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Increasing cell membrane potential and GABAergic activity inhibits malignant hepatocyte growth

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Sun, D., Y. Gong, H. Kojima, G. Wang, E. Ravinsky, M. Zhang, and G. Y. Minuk. Increasing cell membrane potential and GABAergic activity inhibits malignant hepatocyte growth. *Am J Physiol Gastrointest Liver Physiol* 285: G12–G19, 2003; 10.1152/ajpgi.00513.2002.—Increasing hepatocyte membrane potentials by augmenting GABAergic activity inhibits nonmalignant hepatocyte proliferative activity. The objectives of this study were to document *1*) potential differences (PDs) of four malignant hepatocyte cell lines, *2*) $GABA_A$ receptor mRNA expression in the same cell lines, and *3*) effects of restoring malignant hepatocyte PDs to levels approximating those of resting, nonmalignant hepatocytes. Hepatocyte PDs were documented in nonmalignant and malignant (Chang, HepG2, HuH-7, and PLC/PRF/5) hepatocytes with a fluorescent voltage-sensitive dye and $GABA_A$ receptor expression by RT-PCR and Western blot analyses. Compared with nonmalignant human hepatocytes, all four malignant cell lines were significantly depolarized ($P < 0.0001$, respectively). Only PLC/PRF/5 cells had detectable $GABA_A$-β_3 receptor mRNA expression and all cell lines were negative for $GABA_A$-β_3 receptor protein by Western blot analysis. Stable transfection of Chang cells with $GABA_A$-β_3 receptor cDNA resulted in significant increases in PD and decreases in proliferative activity as manifest by decreased [^{3}H]thymidine and bromodeoxyurieine incorporation rates, 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate activity, a lower mitotic index, prolongation of cell-doubling times, and attenuated growth patterns compared with cells transfected with vector alone. Colony formation in soft agar and the number of abnormal mitoses were also significantly decreased in $GABA_A$-β_3 receptor transfected cells. The results of this study indicate *1*) relative to healthy hepatocytes, malignant hepatocytes are significantly depolarized, *2*) $GABA_A$-β_3 receptor expression is absent in malignant hepatocyte cell lines, and *3*) increasing the PD of malignant hepatocytes is associated with less proliferative activity and a loss of malignant features.

cancer; regeneration; membrane potential; proliferation, differentiation, liver disease, hepatocellular carcinoma

HEPATOCYTE PROLIFERATION is a complex process that not only involves the levels of various growth promoters and inhibitors but also where those regulators are located within the cell (32). Although the factor(s) responsible for determining their intracellular distribution remains to be determined, differences in electrical gradients across cell membranes represent one plausible explanation.

Resting hepatocytes have a carefully regulated transmembrane electrical potential difference (PD) of approximately −35 mV (39). In previous studies (42), we documented that after a growth stimulus such as 70% partial hepatectomy, hepatocytes promptly depolarize to PD values of approximately −20 mV. This depolarized state remains in effect until hepatocyte proliferative activity wanes. Whether the PD changes observed represent a cause or effect of hepatocyte proliferation remains unclear. That nuclear translocation of cationic growth promoters does not occur in cells that maintain their hyperpolarized state argues in favor of the former (11). Also supportive are findings that maintenance of hepatocyte PD at resting levels interferes with hepatocyte proliferative activity (25).

Numerous channels, pumps, receptors and the agents influencing the activity of these sites contribute to the PD of hepatocytes (9). Recently, we identified specific GABA receptors on the surface of isolated rat and human hepatocytes that regulate chloride flux (7). Activation of these sites with $GABA_A$ receptor agonists caused prompt hepatocyte hyperpolarization and inhibition of proliferation, whereas exposure to $GABA_A$ receptor antagonists depolarized hepatocytes and enhanced proliferative activity (14, 24, 41). These data suggest that the GABAergic system is an important regulator of hepatocyte PD and proliferative activity.

The purpose of the present study was to test the hypothesis that malignant hepatocytes are permanently depolarized as a result of downregulated or absent GABAergic activity. As a corollary to this hypothesis, we proposed that by restoring hepatocyte PDs toward those of resting hepatocytes (via increasing GABAergic activity) the proliferative and malignant features of these cells would revert to those associated with nonmalignant hepatocytes.

MATERIALS AND METHODS

Materials. DMEM, pyruvate, penicillin, streptomycin, geneticin (G-418), and Fungizone were purchased from GIBCO-BRL (Life Technologies, Burlington, ON, Canada). Cool calf serum and muscimol were purchased from Sigma (St. Louis,

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MO). Chang, Hep G_2, HuH-7 and PLC/PRF/5 cells were obtained from American Type Culture collection (Manassas, VA). Healthy human hepatocytes were isolated by collagenase perfusion of resected liver tissue from three patients undergoing surgical resections for benign hepatic lesions or solitary metastatic tumors to the liver as described by Roberts et al. (29). The pcDNA3.1/V5-His C vector was purchased from Invitrogen (Carlsbad, CA), and the plasmid pRK5-β3 containing the $GABA_A$-β_3 receptor gene was kindly provided by Professor P. Seeburg, (University of Heidelberg, Heidelberg, Germany).

Cell culture. Cells were grown in DMEM and supplemented with (in %) 10 cool calf serum, 1 penicillin (10,000 units/ml)/streptomycin (10,000 μg/ml), 1 Fungizone, and 0.011 sodium pyruvate in a humidified, 37°C incubator in an atmosphere of 95% air-5% CO_2.

Cell PD determinations. A Leica DM IRB fluorescence microscope equipped with a PDMI-2 Open Perfusion Micro-Incubator (Harvard Apparatus, Saint-Laurent, PQ, Canada) was used to measure PDs as described by Loew (19). The incubating temperature was 37°C. Twenty-four-hour-cultured healthy human hepatocytes and malignant hepatocytes were washed with NB buffer [(in mM) 130 NaCl, 5.5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 25 glucose and 20 HEPES buffer adjusted to pH 7.4] three times and then incubated for 10 min with NTB buffer [100 nM tetramethylrhodamine ethyl ester (TMRE) in NB buffer]. After photographs of the fluorescent cells were obtained, they were washed with KB buffer [(in mM) 130 KCl, 5.5 NaCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 25 glucose, and 20 HEPES buffer adjusted to pH 7.4] three times and incubated for 10 min with DTB buffer (100 nM TMRE and 1 μM valinomycin in KB buffer). Photographs were then obtained of the depolarized cells. Fluorescence within the cell was designated intracellular fluorescence (F_{in}) and fluorescence outside the cell, as extracellular fluorescence (F_{out}). To calculate PDs, the Nernst equation was used: $PD = -(RT/ZF)\lambda n(F_{in}\, dF_{out})/(F_{out}\, dF_{in})$, where PD is membrane potential, Z is the charge of the permeable ion, F is Faraday's constant, R is the ideal gas constant, and T is the absolute temperature.

RT-PCR for $GABA_A$-β_3 receptor. Total RNA was extracted from 1×10^6 cells by the commercially available TRIzol method (Invitrogen, Carlsbad, CA). RT reactions (20 ul) consisted of the following: 1 μg RNA, 5× reaction buffer (Clontech, Palo Alto, CA), 0.5 mM dNTP, 0.5 units RNase inhibitor, 20 pmol oligo(dT)18 primer, and 20 units Moloney murine leukemia virus reverse transcriptase (MMLV RT). Reactions were incubated at 42°C for 60 min, and terminated at 99°C for 5 min. Five microliters of the reactions were used for the PCR reaction.

The oligonucleotide primers for PCR reaction were designed against human $GABA_A$ receptor sequences by using an Oligo 5.0 program (National Biosciences, Plymouth, MN). The sequences of human $GABA_A$-β_3 receptor oligonucleotide primers were as follows: forward primer, 5′-AAGGGCTGGTTACCGGAGTGGA-3′; reverse primer, 5′-CGAAGATGGGTGTTGATGG-3′. The PCR amplification was carried out in 30 cycles of denaturation (94°C, 45 s), annealing (57°C, 45 s) and elongation (72°C, 2 min) and with an additional 7-min final extension at 72°C. Finally, 10 ul of the PCR products were run on 2% agarose gels. The product length is 290 bp (7).

SDS-gel electrophoresis and immunoblotting techniques. Cells were harvested by scraping into a protease inhibitor mixture consisting of (in mM) 20 Tris (pH 7.4), 1 PMSF, 1 benzamidine, and 5 EDTA, with 100 μM leupeptin and passed through a 26-gauge needle. Protein concentrations were measured by using the Lowry protein assay (20). Total protein extracts (50 μg) were separated on 12% polyacrylamide-SDS gels and electroblotted to nitrocellulose membranes as described previously (7). Membranes were blocked with 5% skim milk in Tris-buffered saline (0.02 M Tris-base, pH 7.6) for 1 h at room temperature and incubated with rabbit anti-human $GABA_A$-β_3 receptor antibody (5.55 μg/ml) (provided by Dr. W. Sieghart, University of Vienna, Austria) overnight at 4°C. Bands were detected with a horseradish peroxidase-labeled secondary antibody-catalyzed chemiluminescence reaction (Amersham Pharmacia Biotech, Burlington, ON, Canada) (7). Controls included rat brain microsomal protein (Upstate Biotechnology, Lake Place, NY), and membranes were incubated with secondary antibody but without prior incubation with primary anti-GABA-β3 receptor antibody.

Cell proliferation. [^{3}H]thymidine incorporation was determined as described by Luk (21). On the basis of preliminary experiments designed to identify cell concentrations associated with 60–80% confluence at 48 h, 5×10^4 cells were seeded into 6-well plates. After 2 days of culture to allow attachment to plate bottoms, cells were incubated with 1 μCi [^{3}H]thymidine for 2 h at 37C°. [^{3}H]thymidine incorporated into cellular DNA was precipitated by the addition of 10% TCA for 15 min at room temperature. Cells were rinsed and resolublilized in a 0.3 M NaOH and 1% SDS solution and then assayed for radioactivity in a β-scintillation counter.

Bromodeoxyuridine (BrdU) incorporation was determined by seeding 5×10^3 cells into 96-well plates. At subconfluence, cells were incubated with serum-free DMEM. Twenty-four hours later, the medium was switched to DMEM containing BrdU for 2 h. Incorporation of BrdU was determined by using BrdU Labeling and Detection Kit III (Roche Diagnostics, Mississauga, ON, Canada) according to the manufacturer's instructions. Absorbance at 450–490 nm was measured by using a microplate reader (Molecular Devices, Menlo Park, CA).

4-[3-(4-Lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) determinations were performed by using a commercially available kit (Boehringer-Mannheim, Laval, PQ, Canada). Briefly, 1×10^4 cells were seeded into 96-well plates. After 2 days of culture, one-tenth the volume of WST-1 (Boehringer-Mannheim) was added to each well, and the cells were incubated with WST-1 for 4 h in

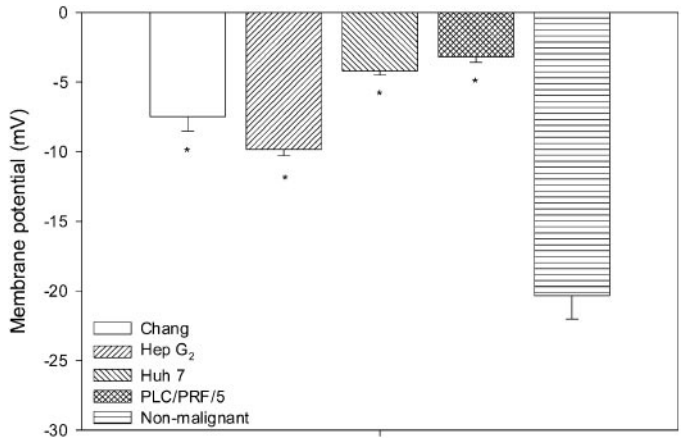

Fig. 1. Hepatocyte membrane potentials (potential difference) in 4 malignant human cell lines and proliferating, nonmalignant human hepatocytes. Nonmalignant hepatocytes were derived from patients undergoing surgical resections for solitary metastases to the liver. Results represent the means ± SE of 4–6 experiments. The differences between each malignant cell line and nonmalignant hepatocytes were significant at P values < 0.0001.

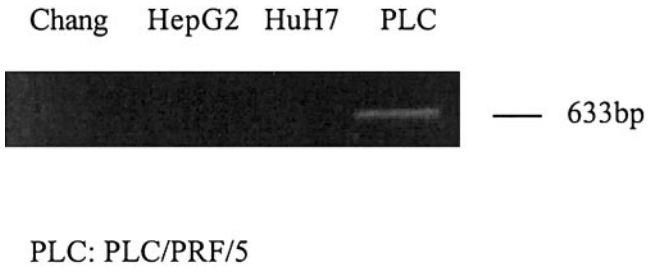

Fig. 2. RT-PCR for human $GABA_A$-β_3 receptor mRNA expression in 4 malignant human cell lines.

37°C. Absorbance at A405–A650 was determined by a microplate reader (Molecular Devices).

Colony formation in soft agar was conducted in 60-mm plates containing two layers of media. The top layer contained DMEM supplemented with 15% cold calf and 0.3% agarose, and the bottom layer contained DMEM supplemented with 15% cold calf and 0.5% agarose. Chang cells and Chang cells transfected with $GABA_A$-β_3 or vector alone were harvested by trypsinization and 0.5×10^4 cells were inoculated into the top layer of agarose. Triplicate plates were incubated at 37°C under 5% CO_2, and the number of macroscopic colonies per plate were counted after 3 wk of culture.

The number of cells undergoing mitoses was expressed as a percentage of 100 cells counted per high-power field (mitotic index). A total of six high-power fields were examined per cell population. The identity of the cell population was not known to the cytologist (E. Ravinsky) calculating the mitotic index.

Plasmid construction. The 1640-bp cDNA containing the $GABA_A$-β_3 receptor coding region was cut with *Xba*I from plasmid pRK5-β3 and cloned into the pcDNA3.1/V5-His C vector producing a plasmid referred to as pcDNA-β3. The cloned cDNA fragment is under the transcriptional control of the immediate early gene of the human cytomegalovirus promoter and the vector contains polyadenylation signals and ampicillin- and zencin-resistant genes (30). pcDNA-β3 plasmids were isolated, purified, and sequenced to confirm that the cDNA of the $GABA_A$-β_3 receptor was in frame with the cytomegalovirus promoter.

Stable transfection. Briefly, 1×10^6 cells in a 3.5-cm dish were transfected with 1 μg of linearized pcDNA-β3 or pcDNA vector alone by using LipofectAMINE (GIBCO/BRL) according to manufacturer's instructions. Stable transfected cells were established in the presence of G418 (800 μg/ml), and resistant clones were isolated by using cloning cylinders and were maintained under G418 selection (200 μg/ml). Clones were analyzed individually by RT-PCR and Western blotting for levels of $GABA_A$-β_3 receptor mRNA and protein expression, respectively (7).

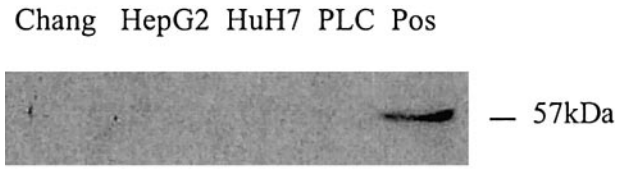

Fig. 3. Western blot analysis for $GABA_A$-β_3 receptor protein expression in 4 malignant human cell lines.

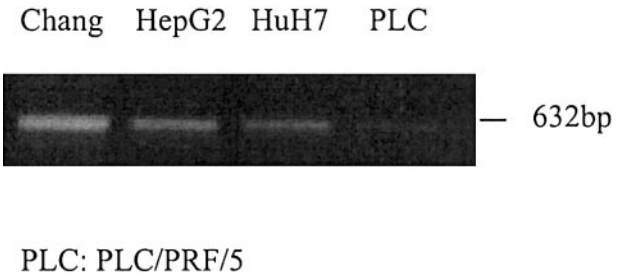

Fig. 4. RT-PCR for human $GABA_A$-ϵ receptor mRNA expression in 4 malignant cell lines.

DNA analysis. 1×10^6 cells were stained with propidium iodide as described by Diez-Fernandez et al. (6). Emitted fluorescence was assayed in a FACScan flow cytometer (Becton-Dickinson). A double discriminator module was used to distinguish between single nuclei and nuclear aggregation.

Statistical analyses. ANOVA followed by paired Student's *t*-test for parametric data and a Wilcoxon rank sum test for nonparametric data were performed where appropriate. *P* values <0.05 were considered significant. All experiments were performed on at least three occasions, and each proliferation assay was documented in 3–5 wells/plates unless otherwise stated. The results provided represent the means ± SE for all data sets generated.

RESULTS

Cellular PDs. As shown in Fig. 1, relative with resting (-25.1 ± 1.5 mV) and proliferating (-20.1 ± 1.6 mV) nonmalignant hepatocytes, the PDs of all four malignant hepatocyte cell lines were markedly depolarized (Chang: -7.5 ± 1.0, Hep G_2: -9.8 ± 0.5, HuH-7: -4.2 ± 0.3, and PLC/PRF/5: -3.2 ± 0.4 mV; $P < 0.0001$ for each malignant cell line vs. nonmalignant hepatocytes). The depolarized state was stable and did not fluctuate beyond 0.5–1.0 mV over a 4-day period of repeated testing (data not shown).

$GABA_A$ receptor expression. The human liver expresses two of the 15 known $GABA_A$ receptor subtypes (β3 and ϵ) (7, 33). Of the two, only β3 has the capacity to form GABA-gated channels (33). The results of RT-PCR for $GABA_A$-β3 receptor mRNA expression are shown in Fig. 2. In Chang, Hep G_2, and HuH-7 cells, expression was undetectable, whereas in PLC/PRF/5 cells, expression was evident. Sequence analysis of the $GABA_A$-β3 receptor mRNA expressed in PLC/PRF/5

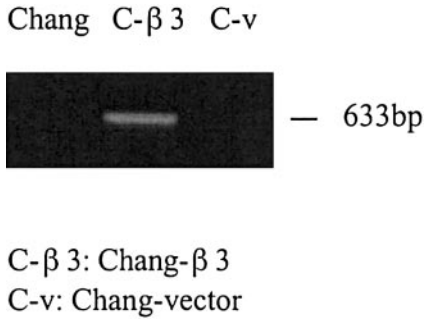

Fig. 5. RT-PCR for human $GABA_A$-β_3 receptor mRNA expression after stable transfection with $GABA_A$-β_3 receptor cDNA in a pcDNA 3.1 vector/V5-His C vector.

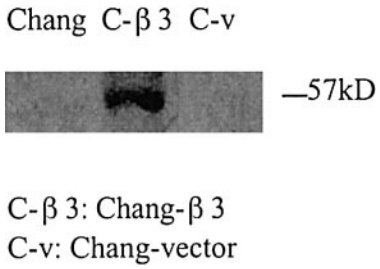

Fig. 6. Western blot analysis for human $GABA_A$-β_3 receptor protein expression after stable transfection with $GABA_A$-β_3 receptor cDNA in a pcDNA 3.1 vector/V5-His C vector.

cells revealed a nonrelevant, single mutation (G to A) at nt 1058. The absence of $GABA_A$-β3 receptor mRNA expression in the three receptor-deficient malignant cell lines was confirmed at the protein level by Western blot analyses. In the case of PLC/PRF/5 cells, a discordance existed in that despite the presence of the transcript, $GABA_A$-β3 receptor protein expression was absent by Western blot analysis (Fig. 3). $GABA_A$-ϵ receptor mRNA and protein expression were present in all four malignant cell lines (Fig. 4).

Transfection studies. To determine what impact restoration of $GABA_A$-β3 receptor mRNA expression has on cellular PD and the proliferative activity of receptor-deficient cells, Chang cells were transfected with $GABA_A$-β3 receptor cDNA in pcDNA 3.1/V5-His C vector or vector alone. Restoration of $GABA_A$-β3 receptor mRNA and protein expression after transfection were confirmed by RT-PCR and Western blot analyses, respectively, but remained undetectable in cells transfected with vector alone (Figs. 5 and 6). After transfection, the PDs of $GABA_A$-β3 receptor cDNA transfected cells increased (became more hyperpolarized) from a baseline PD of -7.5 ± 1.0 to a PD of -12.9 ± 0.4 mV ($P < 0.0001$), whereas the PDs of cells transfected with vector alone remained unaltered.

Proliferative activity. Synthesis (S) phase proliferative activity was documented by [^{3}H]thymidine and BrdU incorporation rates, whereas mitosis (M) phase activity was documented by WST-1 activity, mitotic index, and FACScan DNA analyses. In addition, cell doubling times and counts over a 10-day culture period were calculated. As shown in Table 1, S phase activity was significantly decreased in $GABA_A$-β3 receptor cDNA-transfected Chang cells compared with Chang cells transfected with vector alone. However, an even greater decrease was observed in M phase activity. Of

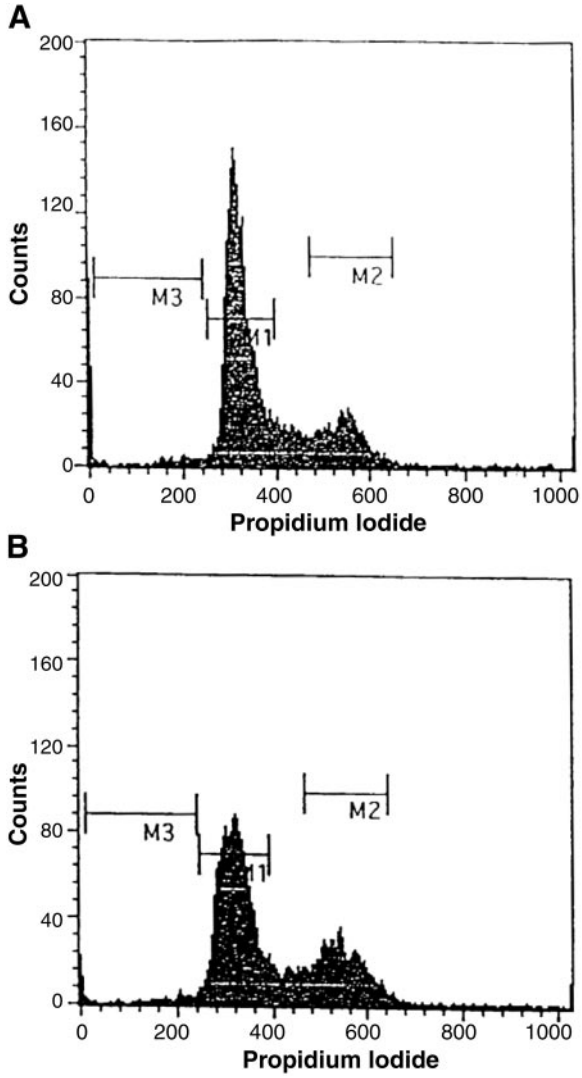

Fig. 7. Representative DNA analysis by FACScan of Chang cells transfected with $GABA_A$-β_3 receptor cDNA or vector alone. In this scan, the percentage of cells in G_0/G_1 (M1) and G_2/M phases (M2) of the cell cycle were 61 and 19%, respectively in vector transfected cells (*A*) and 42 and 25%, respectively in $GABA_A$-β_3 receptor cDNA transfected cells (*B*).

note, the percentage of $GABA_A$-β3 receptor cDNA-transfected cells undergoing mitosis after $GABA_A$-β3 receptor cDNA transfection (34%) was similar to that reported for nonmalignant hepatocytes in culture (6).

The results of DNA analyses by FACScan supported WST-1 and mitotic index findings. Specifically, the

Table 1. *Cell proliferative activity*

	S Phase		M Phase		Whole Cell
	[^{3}H]thymidine, $\times 10^5$ dpm/mg DNA	BrdU, OD	WST-1, OD	Mitotic index, %	Doubling time, h
Chang-vector	1.43 ± 0.10	0.159 ± 0.018	0.85 ± 0.09	46.7 ± 1.6	31.5 ± 2.4
Chang-β3	1.13 ± 0.01	0.105 ± 0.021	0.49 ± 0.03	33.7 ± 0.3	53.2 ± 3.6
P value	<0.05	<0.01	<0.0001	<0.001	<0.0005

Values are means ± SE. BrdU, bromodeoxyuridine; OD, optical density; WST-1, 4-[3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate.

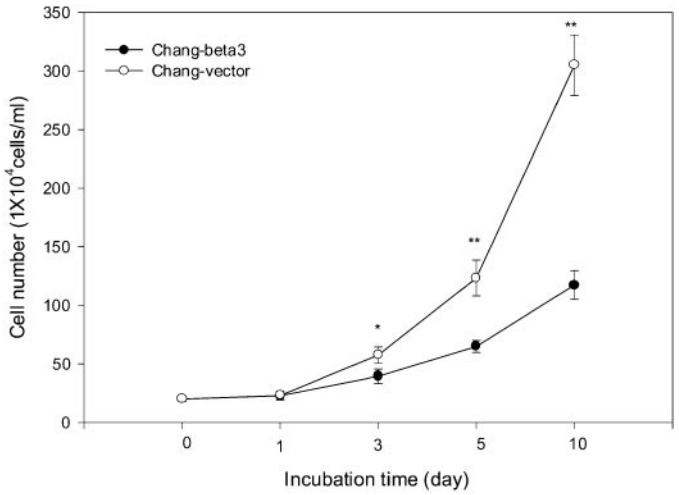

Fig. 8. Growth curves for Chang cells transfected with $GABA_A$-β3 receptor cDNA or vector alone over a 10-day culture period. (*$P <$ 0.01 and **$P < 0.001$).

proportion of Chang cells transfected with $GABA_A$-β3 receptor cDNA in the G_0/G_1 phase of the cell cycle was decreased, whereas those in the G_2/M phase were increased relative to Chang cells transfected with vector alone (Fig. 7).

The doubling time of Chang cells transfected with vector was 31.5 ± 2.4 h (Table 1). In Chang cells transfected with $GABA_A$-β3 receptor cDNA the doubling time increased to 53.2 ± 3.6 h ($P < 0.0005$).

Over a 10-day period, Chang cells transfected with vector alone grew at exponential rates (Fig. 8). However, Chang cells transfected with $GABA_A$-β3 receptor cDNA grew at a slower rate such that by *day 5*, cell counts in $GABA_A$-β3 receptor cDNA-transfected cells were only 50% that of Chang cells transfected with vector alone and 30% on *day 10* of culture.

As shown in Fig. 9, colony formation in soft agar was significantly decreased in Chang cells transfected with $GABA_A$-β3 receptor cDNA compared with cells transfected with vector alone ($P < 0.005$).

There were fewer abnormal mitoses (abnormal spindle patterns) in $GABA_A$-β3 receptor cDNA-transfected cells (9%) compared with Chang cells transfected with vector alone (18%). Chang cells transfected with vector alone also had more multinucleated cells with macronucleoli compared with those transfected with $GABA_A$-β3 receptor cDNA. Representative cells are shown in Fig. 10.

Receptor augmentation. To determine whether further hyperpolarization could be achieved in Chang cells transfected with $GABA_A$-β3 receptor cDNA, these cells together with nontransfected Chang cells and Chang cells transfected with vector alone were exposed to 50 μM muscimol, a specific $GABA_A$ receptor agonist for 48 h. As shown in Fig. 11, muscimol had little or no effect on the PDs of nontransfected Chang cells and Chang cells transfected with vector alone but increased by ~40% the PDs of Chang cells transfected with $GABA_A$-β3 receptor cDNA (premuscimol: −12.0 ± 0.9 vs. postmuscimol: −16.4 ± 0.8, $P < 0.01$).

To determine whether the addition of muscimol and/or other $GABA_A$ receptor agonists might have therapeutic value in inhibiting the growth of malignant hepatocytes with restored $GABA_A$-β3 receptor mRNA expression, proliferative activity was documented in Chang cells transfected with $GABA_A$-β3 receptor cDNA or vector alone after exposure to varying concentrations of muscimol (0–100 μM). As shown in Fig. 12, a dose-dependent decrease in proliferative activity was documented in $GABA_A$-β3 receptor cDNA-transfected cells, whereas in Chang cells transfected with vector alone, proliferative activity remained essentially unaltered.

DISCUSSION

The results of this study indicate that malignant human hepatocyte cell lines are depolarized relative to isolated, nonmalignant human hepatocytes. The results also indicate that $GABA_A$-β3 receptor mRNA expression is absent in at least some of these cell lines. Of potential therapeutic value were findings that restoration of PD values toward those documented in resting, nonmalignant hepatocytes was associated with a loss or attenuation of malignant features including decreased proliferative activity, slower growth rates, less colony formation in soft agar, and fewer numbers of cells with abnormal mitoses.

Other investigators have reported (23) that malignant cells are significantly depolarized in situ and in cell culture systems. In the case of thyroid cancer, PDs of malignant thyroid cells were ~50% depolarized compared with nonmalignant thyroid cells (13). Similar extents of depolarization have been documented in glioma, cervical, breast, and gastric carcinoma cells (16, 23, 28, 35). The only previous study describing PDs of malignant hepatocytes was reported by Binggeli and Cameron (1), who documented PD values of −19.8 ± 7.1 mV in rat hepatoma tissue compared with −37.1 ± 4.3 mV in normal rat liver. The higher PD values in

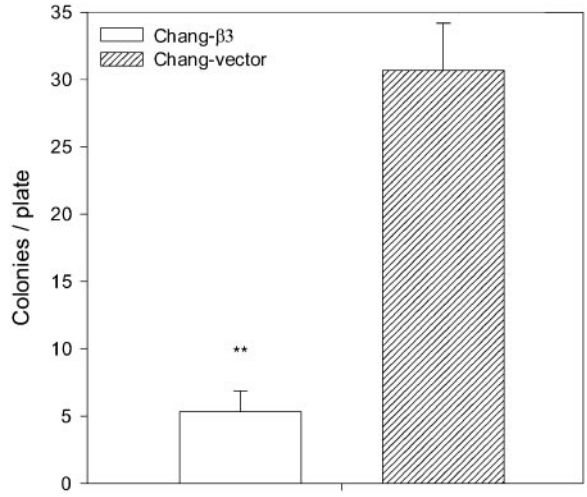

Fig. 9. Number of colonies growing in soft agar after inoculation (0.5×10^4 cells) with Chang cells transfected with $GABA_A$-β3 receptor cDNA or vector (**$P < 0.0005$).

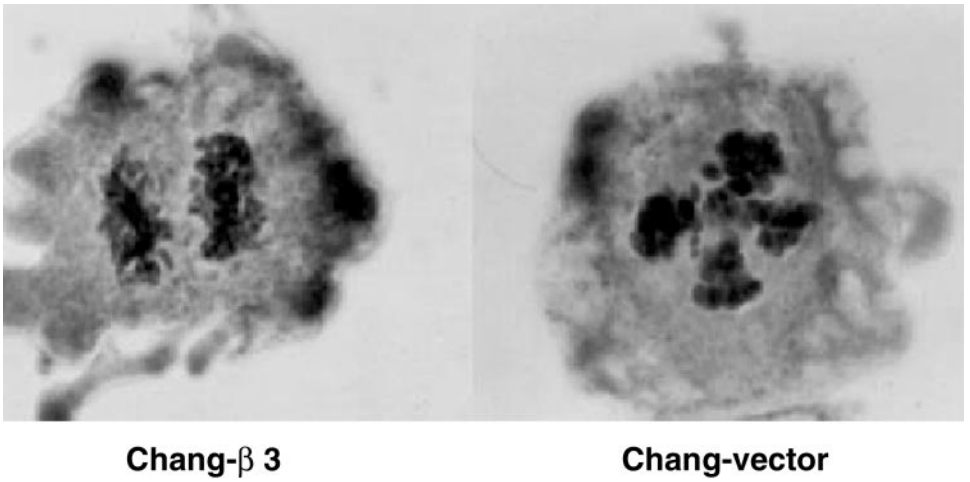

Fig. 10: Representative photomicrograph of Chang cells transfected with $GABA_A$-β_3 receptor cDNA (*left*) or vector alone (*right*). There were 50% fewer abnormal mitoses in Chang cells transfected with $GABA_A$-β_3 receptor cDNA (9 vs. 18%, respectively).

that study reflecting the authors' use of in situ determinations that are known to be associated with higher PD readings than those obtained from isolated cells or transformed cell lines as were employed in the present study (27).

Whether the depolarized state of malignant cells represents a cause or effect of malignant transformation remains to be determined. In favor of the former are findings that hepatocytes derived from cirrhotic livers, which can be considered a potentially premalignant state, are depolarized compared with hepatocytes derived from healthy livers (3). More compelling, however, are data from the present study indicating that malignant features including [^{3}H]thymidine and BrdU incorporation rates, WST-1 activity, mitotic indexes, doubling times, growth patterns, colony formation in soft agar, and the number of abnormal mitoses are significantly decreased and doubling times significantly prolonged when cells are hyperpolarized by augmenting $GABA_A$ receptor expression. Clearly, a prospective study wherein PDs and GABA receptor expression are documented during well-defined stages of malignant transformation is required to further support a causative role.

Whereas PD determinations have been reported in various malignant cell lines and tissues, to our knowledge, direct determinations of $GABA_A$ receptor expression have hitherto not been described. Nonetheless, our finding of absent $GABA_A$-β_3 receptor mRNA expression was not unexpected as in previous studies (10, 12) we described the absence and/or marked downregulation of the sodium-dependent GABA transporter system in seven human HCC tissues and GABA-transporter expression tends to parallel $GABA_A$ receptor activity.

Despite the lack of documentation of GABA receptor expression in malignant cells and/or tissues, we and others (2, 4, 15, 17, 34, 36, 40) have reported that

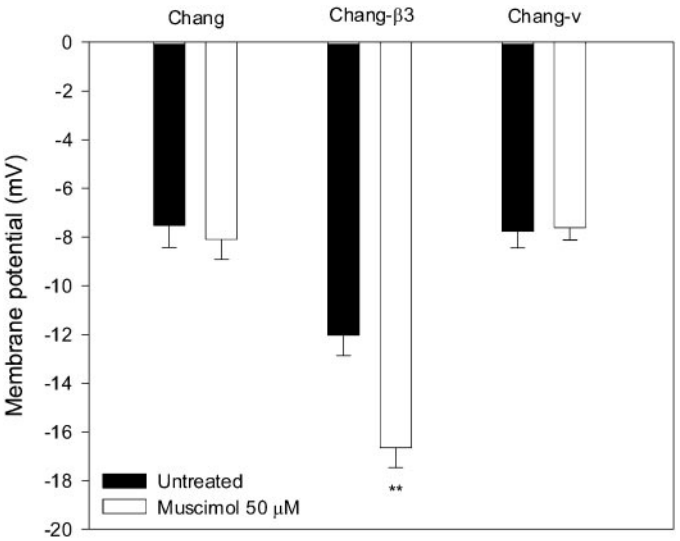

Fig. 11. Effect of the $GABA_A$ receptor agonist (50 μM muscimol) on membrane potentials (potential difference) of Chang cells, Chang cells transfected with $GABA_A$-β_3 receptor cDNA, and Chang cells transfected with vector alone. Only Chang cells transfected with $GABA_A$-β_3 receptor cDNA became more polarized after exposure to muscimol (*$P < 0.01$).

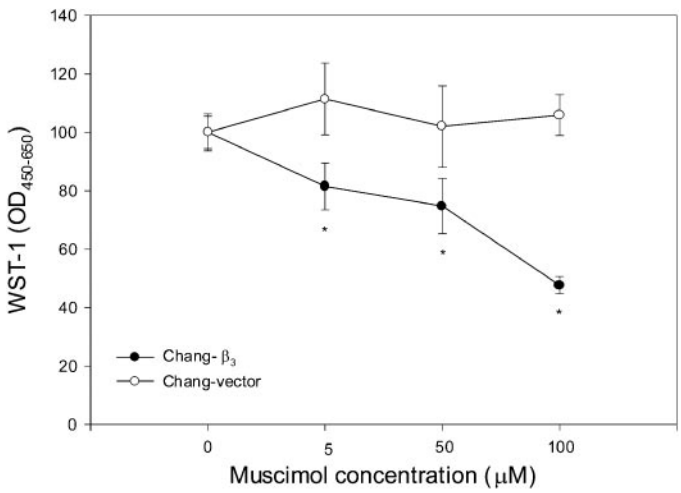

Fig. 12. Effect of increasing concentrations of muscimol on the proliferative activity of Chang cells transfected with $GABA_A$-β_3 receptor cDNA or vector alone. Proliferative activity was significantly decreased in a dose-dependent fashion in $GABA_A$-β_3 receptor cDNA transfected cells (*$P < 0.0001$). WST-1, 4-[3-(4-Lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

augmentation of GABAergic activity, by the administration of high concentrations of GABA, GABA receptor agonists, or transient transfection with $GABA_A$ receptor c-DNA has an inhibitory effect on cell proliferative activity and in the case of malignant hepatocytes, α-fetoprotein mRNA expression. Results of the present study involving stable $GABA_A$-β_3 receptor cDNA-transfections indicate the same effect can be achieved by restoring $GABA_A$ receptor expression in malignant hepatocytes.

Although the precise mechanism(s) whereby restoration of hepatocyte PD toward those of resting values results in decreased proliferative activity has yet to be elucidated, the findings of more pronounced inhibition of M phase (decreased WST-1 expression and a lower mitotic index) relative to S phase (decreased [^{3}H]thymidine and BrdU incorporation) activity in $GABA_A$-β_3 receptor transfected cells suggests that cell cycle arrest is occurring predominantly in the G_2 phase of the cell cycle. The G_2/M increase on FACScan supports this interpretation. Further studies are required to determine what impact cell PDs have on the polar activities of centrosomal segregation, mitotic spindle formation, and their respective migration toward the outer membranes of dividing cells.

Not all malignant hepatocyte cell lines had absent $GABA_A$-β_3 receptor mRNA expression despite a uniformly depolarized state. Indeed, mRNA expression in PLC/PRF/5 cells was similar if not increased relative to healthy, human hepatocytes. That sequencing of the transcript failed to identify mutations resulting in functional changes at the cell membrane level suggests that either posttranscriptional factors are responsible for the absence of $GABA_A$-β_3 receptor expression at the protein level or that GABAergic activity is intact and the depolarization in this cell line reflects disturbances in other regulators of hepatocyte PD such as volume-activated chloride channels, K_{ATP} channels or the Na^+/K^+ ATPase pump (38, 22, 31).

The $GABA_A$-ϵ receptor is the only $GABA_A$ receptor subtype that does not form GABA-gated chloride channels (33). Thus unlike the β_3 receptor subtype, the ϵ receptor subtype has no inherent electrogenic properties. Recent data suggest its role is in regulating the distribution and display of other $GABA_A$ receptor subtypes throughout the cell (5).

Results of the present study raise interesting possibilities regarding new approaches to the treatment of HCC. For example, if depolarization is an essential feature of malignant cells, then interventions that result in tumor hyperpolarization would be expected to have an inhibitory effect on tumor growth. In the case of HCC, such interventions appear to require gene therapy with the $GABA_A$-β_3 receptor gene as the prevalence of receptor-deficient or mutated cells appears to be high, all four malignant hepatocyte cell lines in this study. In addition to the general problems associated with gene therapy, including potential host toxicity and the need to efficiently transfect the majority if not all malignant cells, there are certain issues related to activation of $GABA_A$ receptors in the liver that will need to be addressed. Specifically, GABA is rapidly cleared from the systemic circulation and presently available $GABA_A$ receptor agonists have only a transient effect on receptor activity (8, 24, 37). Whether inhibition of hepatic GABA metabolism, interference with GABA clearance by hepatocytes, and/or manipulation of nonGABAergic electrogenic systems, such as increasing Na^+/K^+ ATPase activity, can be exploited to achieve the desired PD changes remains to be determined (18, 26). It should also be noted that augmentation of GABAergic activity in the liver inhibits healthy hepatocyte proliferation, an important survival mechanism in patients with advanced liver disease (14, 41).

In conclusion, the results of this study indicate that malignant hepatocytes exist in a significantly depolarized state. This depolarized state is associated with an absence of $GABA_A$-β_3 receptor expression. The results also indicate that increasing the PD of malignant hepatocytes by transfecting receptor-deficient cells with $GABA_A$-β_3 receptor cDNA, is associated with a loss or attenuation of malignant features. The later findings raise the possibility that if causative, interventions directed toward altering cell membrane potentials, an approach we have termed electrogenics, would represent a new therapeutic strategy in the treatment of hepatocellular carcinoma.

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