease in both steady-state mRNA and protein levels of all three TGFβ receptor types (34). As hepatocyte proliferation appears to correlate with a down-regulation of the three TGFβ receptors, the reduction in mRNA levels of the TGFβ receptors in the PB-promoted tumors reported here could be related to cellular proliferation. However, the expression of the three TGFβ receptors is not decreased in DEN-initiated tumors, suggesting that the decrease in TGFβ receptors in PB-promoted tumors is not a consequence of increased proliferation.

The apoptotic index has been shown to be decreased in PB-promoted tumors when compared with DEN-initiated non-promoted tumors (35). As TGFβ induces apoptosis in both normal and malignant hepatocytes (35,36), it is possible that an effect of the decreased TGFβ receptor mRNA levels in the DEN/PB and DEN/PB/OFF tumors is that these tumors become refractory to apoptotic signals due to decreased binding of TGFβ.

In conclusion, this study demonstrates that liver tumor promotion by PB involves selection for initiated cells with decreased levels of TGFβ receptors types I–III. These tumor cells appear resistant to the growth inhibitory environment due to a reduced ability to respond to the negative growth signals mediated by TGFβ. This study supports considering new risk assessment models based on negative selection (37), which may better predict the risk posed to humans by chemicals which act as tumor promoters.

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