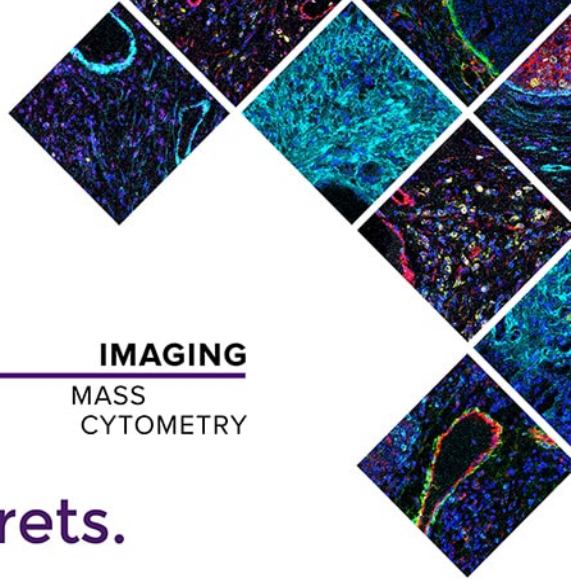
FLUIDIGM®



IMAGING
MASS
CYTOMETRY

Uncover spatial biology secrets.

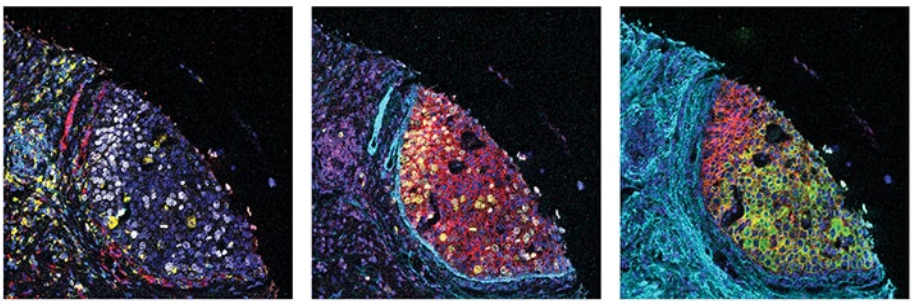
Gain disease insights.

Imaging Mass Cytometry™ (IMC™) is a vital tool that allows researchers to gain an appreciation of the role of heterogeneity in cell function. Researchers globally are using IMC to assess tumor-immune interactions and get deep single-cell insights into the spatial relationships of cells, pathways and phenotypes in the tissue microenvironment.

- Better understand disease and response to treatment.
- Stratify subjects by linking high-plex data to outcomes in clinical studies.
- Uncover novel therapeutic targets.

Don't miss out because you think high-multiplex imaging is early stage technology or too difficult to perform. Learn how your colleagues are already answering important clinical research questions in the context of disease.

With nearly 100 peer-reviewed publications across a wide variety of disease areas and applications, there's already a wealth of information to help you get started. Or you can quickly get going with IMC and mass cytometry services offered through our expert Therapeutic Insights Services group.



Selected markers show the spatial context of immune populations and tissue structure in human breast cancer tissue using IMC.

Start realizing deep insights now.

Visit us at **fluidigm.com/IMCvalue** to see how others are using IMC.

For Research Use Only. Not for use in diagnostic procedures.

Changes in Alpha-1 and Beta-2 Adrenoceptor Density in Human Hepatocellular Carcinoma

Maurizio Bevilacqua, MD,* Guido Norbiato, MD,*
Enrica Chebat, MD,* Gabriella Baldi, PhD,* Pierluigi Bertora, MD,*
Enrico Regalia, MD,† Giovanni Colella, MD,† Leandro Gennari, MD,†
and Tarcisio Vago, PhD*

Catecholamines are involved critically in the mechanisms of liver cell proliferation by acting on hepatic alpha-1 and beta-2 adrenoceptors. To identify the role of these receptors in human hepatocellular carcinoma (HCC), the density was examined of alpha-1 and beta-2 adrenoceptors with their affinity and coupling of beta-2 adrenoceptors to adenylate cyclase in HCC tissue and in nonadjacent/nontumor tissue from the same livers. Studies were also done on healthy livers from age-matched and sex-matched patients undergoing abdominal surgery for nonhepatic diseases. Twenty-two HCC had a decrease of about 72% in alpha-1 adrenoceptor density compared with their nonadjacent/nontumor tissue and a decrease of about 40% compared with healthy controls. Nonadjacent/nontumor tissue from HCC patients had a 125% increase in alpha-1 adrenoceptor density compared with healthy livers. Twenty-three of 24 HCC had an increase of about 180% in beta adrenoceptor density compared with their nonadjacent/nontumor tissue and healthy controls. Beta adrenoceptors were coupled to adenylate cyclase, as evidenced by a guanosine triphosphate-mediated right shift in (−)-isoproterenol competition isotherms and by cyclic adenosine monophosphate (cAMP) production after stimulation with (−)-isoproterenol. The HCC tissue yielded a larger increase in cAMP than nonadjacent/nontumor tissue and healthy controls. The authors conclude that a higher density of alpha-1 adrenoceptors in nonadjacent/nontumor tissue from HCC characterizes the "healthy" part of the liver in HCC patients and that an increase in beta-2 and a decrease in alpha-1 adrenoceptor densities characterize the tumor part of the liver in human HCC. *Cancer* 67:2543–2551, 1991.

IN ADULT HUMANS and animals, hepatocytes have long life spans, ranging from 200 to 400 days or more, but it is a common observation that the proliferation rate of hepatocytes increases after hepatic cell death or loss of liver tissue.[1] Hepatocyte proliferation occurs in viral hepatitis, cirrhosis, hepatotoxic reactions, and massive liver necrosis.[2] In turn, viral hepatitis and cirrhosis are strongly associated with hepatocellular carcinoma (HCC), a usually fatal disease which affects approximately 250 million people in the world.[3]

The mechanism which controls cellular proliferation in HCC is not known. An extensive series of studies on rat liver regeneration after partial resection has disclosed many important findings in this field that might give some insight also into human carcinogenesis, bearing in mind, however, the many differences between nonneoplastic and neoplastic liver cell proliferation.[2] Parenchymal hepatocytes from adult rats exist in a quiescent state, yet undergo coordinate proliferation after partial liver resection. Hepatic growth is a compensatory response to decreased liver mass or cell loss.[4] In these cells an increasing role of oncogenes[4–9] that encode growth factors or their receptors has been identified.

From the *Servizio di Endocrinologia, Ospedale L. Sacco (Vialba), and the †Oncologia Chirurgica "A," Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy.

Address for reprints: Maurizio Bevilacqua, MD, Servizio di Endocrinologia, Ospedale L. Sacco (Vialba), Via GB Grassi 74, 20157 Milano, Italy.

Accepted for publication November 3, 1990.

A major recent advance in this field was the finding that catecholamines directly affect hepatic cell proliferation in rats. In particular, norepinephrine is involved critically in the initiation of DNA synthesis after liver resection.[10,11] The effect is mediated by alpha-1 adrenoceptors, since it is abolished by prazosin. Furthermore alpha-1 adrenoceptors seem to modulate the receptors of epidermal growth factor and transforming growth factor-beta in proliferating hepatocytes.[12,13] Beta-2 adrenoceptors also seem to be involved. In fact, propranolol, a beta-adrenergic blocker, increases liver cell proliferation, which suggests that beta-adrenergic agonists may actually inhibit hepatocyte growth.[14] Regarding beta adrenoceptors, there are data on liver cells from rats treated with carcinogens. In these cells increased cyclic adenosine monophosphate (cAMP) production by stimulation of beta-adrenergic receptors was found.[15] The relevance of these basic findings to *in vivo* liver proliferation is strengthened by the finding that surgical hepatic denervation or pharmacologic alpha-adrenergic blockade results in a reduced incorporation of ^{3}H-thymidine after partial hepatectomy, pointing to a pivotal role for alpha-1 adrenergic receptors not only in *in vitro* experiments but also in *in vivo* conditions.[10] Since the most important determinants of the effects of catecholamines are adrenoceptors[16] and since studies on these structures may afford some clue to a better understanding of the role of catecholamines on hepatic regeneration, we evaluated the status of liver adrenergic receptors in human HCC.

Materials and Methods

Liver samples (0.5 to 5 g of the core of tumor tissue whose necrotic parts were carefully discarded and 1 to 5 g of the nonadjacent/nontumor tissue, taken 6 to 8 cm away from the periphery of the tumor, usually in a different lobe) were obtained from 24 patients with HCC who were operated on for tumor resection. Clinical data, hepatitis B markers (hepatitis B surface antigen [HBsAg], Auszyme Monoclonal Diagnostic Kit, and hepatitis B core antibody, Corzyme Diagnostic Kit, Abbott, North Chicago, IL) for each patient and histologic diagnosis of both tumor and nonadjacent/nontumor liver tissue are reported in Table 1. Twenty-four liver samples (1 to 5 g) were obtained from sex-matched and age-matched patients undergoing major abdominal surgery for extrahepatic diseases (gastrectomy for nonmalignant peptic ulcer, 15 and partial colectomy, nine). Histologic examination revealed mild steatosis in 12 cases, whereas the remaining samples showed no alterations, as previously reported.[17] These patients were HBsAg negative; in previous studies[17] we have not identified an effect of HBsAg infection on human liver alpha-1 and beta adrenoceptors. Medications included antibiotics, fluids, and vitamin and electrolyte supplements. In the 14 days before surgery, no patient in the HCC group or control group received catecholamines or drugs known to interact with adrenergic receptors. Informed written consent was obtained from all the patients before hepatic sample excision.

Plasma Membrane Preparation

Immediately after excision, the liver samples were placed on ice in Falcon tubes (Corning, Milano, Italy) and quickly transferred to our laboratory. The time lapse between tissue excision and the beginning of homogenization procedures never exceeded 40 minutes. The method of membrane preparation was a slight modification of the method of Neville.[18] Liver samples were placed in ten volumes of ice-cold homogenization buffer (0.25 mol/l sucrose, 1 mmol/l $MgCl_2$, 5 mmol/l tris HCl and 1 mmol/l EGTA with 1 mg/ml of bacitracin, pH 7.4). The EGTA was added to prevent the loss of the high-affinity subtype binding sites due to activation of hepatic proteases.[19] The samples were minced with scissors and homogenized with a Polytron homogenizer (Kinematica AG, Luzern, Switzerland) by means of three consecutive 5-second bursts at the setting of 11. The homogenate was centrifuged at 250 × *g* for 30 minutes, and the pellet was discarded. The supernatant was then spun at 50,000 × *g* for 35 minutes and resuspended with ice-cold incubation buffer (50 mmol/l tris HCl and 10 mmol/l $MgCl_2$, pH 7.5). The procedure was repeated twice. The final suspension (2 to 6 mg protein/ml) was immediately used for receptor-binding assays and adenylate-cyclase studies.

Binding Studies

Beta adrenoceptor density on liver plasma membranes was assessed by binding of ^{125}I-pindolol (specific activity, 2200 Ci/mmol; New England Nuclear, Boston, MA), and alpha-1 adrenoceptor density was measured by ^{3}H-prazosin (80 or 82 Ci/mmol; New England Nuclear).

We did ^{125}I-pindolol saturation binding experiments by incubating 20 to 50 μg of membrane preparation with seven to eight increasing concentrations of ^{125}I-pindolol (0.01 to 10 nmol/l in most cases) in the presence or absence of 1 μmol/l (−)-propranolol, in a total volume of 200 μl. In competition experiments we incubated 20 to 50 μg of the membrane preparation with a fixed concentration of ^{125}I-pindolol (approaching the dissociation constant [K_D]) and variable concentrations of displacing drugs. In (−)-isoproterenol competition experiments, incubation was done in the presence or the absence of 0.1 mmol/l guanosine triphosphate (GTP).

We did ^{3}H-prazosin saturation binding experiments by incubating 20 to 50 μg of the membrane preparation with seven concentrations of ^{3}H-prazosin (0.05 to 5 nmol/l in most cases) in the presence or the absence of 2.5 mmol/

TABLE 1. Clinical and Adrenoceptor Binding Data in Human Hepatocellular Carcinoma

				Tumor tissue					Nontumor/nonadjacent tissue				
					Alpha-1		Beta-2			Alpha-1		Beta-2	
Patient no.	Age (yr)	Sex	HBsAg	Histologic type	K_D (pM)	Density (fmol/mg)	K_D (pM)	Density (fmol/mg)	Histologic type	K_D (pM)	Density (fmol/mg)	K_D (pM)	Density (fmol/mg)
1	56	F	−*	Trabecular	97	4	ND	ND	Normal	200	50	ND	ND
2	58	M	−*	Acinar	103	5	215	170	Macronodular cirrhosis	198	49	215	110
3	61	M	+	Trabecular	229	11	83	119	Macronodular cirrhosis	332	109	100	73
4	62	F	+	Trabecular	ND	ND	210	121	Normal	ND	ND	187	41
5	65	M	−*	Trabecular†	408	12	242	228	Normal	543	287	280	141
6	59	M	−*	Trabecular	ND	ND	214	151	Micronodular cirrhosis	ND	ND	234	91
7	71	M	−*	Trabecular	191	33	189	272	Micronodular cirrhosis	606	56	137	135
8	3	F	−	Hepatoblastoma	268	53	395	177	Normal	178	151	257	107
9	53	M	−*	Trabecular	562	16	150	224	Macronodular cirrhosis	664	126	108	66
10	67	M	−*	Trabecular	556	92	150	210	Normal	475	148	183	90
11	67	M	+	Trabecular	628	74	199	172	Normal	248	166	125	134
12	59	F	−*	Trabecular	410	61	209	61	Normal	240	152	168	74
13	70	M	−*	Trabecular	187	30	345	428	Micronodular cirrhosis	215	180	235	182
14	56	M	−	Trabecular	197	41	39	188	Normal	310	175	47	114
15	64	M	−*	Trabecular	431	70	137	215	Micronodular cirrhosis	465	237	131	172
16	65	M	−*	Trabecular	69	17	86	106	Normal	331	192	122	81
17	65	M	−*	Clear cell	310	120	91	40	Micronodular cirrhosis	320	187	104	61
18	58	M	−	Trabecular	58	5	114	213	Macronodular cirrhosis	608	63	127	51
19	59	M	−	Trabecular	59	43	125	509	Normal	117	211	112	133
20	56	M	−	Trabecular	274	113	128	119	Macronodular cirrhosis	182	177	107	85
21	73	F	−	Acinar‡	81	7	125	28	Normal	310	145	133	95
22	65	M	−	Trabecular	459	15	145	204	Micronodular cirrhosis	527	49	142	39
23	57	M	+	Trabecular	100	39	110	128	Normal	162	108	110	125
24	72	M	+	Trabecular	249	17	175	452	Macronodular cirrhosis	170	61	170	63
Mean					269.3	39.9	168.5	197.1		336.4	139.9	153.6	98.4
SD					178.6	35.2	81.9	122.5		168.3	66.3	58.2	39.2

ND: not done.

Normal values in 24 age- and sex-matched controls are as follows: alpha-1 adrenoceptors: Bmax = 61.88 ± 19.69 fmol/mg protein; K_D = 210.14 ± 31.43 pM; beta-2 adrenoceptors: Bmax = 89.67 ± 17.94 fmol/mg protein; K_D = 112.5 ± 32.05 pM (mean ± SD).

* HB core Ab positive.

† Periportal sclerosis.

‡ Pseudoglandular and plurifocal appearance.

l (−)-norepinephrine. In competition experiments we incubated 20 to 50 μg of the membrane preparation with a fixed concentration of ^{3}H-prazosin (approaching the K_D) and variable concentrations of (−)-norepinephrine, in the presence or absence of 0.1 mmol/l GTP. The incubation volume was 200 μl. Superoxide dismutase and catalase were added to the incubation to prevent oxidation of the catecholamines,[21] and they were added also in saturation experiments.

After overnight incubation at 4°C, the assay suspension was diluted with 5 ml of washing buffer (50 mmol/l tris HCl and 10 mmol/l $MgCl_2$, pH 7.5) and vacuum-filtered through Whatman GF/C glass-fiber filters (Whatman, Maidstone, England). Each filter was then washed with 20 ml of washing buffer. The filters were allowed to dry, and bound radioactivity was counted in a gamma-counter (Packard Instruments, Downers Grove, IL) or placed in Filter-Count (Packard Instruments) and counted in a 1500 Tri-Carb liquid scintillation spectrometer (Packard Instruments).

The assay methods described consistently gave a coefficient of variation of less than 5% for ^{125}I-pindolol binding and less than 7% for ^{3}H-prazosin binding.

Adenylate Cyclase Assay

Adenylate cyclase activity in liver plasma membranes was assessed as ^{32}P-cAMP generation from radiolabeled substrate. Briefly, liver plasma membranes (50 μg) were incubated in assay buffer containing 40 mmol/l tris HCl (pH 7.4), 15 mmol/l $MgCl_2$, 0.1 mmol/l EGTA, 1 mmol/l isobutylmethylxanthine, 50 μmol/l GTP, 0.5 mmol/l alpha-^{32}P-adenosine triphosphate (ATP, New England Nuclear) (~10^6 cpm/tube), 20 U/ml of creatine phosphokinase, 20 mmol/l phosphocreatine, 1 mmol/l cAMP, and the various drugs at concentrations indicated in the text, in a final reaction volume of 200 μl. After 10 minutes at 37°C, the reaction was stopped with 150 μl of a solution containing 6% sodium dodecyl sulfate, 90 mmol/l ATP, 3 mmol/l cAMP, and 10,000 cpm ^{3}H-cAMP to monitor the recovery of cAMP from the subsequent separation, and the tubes were boiled for 5 minutes. The amount of ^{32}P-cAMP generated was evaluated by liquid scintillation counting after separation by column chromatography.[21] Assay blanks yielded about 10% of basal activity in all cases, and column recovery of ^{3}H-cAMP that was added after incubation ranged from 60% to 80%. All assay points were the means of quadruplicate values.

Binding Data Analysis

Receptor density (B_{max}) and dissociation constants (K_D) were determined by Scatchard analysis.[22] The ^{125}I-pindolol binding was saturable, linear with respect to the protein concentration in a range of 0.5 to 5 mg/ml, and stereospecific for (−)-isoproterenol and (+)-isoproterenol (data not shown). The specific ^{125}I-pindolol binding, defined as that displaceable by 1 μmol/l (−)-propranolol at a concentration of ^{125}I-pindolol near its K_D and at a protein concentration of 200 to 500 μg/ml, was 85 ± 3% of total binding in tumor tissue, 84 ± 4% in nonadjacent/nontumor tissue, and 83 ± 5% in healthy livers. By analysis of ^{125}I-pindolol displacement by ICI 118,551 (ICI Pharma, Milano, Italy), a beta-2 selective adrenergic antagonist, it was possible to delineate a homogeneous single class of beta-adrenergic binding sites identifiable as beta-2 subtype in four healthy livers, in four tumor tissues, and in four nonadjacent/nontumor tissues.[17,23] The analysis of competition isotherms on ^{125}I-pindolol by different adrenergic agonists confirmed a beta-2 subtype specificity, since the order of potency was (−)-isoproterenol more than (−)-epinephrine much more than (−)-norepinephrine in six healthy livers, in six tumor tissues, and in six nonadjacent/nontumor tissues.[17,23] The ^{3}H-prazosin binding was saturable, linear with respect to the protein concentration between 0.1 and 6 mg/ml, and stereospecific for (−)-norepinephrine and (+)-norepinephrine (data not shown). The specific ^{3}H-prazosin binding, defined as displaceable by 1 mmol/l (−)-norepinephrine at a ^{3}H-prazosin concentration near its K_D and at a protein concentration of 200 to 500 μg/ml, was 68 ± 5% of total binding in tumor tissue, 69 ± 5% in nonadjacent/nontumor tissue, and 67 ± 4% in healthy livers.

Competition isotherms were evaluated.[24] Akaike's[25] test was used to evaluate the best fit to a one-site or two-sites binding model, as previously reported.[26] All assay points were the means of duplicate values.

Protein Determination

Protein content of the plasma membrane suspensions was evaluated by Bradford's assay.[27]

Statistical Analysis

For comparison of a single measurement in the same group, Student's *t* test was used. For comparison among more than two groups, one-way analysis of variance (ANOVA) and Bonferroni's multiple-comparison test[28] were used.

Results

Beta-Adrenoceptor Changes

The HCC yielded consistently higher beta-adrenoceptor density than nonadjacent/nontumor tissue (Table 1; $P < 0.01$, by paired *t* test) and healthy tissue ($P < 0.01$, by ANOVA). The mean increase was approximately 100%; some patients (9, 13, 18, 19, 22, and 24) showed increases

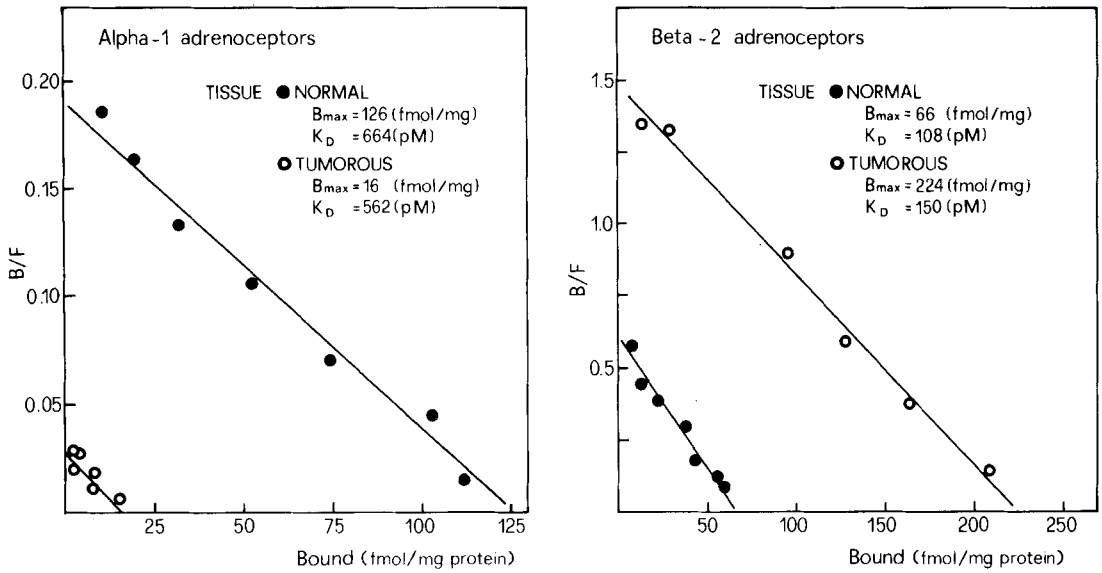


FIG. 1. Scatchard plots of ^{3}H-prazosin (left) and ^{125}I-pindolol (right) binding to plasma membranes from tumor or nonadjacent/nontumor tissue from Patient 9 with hepatocellular carcinoma. Bmax: the number of binding sites; B/F: the bound:free ratio. Data obtained in 24 patients and 24 controls are reported in Table 1.

of about 200%. In three patients (12, 17, and 21), we did not detect an increase in beta adrenoceptors in their tumor tissue; the liver specimen from Patient 21 was characterized by a pseudoglandular appearance and multi-focality of the tumor, a histologic pattern unusual in our series. However the histology of the samples from Patients 12 and 17 was not dissimilar from other cases. No difference of beta-adrenoceptor density was found between cirrhotic and noncirrhotic tissue from HCC, nor between nontumor tissue and healthy livers. The K_D did not differ among

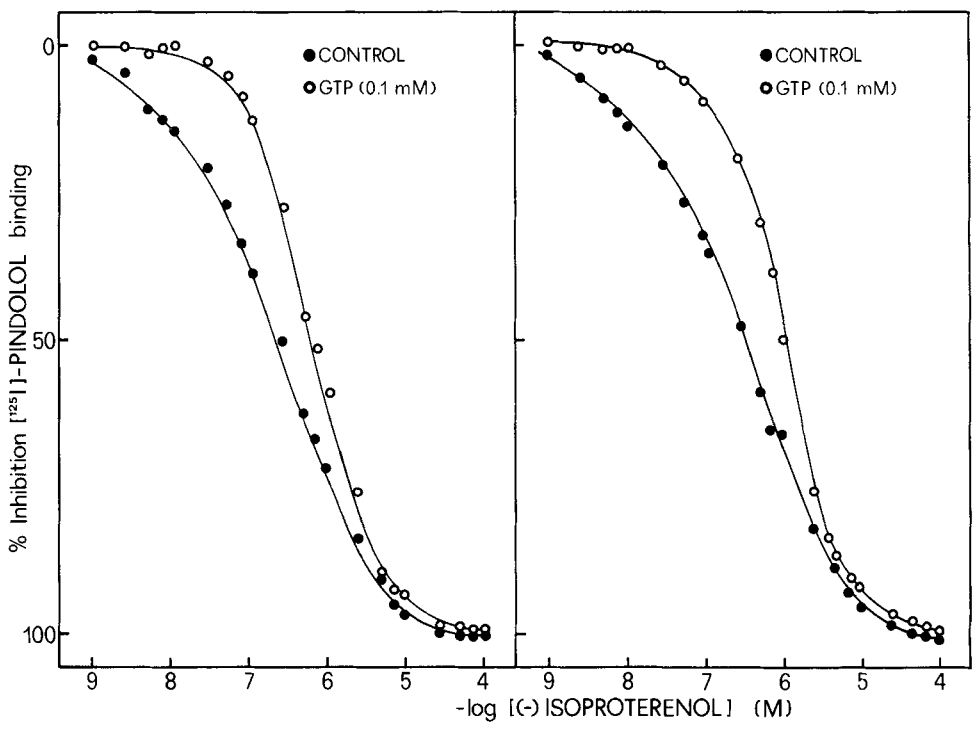


FIG. 2. Competition isotherms for ^{125}I-pindolol binding sites by (−)-isoproterenol in plasma membranes from tumor tissue (left panel) and nonadjacent/nontumor tissue (right panel) from Patient 18 with hepatocellular carcinoma. Competition isotherms were done in the absence (●) or in the presence (○) of 0.1 mmol/l GTP. Left panel (tumor tissue): curve (●) is better explained by a two-site binding model, one with high affinity (beta-H) (pK_i 8.05, 27% of total number of receptors) and the other with lower affinity (beta-L) (pK_i 6.23, 73% of total number of receptors). Addition of GTP causes an evident right shift; curve (○) is explained by a one-site binding model (pK_i 6.61, 100% receptors in low affinity). Right panel (nonadjacent/nontumor tissue): curve (●) is better explained by a two-site binding model, one with high affinity (beta-H) (pK_i 7.99, 25% of total number of receptors). Addition of GTP causes an evident right shift; curve (○) is explained by a one-site binding model (pK_i 6.05, 100% receptors in low affinity). Data obtained in seven patients and in 12 controls are reported in Table 2.

TABLE 2. High and Low Affinity Beta-2 Adrenoceptors in Human Hepatocellular Carcinoma

		Without GTP				With GTP	
Patient no.	Tissue	Beta-H (%)	Beta-L (%)	pK_i-H	pK_i-L	pK_i	Beta-L (%)
5	T	17	83	8.51	6.25	6.28	100
	NT	23	77	7.92	6.20	6.00	98
10	T	29	71	7.83	6.40	6.32	100
	NT	30	70	8.06	6.09	6.05	100
13	T	17	83	8.50	6.41	6.34	100
	NT	20	80	8.09	6.35	6.15	100
14	T	21	79	7.97	6.08	6.32	100
	NT	18	82	8.04	6.12	6.41	100
18	T	27	73	8.05	6.23	6.61	100
	NT	25	75	7.99	6.02	6.05	100
22	T	16	84	8.58	6.12	6.57	100
	NT	18	82	8.20	6.05	6.08	100
23	T	21	79	7.98	6.51	6.10	100
	NT	23	77	8.01	6.80	6.43	
Mean ± SD	T	21.14 ± 5.11	78.86 ± 5.11	8.20 ± 0.31	6.29 ± 0.16	6.36 ± 0.18	100 ± 0.00
Mean ± SD	NT	22.43 ± 4.28	77.57 ± 4.28	8.04 ± 0.09	6.23 ± 0.27	6.17 ± 0.18	99 ± 1.91
Mean ± SD	C	25.08 ± 2.12	74.51 ± 4.13	8.47 ± 0.84	6.35 ± 0.71	6.30 ± 0.52	100 ± 2.05

GTP: guanosine triphosphate; T: tumor tissue; NT: nonadjacent/nontumor tissue; C: control tissue from 12 healthy livers; pK_i: -log of equilibrium dissociation constant.

the groups. Figure 1 illustrates representative Scatchard plots derived from the tumor tissue of Patient 9 and from the nonadjacent/nontumor tissue.

Competition isotherms for (−)-isoproterenol were characterized by a shallow shape (Fig. 2 and Table 2). By a computer-assisted modeling technique, they were best explained by a two-site binding model, with about 25% receptors in the high-affinity and 75% receptors in the low-affinity state. Competition isotherms in the presence of 0.1 mmol/l GTP were right shifted, with the loss of the high-affinity subtype; these were best explained by a one-site binding model. A shallow competition isotherm was obtained in all samples tested, *i.e.*, in seven samples of tumor tissue from HCC and in the corresponding nonadjacent/nontumor tissue (Fig. 2). A right shift after GTP addition was also evident in 12 healthy livers (Table 2).

TABLE 3. Adenylate Cyclase Stimulation in Human Hepatocellular Carcinoma

Patient no.	Tissue	EC_{50} −log[M]	Cyclic AMP production with (−)-isoproterenol, 0.1 mmol/l (pmol/min/mg protein)
5	T	7.00	105
	NT	6.88	52
6	T	6.60	62
	NT	6.69	34
10	T	7.09	63
	NT	6.82	29
13	T	7.00	119
	NT	7.07	48
14	T	7.00	48
	NT	6.85	29
17	T	6.88	18
	NT	7.10	20
18	T	6.80	51
	NT	6.70	15
Mean ± SD	T	6.91 ± 0.17	66.57 ± 34.65*
Mean ± SD	NT	6.87 ± 0.16	32.43 ± 13.60
Mean ± SD	C	7.05 ± 0.09	29.6 ± 7.03

AMP: adenosine monophosphate; T: tumor tissue; NT: nonadjacent/nontumor tissue; C: healthy livers from ten controls; SD: standard deviation.

* Significantly different by Bonferroni's multiple comparison compared with NT and C ($P < 0.01$).

Adenylate Cyclase Activity

Basal adenylate cyclase activity was not different between tumor tissue and nonadjacent/nontumor tissue or healthy livers (Table 3). Isoproterenol significantly increased cAMP production in tumor tissue compared with nonadjacent/nontumor tissue ($P < 0.01$, by paired t test) and healthy livers ($P < 0.01$, by ANOVA). Figure 3 shows a representative dose-response curve of (−)-isoproterenol on adenylate cyclase from the tumor tissue of Patient 5 and nonadjacent/nontumor liver from the same patient.

Alpha-1 Adrenoceptor Changes

The HCC tissue yielded consistently decreased alpha-1 adrenoceptor density with respect to nonadjacent/nontumor tissue ($P < 0.01$, by paired t test) and healthy livers ($P < 0.01$, by ANOVA, Table 1). The mean decrease in alpha-1 adrenoceptor density was about 72%: some patients (3, 5, 9, 16, and 21) displayed changes as high as

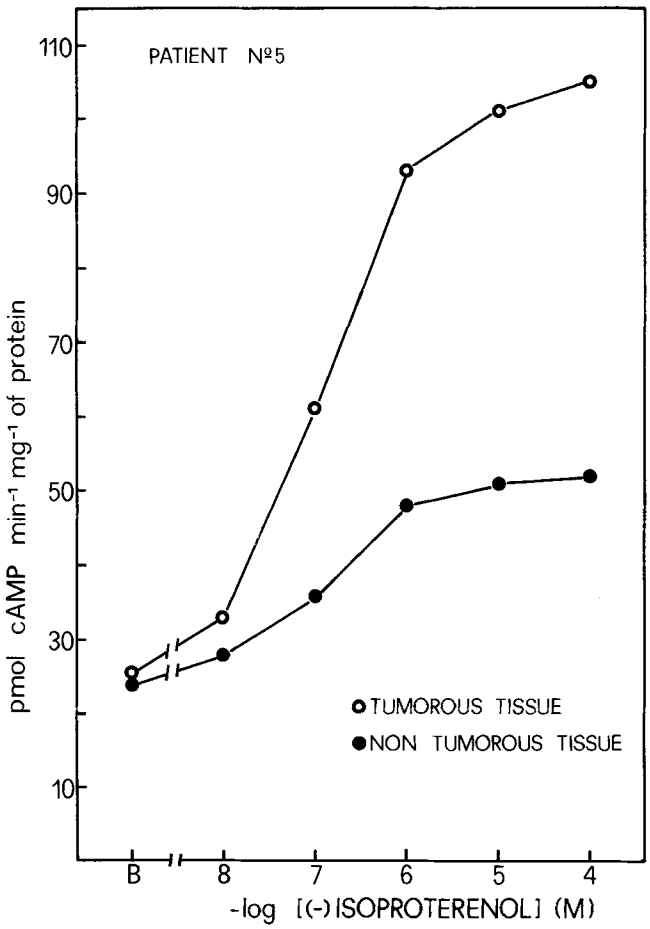


FIG. 3. (−)-Isoproterenol-stimulated adenylate cyclase activity in tumor tissue (○) and in nonadjacent/nontumor liver tissue (●) from Patient 5 with hepatocellular carcinoma. B: cyclic AMP (cAMP) production in the absence of (−)-isoproterenol. In the presence of 0.1 mmol/l (−)-isoproterenol tumor tissue produced 105 pmol/min/mg protein of cAMP and nonadjacent/nontumor tissue 52 pmol/min/mg protein. EC_{50}, expressed as −log [M] (M is the concentration that causes the half-maximal increase in cAMP), is 7.00 in tumor tissue and 6.88 in nonadjacent/nontumor tissue. Data obtained in seven patients and ten controls are reported in Table 3.

90%. A difference was also found between nonadjacent/nontumor tissue from HCC and healthy livers: the nontumor tissue from HCC patients displayed consistently higher alpha-1 adrenoceptor densities than healthy livers, with a mean increase of about 125%. Dissociation constants did not significantly differ among the groups. The decrease in alpha-1 adrenoceptor density in tumor *versus* nonadjacent/nontumor tissue was present in all livers examined (n = 22), and no difference was found between cirrhotic and noncirrhotic livers. Figure 1 illustrates representative Scatchard plots derived from the tumor and nonadjacent/nontumor liver tissue from Patient 9.

Competition isotherms for (−)-norepinephrine were best explained by a one-site binding model in tumor tissue, as evaluated in Patients 2, 3, 6, 10, 11, 17, 18, 21, and 23 (pK_i = 5.1 ± 0.02), in their nonadjacent/nontumor samples (pK_i = 5.0 ± 0.02), and in healthy livers (pK_i = 5.1 ± 0.02; n = 10). In these samples the addition of GTP was not followed by a right shift in any case, suggesting that alpha-1 adrenoceptors exist in a low-affinity state. In the presence of 0.1 mmol/l GTP, pK_i was 5.05 ± 0.2 in tumor tissue, 4.98 ± 0.04 in nonadjacent/nontumor tissue, and 5.01 ± 0.1 in healthy livers.

Discussion

Our principal findings were: (1) membrane preparations from nontumor tissue of HCC livers yielded increased alpha-1 adrenoceptor density compared with healthy livers and (2) membrane preparations derived from the tumor tissue of HCC patients had decreased alpha-1 and increased beta-2 adrenoceptor density compared with nonadjacent/nontumor liver and healthy livers. In the course of these studies we did not find a systematic effect of coexisting liver cirrhosis (12 of 24 patients had micronodular or macronodular cirrhosis). Furthermore, we confirmed previous studies[17,23] which did not find in humans a gender dependence of beta adrenoceptors that is present in rodents.[29]

Alpha-1 Adrenoceptors

The increased density of alpha-1 adrenoceptors in the nontumor part of HCC might be clinically relevant. In rat liver parenchymal cells and in *in vivo* studies, norepinephrine stimulates liver cell proliferation through stimulation of alpha-1 adrenoceptors,[10,11] and alpha-1 adrenoceptors are involved in the regulation of epidermal growth factor and transforming growth factor-beta in hepatocytes.[12,13] It appears, therefore, that, in the healthy part of HCC livers, the increased alpha-1 adrenoceptor density might play a role in the control of hepatocyte proliferation. In tumor tissue from HCC, we found a striking decrease in alpha-1 adrenoceptors. It seems that neoplastic degeneration strongly affects alpha-1 adrenoceptors. A decrease in alpha-1 adrenoceptors also has been found in rat hepatocytes after hepatic resection[30,31] or in primary culture,[32,33] suggesting that the loss of alpha-1 adrenoceptors is a characteristic of rapidly proliferating cells.

Beta Adrenoceptors

In tumor tissues we found an increased density of beta adrenoceptors, normal coupling to G-protein (as evidenced by a normal right shift caused by GTP in isoproterenol competition isotherms),[16] and increased cAMP production by (−)-isoproterenol. The increase in beta-2

adrenoceptors and cAMP production in tumor tissue from HCC is important pathologically. Beta adrenoceptor-related cAMP production is highly involved in liver cell proliferation in normal conditions.[34] A lower level of hepatocyte differentiation is associated with an increased expression of beta-2 adrenoceptors.[32,33] Beta adrenoceptors and increases in adenylate cyclase activity may affect cellular proliferation.[35] In fact, cAMP mediates the mitogenic and protooncogene-inducing effects of epidermal growth factor in hepatoma cell lines.[35]

On the basis of our experimental data and literature reports, an increased density of beta adrenoceptors and an increased activity of adenylate cyclase in HCC might play a role in hepatic carcinogenesis. Regarding beta adrenoceptors, there are data on liver cells from rats treated with carcinogens. In these cells increased cAMP production by stimulation of beta adrenoceptors was found.[15]

The mechanism that accounts for the decreased density of alpha-1 adrenoceptors and the increased density of beta adrenoceptors in tumor tissue from HCC is not clear, but it might be related in some way to different exposure to catecholamines. Since elevated plasma norepinephrine levels have been previously documented in partial hepatectomized rats[10] and in patients with decompensated liver cirrhosis,[36] chronic exposure to high levels of circulating catecholamines and/or an increase in liver sympathetic outflow may provide one plausible explanation for decreased alpha-1 adrenoceptor density. However, this explanation is unlikely since none of the cirrhotic patients evaluated by us was decompensated, as evidenced by the absence of ascites, and it does not explain the downregulation of hepatic alpha-1 adrenergic receptors in tumor tissue from noncirrhotic patients. Moreover, as stated above, we found no differences in hepatic adrenoceptors between cirrhotic and noncirrhotic patients. Therefore an increase in catecholamine levels does not explain the increased density of beta adrenoceptors in HCC. Elevated catecholamines cannot therefore adequately explain our results.

Local factors may also be important in receptor regulation. It is possible that different catecholamine turnover in tumor compared with nontumor tissue may be involved in alpha-1 and beta adrenoceptor regulation in human HCC. Alternatively, since alpha-1 and beta-2 adrenoceptors are distributed heterogeneously on the hepatocyte surface,[37] a loss of polarity of the hepatocyte during neoplastic differentiation might account for our results.

In summary, we found quantitative changes in adrenergic receptors in livers from HCC patients. An increased density of alpha-1 adrenergic receptors in the nontumor part of the HCC liver might predispose the cells to proliferation. The decreased density of alpha 1 adrenoceptors and increased density of beta-2 adrenoceptors in tumor tissue might play a role in the process of hepatic carcinogenesis.

REFERENCES

1. Bucher NLR, Malt MA. Regeneration of liver and kidney. Boston: Little Brown; 1971; 17–176.
2. Smuckler EA, James JL. Irreversible cell injury. *Pharmacol Rev* 1984; 36:775–790.
3. Kew MC. The development of hepatocellular carcinoma in humans. *Cancer Surv* 1986; 5:719–739.
4. Fausto N, Mead JE. Biology of disease: Regulation of liver growth: Protooncogenes and transforming growth factors. *Lab Invest* 1989; 60: 4–13.
5. Goyette M, Petropoulos CJ, Shank PR, Fausto N. Expression of a cellular oncogene during liver regeneration. *Science* 1983; 219:510–513.
6. Goyette M, Petropoulos CJ, Shank PR, Fausto N. Regulated transcription of c-ki-*ras* and c-*myc* during compensatory growth of rat liver. *Mol Cell Biol* 1984; 4:1493–1498.
7. Thompson NL, Mead JE, Braun L, Goyette M, Shank PR, Fausto N. Sequential protooncogene expression during rat liver regeneration. *Cancer Res* 1986; 46:3111–3115.
8. Kruijer W, Skelly H, Botteri F *et al.* Proto-oncogene expression in regenerating liver is stimulated in cultures of primary adult hepatocytes. *J Biol Chem* 1986; 261:7929–7932.
9. McGowan JA. Hepatocyte proliferation in culture. In: Guillouzo A, Guguen-Guillouzo C, eds. Research in Isolated and Cultured Hepatocytes. London: John Libbey, 1986; 14–38.
10. Cruise JL, Knechtle SJ, Bollinger RR, Kuhn C, Michalopoulos G. Alpha 1-adrenergic effects and liver regeneration. *Hepatology* 1987; 7:1189–1194.
11. Cruise JL, Houck KA, Michalopoulos G. Induction of DNA synthesis in cultured rat hepatocytes through stimulation of alpha 1-adrenoceptor by norepinephrine. *Science* 1985; 227:749–751.
12. Cruise JL, Cotecchia S, Michalopoulos G. Norepinephrine decreases EGF binding in primary rat hepatocyte cultures. *J Cell Physiol* 1986; 127:39–44.
13. Houck KA, Cruise JL, Michalopoulos G. Norepinephrine modulates the growth-inhibitory effect of transforming growth factor-beta in primary rat hepatocyte cultures. *J Cell Physiol* 1988; 135:551–555.
14. Morley CGD, Royse VL. Adrenergic agents as possible regulators of liver regeneration. *Int J Biochem* 1981; 13:969–973.
15. Christoffersen T, Berg T. Altered hormone control of cyclic AMP formation in isolated parenchymal liver cells from rats treated with 2-acetylaminofluorene. *Biochim Biophys Acta* 1975; 381:72–77.
16. Lefkowitz RJ, Caron MC. Regulation of adrenergic receptor function by phosphorylation. *J Mol Cell Cardiol* 1986; 18:885–895.
17. Bevilacqua M, Vago T, Norbiato G, Chebat E, Baldi G, Meroni R. Identification and characterization of alpha-1 and beta-2 adrenergic receptors in human liver. *Eur J Clin Invest* 1987; 17:330–335.
18. Neville DM Jr. Isolation of an organ-specific protein antigen from cell-surface membrane of rat liver. *Biochim Biophys Acta* 1968; 154: 540–550.
19. El-Refai MF, Exton JH. Effects of trypsin on binding of ^{3}H-epinephrine and ^{3}H-dihydroergocriptine to rat liver plasma membranes: Evidence for interconversion of binding sites. *J Biol Chem* 1980; 255: 5853–5858.
20. Mahan LC, Insel PA. Use of superoxide dismutase and catalase to protect catecholamines from oxidation in tissue culture studies. *Anal Biochem* 1984; 136:208–216.
21. Salomon Y, Londos C, Rodbell M. A highly sensitive adenylate cyclase assay. *Anal Biochem* 1974; 58:541–548.
22. Scatchard G. The attraction of proteins for small molecules and ions. *Ann N Y Acad Sci* 1949; 51:660–672.
23. Kawai Y, Powell A, Arinze IJ. Adrenergic receptors in human liver plasma membranes: Predominance of beta 2 and alpha 1 receptor subtypes. *J Clin Endocrinol Metab* 1986; 62:827–892.

24. Leatherbarrow R. Enzfitter Manual. Amsterdam: Elsevier, 1987; 54–62.

25. Akaike H. A new look at the statistical model identification. *IEEE Transac Biomed Enging* 1974; AC-19:716–723.

26. Vago T, Bevilacqua M, Norbiato G *et al.* Identification of alpha 1 adrenergic receptors on sarcolemma from normal subjects and patients with idiopathic dilated cardiomyopathy: Characteristics and linkage to GTP-binding protein. *Circ Res* 1989; 64:474–481.

27. Bradford N. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976; 72:248–252.

28. Wallenstein S, Zucker CL, Fleiss JL. Some statistical methods useful in circulation research. *Circ Res* 1980; 47:1–9.

29. Studer RK. Sexual dimorphism in adrenergic regulation of hepatic glycogenolysis. *Am J Physiol* 1987; 252:E467–476.

30. Huerta-Bahena J, Villalobos-Molina R, Corvera S, Garcia-Sainz J. Sensitivity of liver cells formed after partial hepatectomy to glucagon, vasopressin, and angiotensin II. *Biochim Biophys Acta* 1983; 763:120–124.

31. Sandnes D, Sager G, Sand T-E G, Refsnes M, Jacobsen S. Elevated level of beta-adrenoceptors in intact hepatocytes from partially hepatectomized rats. *Pharmacol Toxicol* 1988; 62:199–202.

32. Okajima F, Ui M. Conversion of adrenergic regulation of glycogen phosphorylase and synthase from an alpha- to a beta-type during primary culture of rat hepatocytes. *Arch Biochem Biophys* 1982; 213:658–668.

33. Kunos G, Ishac EJN. Mechanism of inverse regulation of alpha 1- and beta-adrenergic receptors. *Biochem Pharmacol* 1987; 36:1185–1191.

34. Boynton AL, Whitfield JW. The role of cyclic AMP in cell proliferation: A critical assessment of the evidence. *Adv Cyclic Nucleotide Res* 1983; 15:193–294.

35. Squinto SP, Block AL, Doucet JP. Epidermal growth factor induction of cellular proliferation and protooncogene expression in growth-arrested rat H4IIE hepatoma cells: Role of cyclic adenosine monophosphate. *Mol Endocrinol* 1989; 124:433–446.

36. Nicholls KM, Shapiro MD, Van Putten VJ *et al.* Elevated plasma norepinephrine concentrations in decompensated cirrhosis: Association with increased secretion rates, normal clearance rates and suppressibility by central volume expansion. *Circ Res* 1985; 56:457–461.

37. El-Refai MF, Chan TM. Evidence for heterogeneous distribution of alpha 1, alpha 2 and beta-adrenergic binding sites on rat-liver cell surface. *Biochim Biophys Acta* 1984; 775:356–364.