Production of NGF by mouse hepatocellular carcinoma cells and expression of TrkA in tumor-associated arteries

Kan Kishibe [1], Yoshihisa Yamada [2] and Katsuhiro Ogawa [1]

Department of Pathology , Asahikawa Medical College

2-1-1-1 East, Midorigaoka, Asahikawa 078-8510[1] and

First Institute of New Drug Discovery, Ohtsuka Pharmaceutial Co., Tokushima

771-0192 [2], Japan

Running Head: NGF PRODUCTION BY HCC

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Abbreviations: HCC; hepatocellular carcinoma, CM; conditioned medium, NPY; neuropeptide Y

Correspondence: To Katsuhiro Ogawa at the Department of Pathology, Asahikawa Medical College, 2-1-1-1 East, Midorigaoka, Asahikawa 078-8510, Japan. Fax: 81-166-68-2379; E-mail: ogawak@asahikawa-med.ac.jp

Abstract

The microarray analysis has revealed that NGF is specifically elevated in mouse hepatocellular carcinomas. The present study aimed to elucidate expression of NGF and its receptors (TrkA and p75NTR) during hepatic carcinogenesis, regeneration, development and primary hepatocyte culture in mice. Although NGF was negative or very weakly expressed in adult and developing livers, it was markedly elevated in neoplastic hepatocytic lesions from early stages of carcinogenesis. Appreciable levels were also detected in regenerating livers and hepatocyte cultures. The conditioned medium of hepatocellular carcinoma cells caused PC12 neurite outgrowth, but this was reduced with pretreatment of the conditioned medium with the anti-NGF antibody or NGF antisense expression in hepatocellular carcinoma cells. Although neither TrkA nor p75NTR was detectable in either hepatocellular carcinoma or normal hepatic cells, TrkA was demonstrated in the walls of tumor associated arteries that contain abundant nerve fibers. This study demonstrated that NGF is expressed by hepatocytes during carcinogeneis, regeneration and primary culture, but may have cells other than hepatocytes as the target. TrkA expression and abundance of nerve fibers in the walls of tumor-associated arteries suggest some possible role for NGF in angiogenesis.

Introduction

Although the hepatocellular carcinoma (HCC) is one of the most common types of cancer in the world, the molecular mechanisms involved in HCC development are still poorly understood. Since specific transcription or suppression of genes in cancer cells may be closely related to transformed phenotypes, we have compared gene expression patterns between normal livers and HCC in mice using a cDNA array method, allowing quantitation of the expression of many genes.[1] NGF was found to be specifically expressed in HCC together with a number of other genes.

NGF is a prototypical member of the neurotrophin family, which is essential for survival, differentiation and maintenace of neuronal cells. Although NGF is formed by α, β and γ subunit dimers in the mouse salivary gland, it is composed of only α and β subunits in most other tissues.[2, 3] Biological activity of NGF is confined to the β subunit, while the α and γ subunits are thought to be related to processing and/or protection from proteolytic degradation. NGF can interact with two types of membrane binding sites: TrkA and p75NTR, receptively high and low affinity receptors with and without protein kinase activity.[4-6] Binding of NGF to TrkA results in intracellular signaling including the ras/ERK and phosphatidylinositol-3 kinase cascades, leading to differentiation and survival of neuronal cells. p75NTR,

on the other hand, structurally resembling members of the p55 tumor necrosis factor receptor family, activates Jun-N-terminal kinase and ceramide to promote apoptosis.[7-10] Recent evidence has indicated, however, that TrkA and p75NTR can dimerize in response to NGF binding, TrkA homodimers and TrkA-p75NTR heterodimers promoting cell survival, whereas p75NTR homodimers mediate cell death.[11]

In recent years, many findings have indicated that NGF is involved in aspects of tumor biology such as growth, invasion and metastasis. [12-20] TrkA and/or p75NTR as well as NGF have been documented to be expressed in various cancer cells, indicating the existence of autocrine stimulation. There is evidence that the $p185^{HER2}$ receptor kinase, which is encoded by the *c-erb*B2 proto-oncogene and frequently overexpressed in human breast cancer cells, can cooperate with TrkA to activate the MAPK pathway. [21] Furthermore, p75NTR may be downregulated in some cancer cells without any alteration in the amount of TrkA,[17, 19] suggesting a reduction in growth-adverse signal from NGF.

In the present study, we investigated NGF as well as TrkA/p75NTR expression in the mouse liver under various conditions in order to assess the possibility of NGF autocrine and/or paracrine mechanism operating during hepatic carcinogenesis. Included was an examination of the nerve system of the liver during hepatic carcinogenesis.

Materials and Methods

Treatment of Animals

To induce HCC, male B6C3F$_1$ mice (Clea Japan, Tokyo, Japan) were intraperitoneally administered diethylnitrosamine (WAKO, Osaka, Japan) at a dose of 5μg/g body weight at the age of 2 weeks,[22] and maintained thereafter on laboratory chow (Clea) and water *ad libitum*. HCC samples were removed from the surrounding normal liver tissues after 8-12 months, frozen in liquid nitrogen and stored at -80°C until use. For some mice, the livers were perfusion-fixed with buffered formalin solution for immunohitochemical studies. For liver regeneration, B6C3F1 male mice at 10 weeks age were two-thirds hepatectomized, and the livers were removed at various time points after the operation. Normal hepatic tissues at different developmental

stages were also sampled from mice of various ages from the fetus to the adult.

Cell Cultures

For primary cultures, hepatocytes were isolated by the in situ collagenase perfusion method,[23] suspended in 45% Percoll solution (Amersham-Pharmacia, Uppsala, Sweden) and centrifuged for 15 min at 1000 rpm. Cell viability was examined by the trypan blue-dye exclusion test, and samples with more than 90% viability were used for primary culture. The cells were cultivated in Williams'E medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 10^{-7} M epidermal growth factor, 10^{-7} M transferrin, 10^{-7} M insulin, 10^{-7} M dexamethasone, 10^{-5} M aprotinin, 5 units/ml penicillin and 100 μg/ml streptomycin. HCC cell lines were established from primary HCC cell cultures as described elsewhere[24] and cultivated using the same medium as for the primary cultures.

Establishiment of HCC Cell Lines with Expression of Antisense NGF

A 233 bp NGF fragment (nt 72-305, K01759.1 GI:200051) was amplified from mouse HCC cDNA by PCR using a primer set (forward: 5'-AACATGCTGTGCCTCAAGCCAGT-3'

and reverse:5'-CGCAGTGATCAGAGTGTAGAAC-3') and cloned into a mammalian expression vector (pEF6/V5-His-TOPO) using an eukaryotic TA cloning kit (Invitrogen, Carlsbad, CA). The direction of the insert was determined by PCR using primers for the vector and insert sequences, and vectors carrying antisense or sense NGF were transfected into one of the HCC cell lines (HCC1) using Lipofectin (Gibco-BRL). Cells stably expressing the introduced gene were selected with

blastocidine (5 mg/ml), cloned using a cloning ring and expanded. Expression of NGF mRNA was examined by RT-PCR for many clones, and NGF protein levels in the conditioned medium (CM) of cells with and without NGF mRNA expression were further examined by ELISA using an NGF detection kit (Clontec, Polo Alto, CA) according to the manufacturer's instructions.

Isolation of RNA

Messenger and total RNAs were respectively isolated using a FastTrack 2.0 kit (Invitrogen) and the TRIzol reagent (Gibco-BRL) according to the manufacturer's protocol. Total RNA was further treated with DNase I (Message Clean kit; GenHunter, Nashville, TN) to remove contaminating DNA.

cDNA Array Analysis

Mouse cDNA expression array membranes were purchased from Clontech. The mRNA samples were reverse-transcribed into cDNA using a mixture of array gene-specific primers and dNTP including ^{32}P-dATP. The cDNA was purified using Chroma spin-200 DEPC-H_2O columns (Clontech), hybridized to the array membranes according to the manufacturer's manual, exposed to a phosphorimaging screen at room temperature for 30 min and then scanned using BAS2000 (Fuji Photo Film, Tokyo, JAPAN). A grid was applied to the images of hybridization spots, followed by quantification of spot intensity using BAStation ver. 1.31 (Fuji Photo Film) software. Background signals were defined as the average of the hybridization signals produced

by nine negative controls on the array. All hybridization signals were normalized by those of β actin and glyeraldehyde 3-phosphate dehydrogenase.

RT-PCR

Total RNA was reverse-transcribed by the use of an oligo$(dT)_{16}$ primer and unlabeled dNTP. Hot-start PCR was performed using a 1μl aliquot with 0.2 μM of each specific sense and antisense primer (Table1), 200 μM dNTP and 0.25 units of Taq polymerase (Ampli Taq Gold; Perkin-Elmer Applied Biosystems, Foster City, CA) in 20 μl PCR buffer with a first prewarming step at 94°C for 10 min, followed by 25-40 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final elongation step of 5 min at 72°C. The primers for βNGF were designed to amplify all splicing variants forms of βNGF mRNA.[25] The sequence for the mouse TrkA cDNA was based on mouse EST W97049 which shows 93% nucleotide and 97 % amino acid sequence homology to a rat TrkA cDNA.[26] For the internal control, β2 microglobulin cDNA was co-amplified with each sample. The PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

Northern Blot Analysis

Total RNAs were resolved by electrophoresis using denaturing formamide/agarose gels, transferred to Hybond N^+ membranes (Amersham, Buckinghamshire, United Kingdom), and hybridized with α and βNGF cDNA probes labeled with ^{32}P-dCTP (DuPont NEN, Wilmington, DE) using a random priming kit (Takara, Shiga, Japan). After washing by a

standard protocol, the membranes were exposed to X-ray films (Kodak X-OMAT, Roc hester, NY) at -80°C for 2 hr to 1 week.

Immunohistochemistry

Liver tissues perfusion-fixed using a 10 % phosphate-buffered formalin solution were further fixed in the same fixative overnight, processed for embedding in paraffin and cut into 4 μm-thick serial sections. One section was stained with H&E, and the others were used for immunohistochemical studies. Focal hepatocellular lesions were classified into preneoplastic foci, adenomas and carcinomas as described previously.[27] Depraffirized sections were microwaved for 5 minutes twice in acidic citrate buffer (pH 6) before application of anti-βNGF, TrkA and p75NTR (each 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-neuropeptide Y (NPY) antibodies (1:6000 dilution; Dia Sorin, Stilwater, MN). Antibody binding was visualized using a Histofine kit (Nichirei, Tokyo, Japan).

NGF Bioassay

HCC and normal hepatic cells were cultured in the RPMI1640 medium containing 5% fetal bovine serum, 10% horse serum, 5 units/ml penicillin and 100 μg/ml streptomycin for 2 days, and the CM was collected and filtered. PC12 rat pheochromocytoma cells were provided by the Health Science Resources Bank (Osaka, Japan) and cultured for 12 h in 24-well tissue culture plates using RPMI1640 medium without serum, followed by incubation with the CM from HCC cell lines. As a positive control, PC12

cells were incubated with 50 ng/ml mouse NGF (Chemicon, Temecula, CA). A NGF neutralization antibody (Roche, Basel, Switzerland) was used to block the neurite outgrowth reaction. For this purpose the CM was incubated with 1 μg/ml anti-NGF antibody for 1h at 37 C. Random fields were scored for neurite outgrowth 4 days after addition of CM, and a minimum of 500 cells were counted per test well. Cells were considered positive when they had processes extending more than one cell body diameter.

MTT Assay

Changes in the numbers of viable HCC and PC12 cells were examined with MTT assay.[28] The parental, sense and antisense HCC cells were cultivated in WE medium with 10 % FBS without any growth factors. On the other hand, PC12 cells were cultivated in the CM (serum free RPMI1640 medium) of the parental, sense and antisense HCC cells, or serum- or NGF-supplemented RPMI1640 medium. Cells were plated onto 24-well plates, followed by incubation with the MTT reagents for 2 h and subsequent colorimetic analysis at various time points.

Apoptosis Assay

The parental, sense and antisense HCC cells were cultivated with the serum free RPMI medium for 1 day, and the CM was collected. PC12 cells were then cultivated with the CM of HCC cells or the RPMI1640 medium supplemented with or without the serum or NGF for 4

days, removed from dishes by pipetting, pelletted by centrifugation and fixed with the phosphate-buffered 10 % formalin solution containing 1.25 ng Hoechst33248/ml. The cells were spread on slide glasses and observed under a fluorescent microscope. Apoptotic index was determined by examining more than 1000 cells.

Statistics

The data were statistically evaluated with JSTAT software using the two tailed χ^2 test. Significance was concluded with a P value < 0.05.

Results

Expression of NGF in HCC

A survey of differential gene expression patterns between normal liver and HCC tissues by cDNA array analysis identified the α and βNGF to be respectively expressed 15.7 and 4.0 times higher in HCCs than in normal liver (Figure 1a). RT-PCR analysis revealed that, although both α and βNGF were negative after 35 cycles of PCR in normal liver, they were weakly positive after 40 cycles. On the other hand, all of 5 HCC tissues and 6 cell lines were positive for αNGF after 27 and for βNGF after 35 cycles (Figure 1b). DNA sequencing of the PCR products revealed the consistence with mouse α and βNGF (data not shown). No γNGF was detected in any sample (data not shown). Northern blot analysis also detected α and βNGF expression in the HCC tissues, but not in the normal liver (Figure 1c). Immunohistochemical staining revealed βNGF to be positive in all hyperplastic foci, adenomas and HCC with various intensities, but negative in normal hepatic tissues (Figure 1d). The staining was localized in the cytoplasm of tumor cells.

Bioassay of NGF Produced by HCC Cells

To examine for biological activity of NGF, rat PC12 pheochromocytoma cells were incubated with the CM of HCC cells. Neurite outgrowth was observed from the 2nd day of incubation, becoming dominant on the 4th day (Figure 2b, c). All 6 HCC cell lines examined exhibited neurite outgrowth

activity (Figure 2e), equivalent to almost 50 ng/ml NGF in 3 lines and 45-70 % of this in the other 3 lines. Incubation of the CM with the neutralizing NGF antibody markedly reduced the neurite outgrowth activity (Figure 2d, e). However, although neurite outgrowth by NGF itself was reduced to almost the basal level, the degree of inhibition was much less (23-47% remaining) with the CM of HCC cell lines. Neurite outgrowth was also observed with the CM of primary normal hepatic cell cultures 4 days after the start of cultivation (data not shown).

Effect of Antisense NGF on HCC Cells

Suppression of NGF mRNA production was confirmed by RT-PCR in an HCC cell line introduced with the antisense NGF (Figure 3a), and the NGF protein level in the CM was demonstrated to be reduced to 1/8 of that in the parent cells (Figure 3b). Neurite growth assays using PC12 cells revealed reduction of the CM activity of antisense HCC cells to 30 % of that with the original cells (Figure 3c). Interestingly, the viable cell numbers were decreased (Figure 3d), and apoptotic cell numbers were increased, when PC12 cells were treated with the CM of the antisense cells (Figure 3e, f). However, there was no difference in proliferation activity of parental, antisense and sense HCC cells (Figure 3g).

Expression of NGF under Conditions Other than Carcinogenesis

To explore the possibility that NGF may be expressed under conditions other than hepatic carcinogenesis, developing and regenerating livers, and primary hepatocyte cultures were examined. RT-PCR

analysis demonstrated no detectable βNGF in the livers of fetus (18 ED) and newborn (0-14 days), although the adult (8 weeks) liver was positive with 40 cycles of PCR (Figure 4a). βNGF was detected by 35 cycles of PCR 2 days after two-thirds hepatectomy at least until the 10 day time point (Figure 4b) and was also expressed in primary cultured normal hepatocytes on the 2nd day after cultivation with a peak on the 4th day (Figure 4c).

Expression of TrkA in Tumor-Associated Arteries

RT-PCR analysis of TrkA and p75NTR did not detect any expression in normal livers, HCC tissues or cell lines (Figure 5a), although mouse brain tissue was positive. [29] The receptors were also not detected in regenerating livers (Figure 5b). Immunostaining for TrkA and p75NTR did not detect any positivity in the normal liver. On the other hand, thickened Glisson's sheaths including veins, arteries and interlobular bile ducts were frequently observed at the edges or periphery of hepatic tumors (Figure 5c). This feature was apparent in HCC and large adenomas, and was less prominent or absent in foci and small adenomas. Immunohistochemical staining demonstrated TrkA exclusively in the walls of arteries associated with tumors, presumably in smooth muscle cells, whereas it was negative in other cell types including tumor cells (Figure 5c, d). p75NTR was generally negative in hepatic tumors (data not shown).

Increase in Nerve Fibers in Blood Vessels Adjacent to HCC Tissues

To investigate the possibility that NGF produced by HCC cells may promote the innervation of HCC tissues, immunohistochemical NPY staining was performed to visualize nerve fibers. In the normal liver, NPY positive fibers were observed in the Glisson's sheath, mainly associated with the walls of hepatic arteries with a few in the walls of portal vein and interlobular bile ducts (Figure 6a). No NPY positivity was detected within the parenchyma of hepatic lobules and around the terminal hepatic veins. On the other hand, blood vessels, especially arteries, associated with large adenomas and HCC contained abundant NPY positive nerve fibers, although nerve fibers were generally not detectable in the tumor parenchyma (Figure 6b, c).

Discussion

This study demonstrated, to our knowledge for the first time, that NGF is expressed in mouse HCC tissues and cell lines at much higher levels than in the normal liver. Moreover, immunostaining demonstrated NGF to be expressed not only in HCC, but also in early preneoplastic lesions such as foci and adenomas, indicating this to be a very early change during mouse hepatic carcinogenesis.

Production of NGF by HCC cells was further confirmed by the neurite outgrowth reaction of PC 12 cells with CM. The activity was variable among individual cell lines, and, although the neurite outgrowth activity of pure NGF was almost completely inhibited by the neutralizing anti-NGF antibody, this was not the case with the CM, indicating the possible existence of other factors that can promote neurite outgrowth. Since other factors such as FGF[30-32] and IL-6[33] have the ability to promote neurite outgrowth of PC12 cells, their presence in HCC cell CM should be investigated.

The RT-PCR analysis revealed NGF expression to be also elevated in regenerating livers from 2 days after two-thirds resection, consistent with the report of Nemoto et al.[34] who demonstrated the NGF levels as well as other neurotophins and their receptors to be increased in lead nitrate-induced rat liver hyperplasia. However, in the present study, no NGF was detected in the livers of fetal and newborn mice. Since hepatic cells are proliferating in the fetal and newborn periods more rapidly than in adults, NGF

expression may thus not simply be a marker for hepatic cell division. NGF was also elevated in primary cultures of normal hepatic cells from 2 days after the start of cultivation, in line with the shared characteristics with hepatic tumor cells.[35] NGF expression may thus be a kind of adaptive reaction of hepatic cells to the culture conditions.

Expression of the NGF receptors, Trk A and p75NTR, as well as NGF has been reported in various cancers, suggesting that the NGF autocrine or paracrine pathway may have a role in tumorigenesis.[12-20] However, the pres ent study failed to demonstrate either receptor at detectable levels in HCC tissues or cell lines. Furthermore, there was no change in the proliferating activity of HCC cells with suppression of NGF expression by the antisense NGF. It is therefore conceivable that NGF produced by HCC cells may not be directed of the tumor cell themselves but rather other liver components.

TrkA expression in the arterial walls associated with HCC and large adenomas suggests that arterial smooth muscle cells are a possible target of NGF. Expression of TrkA in vascular walls has been documented in various tissues.[36-38] Although normal hepatic tissues are mainly supplied with blood from the portal vein, hepatic tumors receive their blood predominantly through the hepatic artery, indicating a switch during hepatic carcinogenesis. [39] On the other hand, it has been suggested that NGF plays an important role during wound healing. Topical application to sites of wounding accelerates healing by thickening of granulation tissues as well

as reepithelization,[40] and NGF expression is elevated in various cells such as keratinocytes, fibroblasts and endothelial cells.[41-43] Since angiogenesis is a hallmark of granulation tissues, NGF may have a role for vascular growth as well as other growth factors.[44]

The finding of abundant nerve fibers mainly associated with the walls of tumor arteries is also of interest. It has been reported that granulation tissues during wound healing are associated with increased innervation mainly along the blood vessels.[41-43] Furthermore, neuronal cells can produce angiogenic factors such as VEGF[45] and NPY, [46] the latter having the potential to promote vessel sprouting, endothelial cell adhesion, migration and proliferation, and capillary tube formation.[47] Transcription of NPY is upregulated in PC 12 cells by treatment with NGF.[48] Furthermore, vascular endothelial and smooth muscle cells express the NPY receptor together with its activating enzyme (dipeptidyl peptidase IV).[46, 48] It is thus possible that NGF produced by HCC cells may contribute to increase of nerve fibers which may be also related to the development of tumor arteries.

Proliferation of hepatic cells is known to be influenced by the nervous system. Nerve fibers are increased in number and size, mainly in association with the interlobular hepatic arteries, after partial hepatectomy,[49] and bilateral vagotomy markedly inhibits hepatic regeneration.[50] Our observation that NGF is elevated in livers after partial hepatectomy suggests that this factor has a role in the development

of nerve fibers which may contribute to regeneration of the vasculature. However, we found no nerve fibers in the tumor parenchyma, consistent with previous electron microscopic and immunohistochemical studies of primary and metastatic tumors in man and rodents.[51] This suggests some inhibitory mechanism against innervation presumably by tumor cells themselves or associated vascular elements. Decrease in the cell number in association with occasional apoptosis in PC12 cells by treatment with the CM of HCC cells in which NGF was suppressed by the antisense NGF suggests the possibility that apoptotic factors are produced together with neurotrophic factors by HCC cells, although further studies are needed to this context.

Another possible target of the NGF produced by HCC cells may be hepatic stellate cells, because they express p75NTR which mediates apoptotic signals from NGF.[52, 53] As hepatic stellate cells can produce growth inhibitory factors for HCC cells such as TGFβ [54, 55] and HGF,[56, 57] it is possible that NGF could exert an influence on hepatic stellate cells.

In conclusion, the NGF expression is an early change during mouse hepatic carcinogenesis. However, failure of detection of NGF receptors in HCC cells suggests that NGF may not be an autocrine factor, but rather may act as a paracrine factor. Role(s) of NGF produced by HCC cells require further investigation according to biological processes during carcinogenesis such as angiogenesis, nerve development and apoptosis.

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Figure Legends

Figure 1

Expression of NGF by HCC. a) cDNA array analysis. αNGF (solid arrowheads) is present in an HCC at 15.7 times the normal liver level, and βNGF (open arrowheads) at 4.0 times. b) RT-PCR analysis of α and βNGF. Both proved detectable in all HCC tissues and cell lines, but not normal liver, by 35 cycles of PCR. β2-m, β 2-microglobulin as an internal control. c) Northern blot analysis of α and βNGF. Expression is apparent in HCC tissue, but not in normal liver. d) Immunohistochemical staining of βNGF. βNGF is positive in adenoma cells, but negative in the surrounding normal liver.

Figure 2

Bioassay of NGF produced by HCC cells using PC12 cells. Although PC12 cells showed only a few neurites without NGF (a), they demonstrated prominent neurite outgrowth after treatment with NGF (b) or the CM of HCC cells (c). However, when the CM was treated with the neutralizing anti NGF antibody, the neurite outgrowth was markedly inhibited (d). e) Neurite outgrowth in PC12 cells with the CM of 6 HCC cell lines. Although the activity of pure NGF was almost completely inhibited by the neutralizing anti-NGF antibody, the degree of inhibition was less prominent with the HCC cells. Open columns: CM without treatment with the anti-NGF antibody; and closed columns: CM with the antibody treatment.

Figure 3

Establishment and properties of HCC cells introduced with antisense NGF. a) RT-PCR for βNGF mRNA. Although the parental (HCC) and sense cells (S) express βNGF mRNA, the antisense cells (AS) are negative. b) The NGF level in the CM of antisense cells (AS) is markedly reduced as compared to the parental (HCC) and sense cells (S). WE; Williams' E medium supplemented wih 10 % fetal bovine serum. c) Neurite outgrowth activity of antisense cells (AS) is markedly decreased as compared to the parental (HCC) and sense cells (S). d) Changes in the numbers of viable PC12 cells after treatment with the CM of parental (HCC), antisense (AS) and sense cells (S) relative to the number at the start of cultivation. The number of viable PC12 cells is decreased in the antisense case. e) Although apoptotic cells are rare in PC12 cells treated with parental HCC cell CM (above), they are frequently seen in the PC12 cells treated with the antisense cell CM (arrows; lower). Hoechst33248 staining. f) Apoptotic index of PC12 cells is increased by cultivation with serum free RPMI medium (-) as compared to the medium supplemented with serum (+) or NGF. However, the apoptotic index is much increased when PC 12 cells were treated with the antisense cell CM (AS). Parental HCC cell CM (HCC), and S sense cell CM (S). g) Growth curves of the parental (HCC), antisense (AS) and sense cells (S). Note the lack of any difference between them.

Figure 4

RT-PCR analysis of βNGF expression in developing and regenerating livers, and cultured hepatocytes. a) βNGF is weakly positive in adult liver after 40 cycles of PCR,

but is not detectable in fetal and newborn livers. b) βNGF is detectable in regenerating livers from the 2nd day after two-thirds hepatectomy at least until the 10th day. c) Primary cultured hepatocytes also express βNGF from 2 days after the start of cultivation with a peak on the 4th day.

Figure 5

RT-PCR for TrkA and p75NTR (a, b) and immunostaining for TrkA (c, d). Neither receptor is evident in HCC tissues and cell lines (a) or regenerating liver (b), although both are detectable in the brain. c) TrkA is expressed in the wall of HCC artery, but not in the wall of portal vein (P) or bile duct (B). N; normal liver, H; HCC. d) Higher magnification of c (*). Presumaby smooth muscle cells in the arterial wall show the TrkA staining.

Figure 6

NPY immunostaining in the normal liver and HCC tissue. Brown dots represent NPY positive nerve fibers. a) In the normal liver, only a few nerve fibers are evident in the portal tract. Portal vein (P), bile duct (B) and artery (A). b) In HCC (H), nerve fibers are thick and abundant in the vessel walls, especially at the wall of artery.

Table 1. Sequences of the PCR primers for NGF, TrkA, p75NTR and β2-microglobulin

Targents	Sense primers	Antisense primers	PCR	
			Product size (*bp*)	Cycles
α-NGF	CAGCTGCCCACTGCTATAAC	TGTAGACACTTGGCTCAGTG	546	27
NM-010915	(nt185-204)	(nt711-730)		
β-NGF	GACGCAGCTTTCTATACTGG	GTTAATGTTCACCTCGGCCA	540	35
K01759	(nt231-250)	(nt751-770)		
γ-NGF	CAAGGATGAACCATCTGCTCAG	TGGTGTAGACGCCTGGCATATCA	491	40
X01389	(nt260-281)	(nt728-750)		
TrkA	TGGAGCAGCATCATTGGTGC	CCATAAAAGCAGCCATGACG	261	40
W97049	(nt110-132)	(nt351-370)		
p75NTR	TGCAGAGTATGTCCGCTCCCTGT	AGCAGCCAAGATGGAGCAATAGAC	509	40
AF105292	(nt282-304)	(nt767-790)		
β2-M	CTTCAGTCGTCAGCATGGCT	ATGCTTGATCACATGTCTCG	382	25
NM-009735	(nt39-58)	(nt491-420)		

NOTE. The sequences and numbers of nucleotide position of the primers refer to the GenBank data base (http://www.ncbi.nlm.ncbi.nlm.nih.gov:80/Database/index.html). The number below the gene name is its GenBank accession number.

Fig. **1**

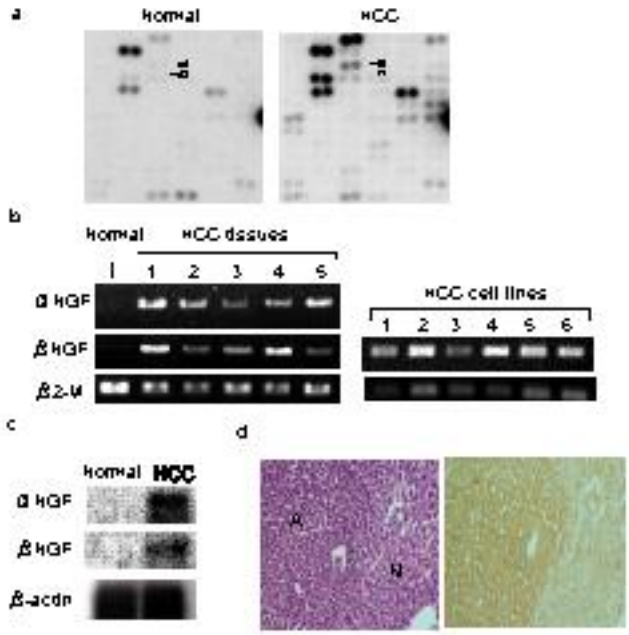
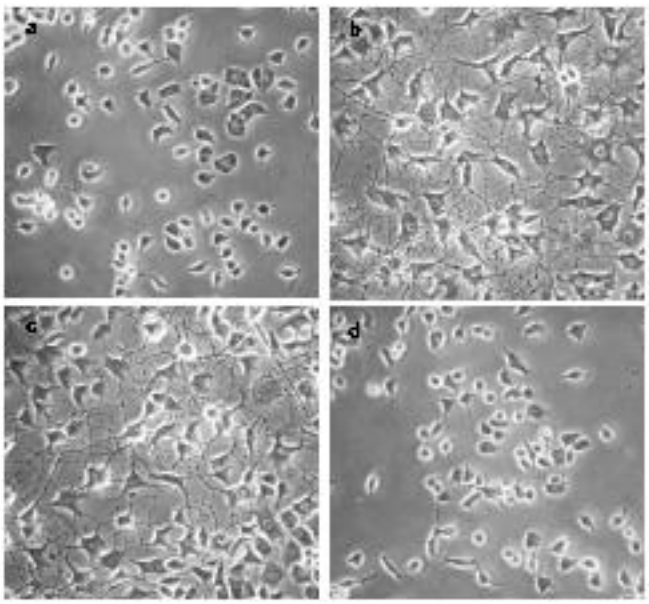

a
Normal
HCC
b
Normal
HCC tissues
1
1
2
3
4
5
α hGF
β hGF
β2-M
HCC cell lines
1
2
3
4
5
6
c
Normal
HCC
α hGF
β hGF
β-actin
d
A
N

Fig. 2



e

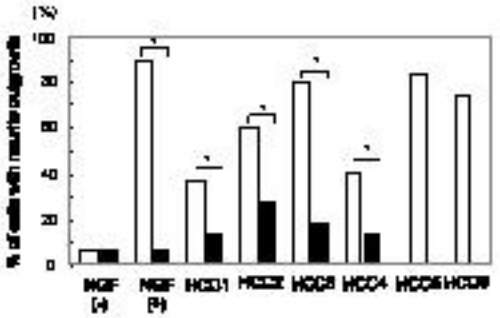


Fig. 3

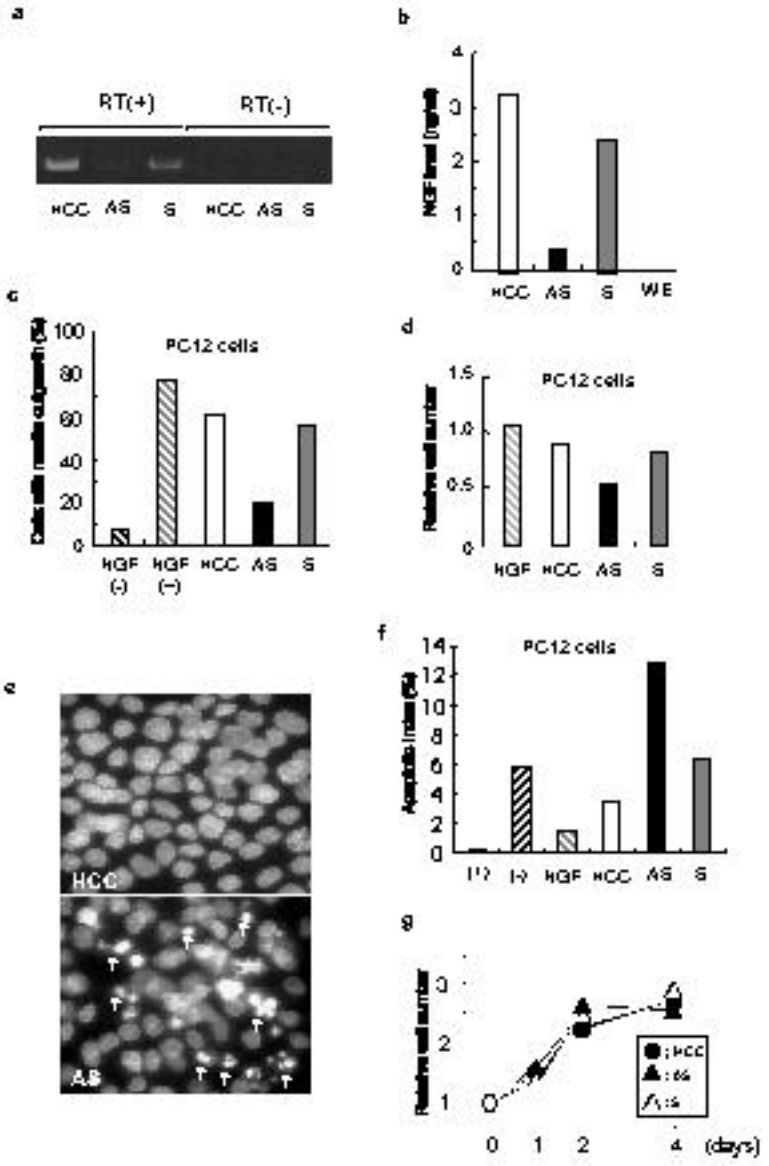

a
RT(+)
RT(-)
HCC AS S HCC AS S
b
HCC AS S WE
c
PC12 cells
HGF (-)
HGF (+)
HCC AS S
d
PC12 cells
HGF HCC AS S
e
HCC
AS
f
PC12 cells
HGF HCC AS S
g
HCC
0 1 2 4 (days)

Fig. 4

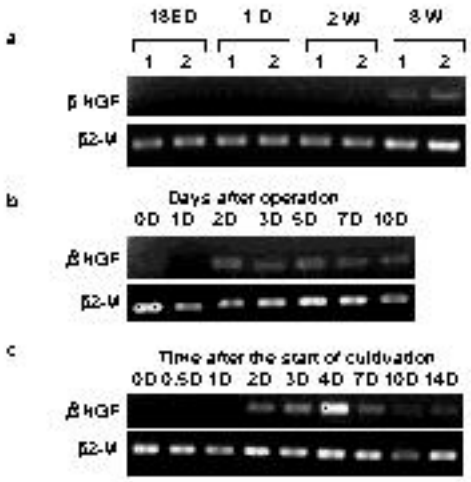

a
18ED
1 D
2 W
8 W
1 2 1 2 1 2 1 2
β HGF
β2-M
b
Days after operation
0D 1D 2D 3D 5D 7D 10D
β HGF
β2-M
c
Time after the start of cultivation
0D 0.5D 1D 2D 3D 4D 7D 10D 14D
β HGF
β2-M

Fig. 5

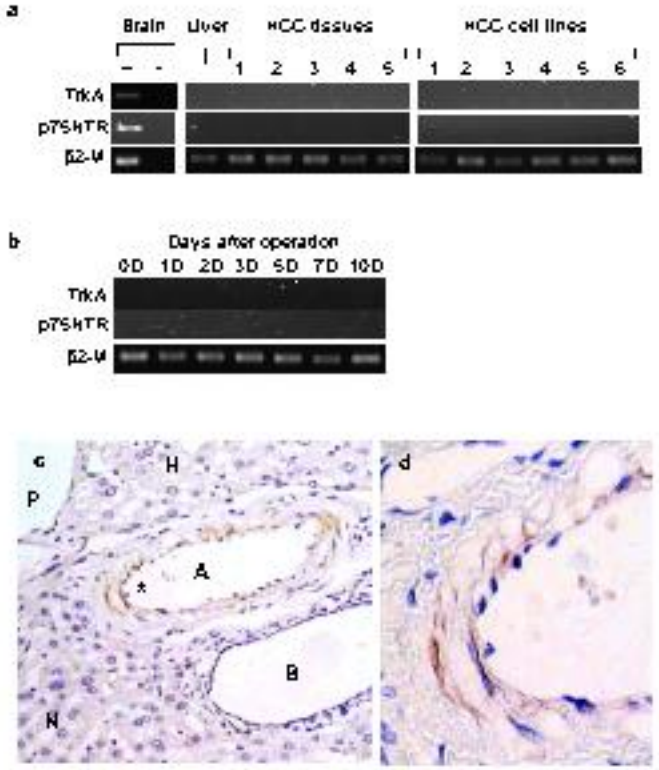

a
Brain
Liver
HCC tissues
HCC cell lines
1 2 3 4 5
1 2 3 4 5 6
TrkA
p75NTR
β2-M
b
Days after operation
0D 1D 2D 3D 5D 7D 10D
TrkA
p75NTR
β2-M
c
H
P
A
*
B
N
d

Fig. 6