Identification of Brain-Derived Neurotrophic Factor as a Novel Functional Protein in Hepatocellular Carcinoma

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Abstract

This study aims to identify a novel molecule that may contribute to hepatocarcinogenesis in a rat orthotopic hepatocellular carcinoma model. The hepatocellular carcinoma model was generated by injection of tumor cells into the left lobe of the liver. Proteomic approaches, including ProteinChip and two-dimensional electrophoresis, were used to identify proteins from serially collected rat serum samples. By both ProteinChip and two-dimensional electrophoresis techniques, the level of a 27-kDa protein was found to be augmented in serum samples during tumor development, decreased after left lobectomy, and reincreased at the time of tumor recurrence. The protein was identified to be brain-derived neurotrophic factor (BDNF). By using specific primers and monoclonal antibody, the expression pattern of BDNF was confirmed in tumor tissue but not in the adjacent nontumorous liver tissue. In addition, the truncated isoform of BDNF receptor-tyrosine protein kinase receptor B was only found in tumor tissue. An in vitro study showed that exogenous BDNF could induce tumor cell proliferation predominantly in relatively small numbers of inoculated cells. Administration of BDNF to tumor cell lines induced significantly increased expression of heat shock protein 90 (Hsp90) and cyclin D1, and blocking the activity of Hsp90 could reverse the up-regulation of cyclin D1 induced by BDNF. The present study revealed that BDNF and its receptor were uniquely expressed in tumor tissue and cell lines of hepatocellular carcinoma but not in nontumorous liver tissue and normal cell line. BDNF could stimulate tumor cell proliferation in a Hsp90-dependent manner. (Cancer Res 2005; 65(1): 219-25)

Introduction

The incidence of hepatocellular carcinoma (HCC) is increasing worldwide because of the spreading of hepatitis B and C virus infection (1). Hepatectomy and liver transplantation are the only two approaches that may cure HCC, but the 5-year survival rate is mainly dependent on the stage of tumor at the time of diagnosis (2–4). The majority of patients with HCC presented with an advanced stage beyond surgical treatment. In addition, chemotherapy and radiotherapy have limited efficacy in hepatocellular carcinoma of an advanced stage (5–7). As the pathogenesis of HCC remains largely unclear, exploring the mechanisms of hepatocarcinogenesis may benefit the diagnosis and therapy of this disease.

Proteomic approaches, such as two-dimensional electrophoresis (8, 9) and ProteinChip (10, 11), provide a possibility to identify novel proteins from tissue, serum, and even urine samples. The major advantages of ProteinChip are its timesaving characteristic and the capacity of comparing serial samples at the same time, which overcome the drawbacks of two-dimensional electrophoresis, whereas two-dimensional electrophoresis makes it possible to pick protein spots of interest from the gel and identify them by mass spectrometry. Therefore, by combining these two techniques, a higher efficiency to identify novel proteins can be achieved.

As the syngeneic tumor model generated in Buffalo rat (12, 13) could reflect the homogeneous pathophysiology of HCC, the genetic and molecular alterations identified by this model might benefit the exploration of hepatocarcinogenesis in human beings. Therefore, we designed a study to identify the proteins that might be related to tumor development and recurrence in the rat orthotopic HCC model.

Materials and Methods

Orthotopic Hepatocellular Carcinoma Model in Rat Liver

Male Buffalo rats weighing 250 to 300 g were purchased from the Charles River Labs (Wilmington, MA). They were maintained under standard conditions and cared for according to the institutional guidelines for animal care. All the animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research, University of Hong Kong. McA RH7777 rat HCC cell line (CRL-1601) was purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained as monolayer culture in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin (Life Technologies, Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO2 in air. A total of 1 × 105 cells were resuspended in 0.2 mL 1× PBS and injected into the left lobe of the liver. To continuously monitor protein alterations during tumor development, serum samples (100 μL) were collected on days 0, 3, 7, 9, 14, 21, and 28 after cell inoculation from the tail veins of the same rats at different time points (five samples for each time point). Another 15 rats were sacrificed on days 0, 7, 14, 21, and 28 (3 rats for each time point) for tissue collection. In addition, 10 other animals were anesthetized with sodium pentobarbital and underwent left lobectomy on day 9 after tumor cell inoculation. Serum samples were collected from the tail veins on day 9 before lobectomy, day 16 (7 days after lobectomy), and day 36 (27 days after lobectomy). The tissue samples were collected from the removed tumor-bearing lobe. When the animals were killed, half of the tumor and adjacent nontumorous liver tissues were fixed in 10% buffered formalin and embedded in paraffin; another half were frozen and stored at −80°C for protein and RNA extraction. The paraffin-embedded tissue was cut into 5-μm-thick sections for histologic studies by H&E staining. Brain, kidney, spleen, heart, lung, and liver tissues were also collected from normal Buffalo rats for RNA extraction.

Proteomic Analysis

ProteinChip. All the reagents for ProteinChip were purchased from Sigma-Aldrich (St. Louis, MO). Rat serum samples were analyzed on H50
ProteinChip arrays that bound proteins through reversed phase or hydrophobic interaction. Five microliters of each rat serum sample were diluted into 5 μL of 9 mol/L urea, 2% CHAPS, and 50 mmol/L PBS (pH 7.2). The H50 ProteinChip array was equilibrated twice with H50 binding buffer (10% acetonitrile, 250 mmol/L NaCl in PBS). After incubation, each serum sample was further diluted with the H50 binding buffer, and 20 μL of each sample were applied to the H50 ProteinChip array surfaces and incubated for 1 hour at ambient temperature. The arrays were washed thrice with the binding buffer for 5 minutes with shaking followed by a final high-performance liquid chromatography-grade water wash. The ProteinChip array was air-dried. Two microliters of a saturated sinapinic acid solution in 50% acetonitrile and 0.5% trifluoro-acetic acid were added to each spot of the ProteinChip array. The chip arrays were analyzed by a PBS II C reader of the ProteinChip Biomarker System (Ciphergen BioSystems, Inc., Fremont, CA). All mass spectra were normalized to have the same total ion current and analyzed by the Biomarker Wizard function of the ProteinChip software version 3.1 (Ciphergen Biosystems).

**Two-dimensional Electrophoresis.** All the reagents for two-dimensional electrophoresis were purchased from Amersham Biosciences (Little Chalfont, United Kingdom) and Sigma-Aldrich. Aliquot of 63 μL rat serum was mixed with 10 μL buffer containing 10% SDS and 2.3% DTT. The mixture was heated at 95°C for 5 minutes followed by a dilution to 500 μL with another buffer (9 mol/L urea, 4% CHAPS, 35 mmol/L Tris, 65 mmol/L DTT, and bromophenol blue). Sixty microliters of final diluted serum sample were then loaded with 390 μL rehydration solution containing 8 mol/L urea, 2% CHAPS, 0.5% immobilized pH gradient buffer, 40 mmol/L DTT, and 0.002% bromophenol blue. Rehydration was allowed to take place in ceramic strip holders for 12 hours at 30 V. Isoelectric focusing was carried out with IPIphor (Amersham Biosciences). The strips were subsequently equilibrated with 10 mL buffer (50 mmol/L Tris-HCl, 6 mol/L urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue) containing 1% DTT for 15 minutes with gentle shaking. Another 15-minute equilibration was done with the buffer containing 2.5% iodoacetamide. After transferring the strips onto 1-mm-thick 12.5% precast Ettan DALT gel, they were sealed with 0.5% agarose. Two-dimensional electrophoresis was carried out at a constant current of 10 mA per gel overnight using Ettan DALT working with connecting to a MultiTemp III thermostatic circulator (Amersham Biosciences) setting at 10°C. Gels were then stained with protein silver stain kit following the manufacturer’s protocol. Gel images were captured by a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA), and the digital images were analyzed using the PDQuest software version 7.1 (Bio-Rad).

**In-Gel Trypsin Digestion and Mass Spectrometry.** A protein spot that was identical in molecular weight in both ProteinChip and two-dimensional electrophoresis with an increasing pattern during tumor development was excised from the gel, destained, and digested by trypsin. The peptide masses were determined by a matrix-assisted laser desorption ionization-time of flight mass spectrometer (Voyager-DE STR Biospectrometry Workstation, Applied Biosystems, Foster City, CA). Peptide mass lists were searched in online databases including National Center for Biotechnology Information and SwissProt, with two online programs: MS-FIT (http://prospector.ucsf.edu) and Mascot (http://www.matrixscience.com).

**Reverse Transcription-PCR and Western Blot**

Total RNA was extracted from the snap-frozen tissue using RNeasy Mini kit (Qiagen, Inc., Valencia, CA). Reverse transcription-PCR (RT-PCR) was used to detect brain-derived neurotrophic factor (BDNF) and tyrosine protein kinase receptor B (trkB) mRNA expression. Primer sequences for rat BDNF and extracelular truncated and intracellular isoforms of trkB were designed according to Tokuyama et al. (14). Primer sequences for human BDNF were designed according to Noga et al. (15). The protein level of BDNF was determined by standard Western blot protocol using 12% SDS-PAGE gel. The
mouse monoclonal anti-human BDNF antibody was purchased from Abcam Ltd. (Cambridge, United Kingdom).

**In vitro Studies**

**Cell Culture.** The *in vitro* studies were done using four cell lines, including CRL-1601 rat HCC, CRL-1439 normal hepatocyte, PLC, and HepG2 human HCC cell lines. The CRL-1601, PLC, and HepG2 cell lines were maintained with 10% FBS-DMEM, whereas CRL-1439 cell lines were cultured with 10% FBS-F-12K.

**Western Blot.** The four cell lines were treated with 2.5% FBS alone or 2.5% culture medium with 25, 50, or 100 ng/mL human BDNF, respectively, for 24 or 48 hours. In addition, the cells were treated by the above different doses of BDNF combined with 1 µg/mL heat shock protein 90 (Hsp90) inhibitor geldanamycin (Sigma-Aldrich), respectively, for 24 hours. The cells were then harvested and total protein was extracted. The protein levels of BDNF, Hsp90, and cyclin D1 were determined by the standard Western blot protocol. Antibodies were purchased from Abcam Ltd., Stressgen Biotechnologies (Palo Alto, CA), and Cell Signaling Technology, Inc. (Beverly, MA), respectively.

**Cell Proliferation Assay.** Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Different numbers of cells from CRL-1601, CRL-1439, PLC, or HepG2 cell lines (5 × 10³, 1 × 10⁴, and 2 × 10⁴) were inoculated in each well of a 96-well culture plate, respectively. The cells were then cultured with 2.5% FBS-DMEM alone or 2.5% FBS-DMEM with 25, 50, or 100 ng/mL human BDNF (Calbiochem, San Diego, CA), respectively, for 24 or 48 hours before MTT was added. The four cell lines also received a combined treatment by different doses of BDNF and geldanamycin, respectively, for 24 hours. After incubation with MTT for 4 hours, the reaction was stopped with 0.04 mol/L HCl (in isopropanol) and measured at A₅₇₀-₆₃₀ nm in a V₂₅ kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA). The cell number was calculated based on a standard curve set up by serial dilutions of a known number of cells at the time of harvest and expressed as mean ± SD.

**Results**

A 27-kDa Serum Protein Was Augmented with Tumor Development, Decreased after Tumor Removal, and Reappeared at the Time of Tumor Recurrence.

In the present study, we induced a rat orthotopic HCC model and serially collected serum samples during tumor development. All animals that received tumor cell injection developed visible tumor nodules. Figure 3. Confirmation of BDNF expression in the rat tumor and nontumorous liver tissues by RT-PCR and Western blot. Both mRNA and protein expression of BDNF was detected in tumor tissue, with a gradually increasing trend during tumor development (days 0–28). mRNA and protein of BDNF were also identified in the recurrent tumor tissue (day 27 after left lobectomy). On the contrary, the nontumorous liver tissue had an undetectable level of BDNF.

**Figure 4.** Determination of BDNF expression in HCC tumor cell lines and normal cell line by RT-PCR and Western blot. It was found that all the three tumor cell lines [one rat HCC cell line (CRL-1601) and two human HCC cell lines (PLC and HepG2)] expressed a relative lower level of BDNF when no treatment was given. However, when exogenous BDNF was administered, the expression of BDNF was significantly up-regulated. In the normal cell line (CRL-1439), there was no noticeable change of endogenous BDNF expression after exogenous BDNF stimulation.

**Statistical Analysis**

Comparison of the cell number between cell lines with or without treatment was done using Student’s *t* test (SPSS 10.0 for Windows, SPSS, Inc., Chicago, IL). *P* < 0.05 was considered statistically significant.

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**Results**

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from day 7 (Fig. 1) and survived to 28 days (the longest observation time). All animals recovered from operation. There were no visible tumor nodules in the remaining liver lobes after left lobectomy. On day 27 after left lobectomy, 6 of the 10 rats presented with visible recurrent tumors. By ProteinChip technique, six serum proteins were found to be up-regulated during tumor development, decreased after lobectomy, and reincreased at the time of tumor recurrence, within which a 27-kDa protein showed a similar expression pattern that was identified by two-dimensional electrophoresis (all the five serum samples at each time point presented with the same pattern; Fig. 2). After mass spectrometry analysis and database searching, the protein was found likely to be BDNF.

BDNF Was Prominently Expressed in Tumor Tissue. RT-PCR and Western blot were done to confirm BDNF mRNA and protein expression in tumor and nontumorous liver tissues. It was found that both mRNA and protein levels of BDNF were highly expressed in tumor tissue, whereas they were undetectable in nontumorous liver tissue. In addition, the levels of BDNF mRNA and protein were increased with tumor development and were also present in the recurrent tumor (Fig. 3).

Expression of BDNF in Tumor Cell Lines Was Enhanced by Exogenous BDNF Stimulation. The rat and two human HCC cell lines initially expressed a low level of BDNF mRNA and protein. However, after culture with BDNF, significantly increased expression of BDNF was detected in all the three tumor cell lines but not in the normal cell line. In addition, the expression levels of BDNF in the three tumor cell lines increased in a dose-dependent manner with exogenous BDNF stimulation (Fig. 4).

Tumor Tissue Expressed a Higher Level of Truncated Isoform of BDNF Receptor-trkB. By RT-PCR, it was found that the tumor tissue highly expressed BDNF receptor-trkB mRNA, and the levels increased with tumor development. Although both tumor and nontumorous liver tissues expressed intracellular isoform of trkB, a higher level was detected in the tumor tissue. Interestingly, the truncated isoform of trkB was only detected in the tumor tissue but not in the nontumorous liver tissue (Fig. 5).

Exogenous BDNF Stimulated Up-Regulation of Cell Cycle–Related Molecules in Tumor Cell Lines. Hsp90 (16) and cyclin D1 (17) were two molecules that were involved in cell cycle modulation. With BDNF stimulation, significantly increased expression of Hsp90 and cyclin D1 was detected in all the tumor cell lines but not in the normal cell line. On the other hand, BDNF did not affect the Hsp90 level in the normal cell line.

Exogenous BDNF Induced Tumor Cell Proliferation. Different numbers of cells from the four cell lines were cultured with various doses of BDNF for 24 or 48 hours. The number of tumor cells increased with BDNF stimulation. A higher dose of BDNF did not further enhance the expression of these molecules (Fig. 6).

**Figure 5.** Detection of BDNF receptor-trkB expression in tumor and nontumorous liver tissues by RT-PCR. Truncated isoform of trkB was uniquely expressed in tumor tissue during the time of tumor development and at the time of tumor recurrence, whereas both tumor and nontumorous liver tissues expressed the intracellular isoforms of trkB, with a higher level in the tumor tissue.

**Figure 6.** Determination of Hsp90 and cyclin D1 expression in tumor and normal cell lines after exogenous BDNF stimulation by Western blot. Significantly higher level of Hsp90 and cyclin D1 expression was detected in all the three tumor cell lines after BDNF stimulation for 24 hours, although the level of Hsp90 and cyclin D1 did not correlate with the doses of BDNF. On the other hand, BDNF did not affect the Hsp90 level in the normal cell line.
cells was significantly increased after a 24-hour culture with BDNF at the doses of 25 or 50 ng/mL, with the highest number observed at the dose of 50 ng/mL. However, a longer incubation time (48 hours) did not further increase the number of cells. Interestingly, the stimulatory effects of BDNF were prominent with the relatively lower numbers of inoculated cells ($5 \times 10^5$ and $1 \times 10^6$) but not with a higher number of cells ($2 \times 10^6$). On the other hand, there were no obvious changes in the normal cell line with BDNF stimulation (Fig. 7).

Blockade of Hsp90 Activity Suppressed BDNF-Induced Tumor Cell Proliferation. When Hsp90 inhibitor geldanamycin was added to the CRL-1601 cell line with BDNF, the proliferation activities of tumor cells were significantly inhibited. A similar pattern was also observed in the two human HCC cell lines. However, Hsp90 inhibition did not affect the cell number in the normal cell line (Fig. 7). By Western blot, it was found that the protein levels of cyclin D1, especially the up-regulated cyclin D1 expression induced by BDNF, were obviously reduced by Hsp90 inhibition in all the three tumor cell lines but not in the normal cell line.

Expression of BDNF in Normal Tissues. The expression of BDNF mRNA in normal rat brain, kidney, spleen, heart, lung, and liver was determined by RT-PCR. BDNF mRNA was found to be highly expressed in brain and spleen and moderately expressed in kidney, heart, and lung but was undetectable in liver (Fig. 8).

Expression of BDNF mRNA in Human Hepatocellular Carcinoma, Cirrhotic, and Normal Liver Tissues. The mRNA levels of BDNF in human HCC, cirrhotic, and normal liver tissues were detected by RT-PCR. A higher level of BDNF mRNA was detected in HCC tumor tissue than adjacent nontumorous (60 cases), cirrhotic (transplantation recipients, 20 cases), and normal liver tissues (liver donors, 7 cases; Fig. 9).

Discussion

In the present study, we used a stable orthotopic rat HCC model. All rats developed visible nodules in the left lobe of the liver on day 7 after tumor cell injection. We serially collected serum samples from the same rats at different time points during tumor development, identified novel proteins, and confirmed their expression in a different set of tissue samples. By ProteinChip technique, we monitored a serum protein with expression gradually up-regulated during tumor development, down-regulated after tumor removal, and re-elevated at the time of tumor recurrence, indicating that this protein was closely related to tumor pathogenesis. We then identified this protein from rat serum by twodimensional electrophoresis and mass spectrometry and found it to be BDNF. Using specific primers and monoclonal antibody, the expression pattern of BDNF was confirmed in the tumor tissue.

BDNF, one member of the nerve growth factor family, plays an essential role in regulating survival, differentiation, and activities of neurons through synaptic modulation (18, 19). BDNF was also found to be expressed in neuroblastoma (20), and the presence of BDNF could enhance neuroblastoma cell survival and its resistance to chemotherapy (21). However, the role of BDNF in other cancers is unclear. Our current study revealed, for the first time, that BDNF was also expressed in liver tumor. This was true not only in rat HCC but also in human HCC, as our clinical study showed that human HCC tissue expressed a higher level of BDNF mRNA than adjacent nontumorous tissues and cirrhotic liver tissues. Corresponding to our findings in the rat model, normal human liver tissues did not express a detectable level of BDNF, suggesting that targeting on BDNF might achieve selective suppression of tumor cell activities.

The present study revealed that BDNF was a functional protein that could induce tumor cell proliferation. Importantly, the stimulatory effects of BDNF were more prominent with a relative lower number of tumor cells ($5 \times 10^5$ and $1 \times 10^6$) than a higher number of cells ($2 \times 10^6$). The reason for the above phenomenon...
remains to be determined. One explanation was that the stimulatory effects of BDNF might approach to a saturating status when the cells were in a relatively higher density. We also did some clinical studies to evaluate the role of BDNF in clinical settings. We observed that serum levels of BDNF in HCC patients were correlated with the status of microsatellites and tumor recurrence (data not shown). These data suggested that BDNF might be a potential prognostic marker in HCC, and blocking the activities of BDNF might prevent HCC recurrence after hepatectomy. However, further studies are needed to explore the therapeutic efficacy of BDNF blockade.

Our present study focuses on investigating the effects of soluble BDNF on tumor development based on the observation that tumor cells expressed the truncated isoform of trkB. Therefore, we administered exogenous BDNF to the tumor cell culture and explored the potential mechanism underneath. We found that exogenous BDNF could enhance endogenous BDNF expression in tumor cell lines and might in turn initiate an endocrine cascade in tumor cells. The molecular basis of the exocrine-endocrine interaction of BDNF in tumor cells is not clear. One possibility is that the exogenous BDNF stimulated tumor cell growth, and the actively proliferating cells might produce more endogenous BDNF. However, further studies are needed to explore the potential mechanism.

Signal transduction by BDNF is initiated by its interaction with receptor-trkB, within which the truncated isoform of trkB plays a more important role (22, 23). Interestingly, only the HCC tumor tissue expressed the truncated isoform of trkB. In addition, the level of intracellular isoform of trkB was also significantly higher in the tumor tissue, suggesting that the abundant expression of trkB, especially the truncated isoform, in tumor cells contributed to the selective signal transduction by BDNF on tumor cell proliferation.

In the present study, we also investigated the possible molecular pathway through which BDNF stimulated tumor cell proliferation. One of the molecules of interest was Hsp90. Hsp90 belongs to the chaperone family that plays essential roles in assisting newly synthesized proteins to fold or to translocate through membranes (24), stabilizing protein conformation (25), and helping eliminate denatured proteins (26). In view of its critical role in cell proliferation and survival (27), we tested the expression pattern of Hsp90 in both tumor and normal cell lines after BDNF stimulation. Interestingly, BDNF induced Hsp90 up-regulation only in tumor cell lines but not in the normal cell line. Blocking the activity of Hsp90 by its specific inhibitor geldanamycin significantly suppressed tumor cell proliferation induced by BDNF. In addition, the augmentation of cyclin D1 by BDNF could be completely reversed by Hsp90 inhibition, indicating that the stimulatory effect of BDNF on HCC cell proliferation was dependent on Hsp90.

In conclusion, the present study showed that BDNF and its receptor are novel molecules involved in tumor development and recurrence of HCC. BDNF could selectively induce tumor cell proliferation, and the stimulatory effect of BDNF on tumor cells is dependent on Hsp90. Further studies on BDNF as a therapeutic target in HCC are warranted and may lead to a novel approach in inhibition of HCC development and recurrence.

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