Jpn. J. Cancer Res. 82, 1263–1270, November 1991

Characterization of High-Molecular-Mass Forms of Basic Fibroblast Growth Factor Produced by Hepatocellular Carcinoma Cells: Possible Involvement of Basic Fibroblast Growth Factor in Hepatocarcinogenesis

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Growth factor(s) with a strong mitogenic effect on BALB/c3T3 cells was purified from an extract of C-Li21 cells, a human hepatocellular carcinoma line, by a combination of heparin-affinity chromatography and reversed-phase high-performance liquid chromatography (HPLC). Two major peaks of mitogenic activity were obtained by reversed-phase HPLC. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the two peaks revealed that one was composed of three proteins with relative molecular masses of 27, 24 and 23 kilodaltons (kD), whereas the other was a single 19-kD protein. Immunoblot analysis showed that all four of these molecules were immunoreactive species of human basic fibroblast growth factor (bFGF). N-Terminal sequence analysis of these molecules revealed that most of them were N-terminally blocked. However, small proportions of the 23- and 19-kD molecules were not blocked, and their respective N-terminal sequences were found to correspond to Gly^{-40}-Gly^{-27} and Pro^{29}-Phe^{40} of human bFGF deduced from the cDNA sequence of a human hepatoma cell line, SK-HEP-1. Expression of bFGF in hepatocellular carcinomas was then investigated by RNA blot analysis. All of the examined hepatocellular carcinoma cells expressed bFGF, and the degree of expression was higher in surgically resected hepatocellular carcinomas than in the corresponding adjacent non-cancerous liver tissue. Transcripts of bFGF were not detected in normal liver. These results suggest that C-Li21 cells produce four molecular forms of bFGF, and that bFGF may be involved in hepatocarcinogenesis. Moreover, it appears that bFGF is a potent mitogen toward primary-cultured hepatocytes, and that high-molecular-mass forms of bFGF produced by C-Li21 cells have stronger mitogenic effects on hepatocytes and are more stable under acidic conditions than the low-molecular-mass form, composed of 146 amino acids.

Key words: Basic fibroblast growth factor — Hepatocellular carcinoma — Hepatocarcinogenesis

Angiogenesis is considered to play an important role in tumorigenesis.[1,2] To date, various angiogenic factors have been identified,[3] and their possible role in the growth of malignant tumors has been suggested. Among these factors, basic fibroblast growth factor (bFGF)[5] is possibly one of the most important, since it strongly stimulates both the proliferation and locomotion of endothelial cells *in vitro* and angiogenesis *in vivo*,[3] and has been identified in a wide variety of tissues[3,4] including malignant tumors such as chondrosarcoma,[5] malignant melanoma[6] and hepatoma.[6,7]

Hepatocellular carcinoma (HCC) is one of several human carcinomas for which the process of development has been well characterized both morphologically[8] and genetically,[9–12] and it is known to become hypervascular with an increase in size.[13] Recently we attempted to culture HCC cells from surgically resected specimens under serum-free conditions in order to study the growth regulation of this tumor in detail, and succeeded in establishing four HCC cell lines, designated C-Li21-24. Unexpectedly, among these lines, C-Li21 cells were found to proliferate in RPMI1640 medium without any supplements (unpublished data), suggesting that these cells produce some autocrine factor(s).

We examined the effects of culture supernatant and extract of C-Li21 cells on several target cells, and found that the cell extract exerted an exceedingly potent mitogenic effect on BALB/c3T3 cells, suggesting that C-Li21 cells produce some growth factor(s) associated mainly with cells. We then isolated this factor from C-Li21 cell extract using several methods including

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[5] Abbreviations used: FGF, fibroblast growth factor; bFGF, basic FGF; rbFGF, recombinant human bFGF; aFGF, acidic FGF; HCC, hepatocellular carcinoma; FCS, fetal calf serum; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; kD, kilodaltons; EGF, epidermal growth factor.

heparin-affinity chromatography and reversed-phase HPLC, based on monitoring of its mitogenic activity toward BALB/c3T3 cells. Subsequent characterization of the molecule suggested that it was a high-molecular-mass form of bFGF, originally identified in another human HCC cell line, SK-HEP-1,[14,15] and that its properties were distinct from those of a lower-molecular-mass form of bFGF composed of 146 amino acids. In this study, we also examined the expression of bFGF in HCC, and considered its possible involvement in hepatocarcinogenesis.

MATERIALS AND METHODS

Purification procedure C-Li21 cells were cultured on 150-mm plastic dishes (Nunc, Roskilde, Denmark) in RPMI1640 medium supplemented with 5% fetal calf serum (FCS). FCS was added in this study, because it facilitates the proliferation of C-Li21 cells and gives a high yield. Cells proliferating at subconfluence were collected by scraping, washed with phosphate-buffered saline (PBS) three times and stored at −80°C until use.

The stored cells were thawed out, suspended in 0.4 *M* NaCl/10 m*M* HEPES (pH 7.4) containing 0.1 m*M* phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), 1 μg/ml antipain (Sigma), 1 μg/ml leupeptin (Sigma) and 1 μg/ml pepstatin A (Sigma), and then sonicated on ice. The resulting cell extract was clarified by centrifugation at 100,000*g* for 30 min at 4°C followed by filtration through 0.45-μm filters. It was then poured onto a column packed with Affi-gel heparin (Bio-Rad Laboratories, Richmond, CA) and equilibrated with 0.4 *M* NaCl/10 m*M* HEPES (pH 7.4). After thorough washing of the column with the same buffer, adsorbed materials were eluted with 2 *M* NaCl/10 m*M* HEPES (pH 7.4). The eluate was diluted to 0.4 *M* NaCl with 10 m*M* HEPES (pH 7.4), filtered through 0.45-μm filters and applied to a Heparin-5PW column (Tosoh, Tokyo) equilibrated with 0.4 *M* NaCl/10 m*M* HEPES (pH 7.4) at a flow rate of 1 ml/min using a Tosoh high-performance liquid chromatography (HPLC) system. After washing of the column with 0.4 *M* NaCl/10 m*M* HEPES (pH 7.4) until the absorbance at 280 nm returned to the baseline, adsorbed materials were eluted with a linear gradient of 0.4–2 *M* NaCl/10 m*M* HEPES (pH 7.4) at a flow rate of 1 ml/min for 120 min. Fractions of 2 ml were collected and assayed for their mitogenic activities toward BALB/c3T3 cells as described below. Biologically active fractions from several chromatography steps were combined and concentrated by ultrafiltration using a YM10 membrane filter (Amicon, Danvers, MA) for the following step.

The concentrated fractions were analyzed by reversed-phase HPLC using a Vydac C_4 column (0.46×25 cm, 5 μm particle size, 300 Å pore size; The Separation Group, Hesperia, CA). They were applied to the column equilibrated with 20% acetonitrile/0.1% trifluoroacetic acid (TFA) and eluted with a multi-linear gradient of 20–60% acetonitrile/0.1% TFA at a flow rate of 0.8 ml/min for 90 min. Fractions of 0.8 ml were collected, assayed for their mitogenic activities and used for further analyses.

Mitogenic assays Throughout the above procedure, growth factor activity was monitored by [^{3}H]thymidine incorporation assay using BALB/c3T3 clone A31-1-1 cells.[16] Briefly, BALB/c3T3 cells suspended in DME medium supplemented with 5% FCS were plated on 96-well plates (Iwaki Glass, Tokyo) at a cell density of 5×10^3/well. Two days later, the medium was replaced with fresh DME medium supplemented with 0.5% FCS, and the cells were cultured for another five days. Samples diluted with 0.1% bovine serum albumin (BSA)/PBS were then added to each well. After incubation for 16 h, cells were labeled with 1 μCi/well [methyl-^{3}H]thymidine (Amersham, Buckinghamshire, UK) for 4 h. Subsequently, the cells were washed with PBS, trypsinized and collected on glass filters. Radioactivity was measured as described previously.[17]

To determine the mitogenic activities of growth factors on hepatocytes, rat hepatocytes were isolated from F344 rats weighing 100–200 g by perfusing the liver with collagenase *in situ* according to the method of Seglen,[18] and subjected to [^{3}H]thymidine incorporation assay. This assay was performed as described previously[19] with some modifications. Briefly, hepatocytes suspended in MCDB107 medium (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo) supplemented with 2% FCS were plated on collagen-coated 96-well plates (Iwaki Glass) at a cell density of 2×10^4/well and incubated for 2 h. After removal of the medium containing floating cells and rinsing of the wells with PBS, fresh MCDB107 medium supplemented with 1 mg/ml BSA, 4 μg/ml oleic acid, 50 μ*M* dithiothreitol, 5μg/ml ethanolamine and 1 μg/ml insulin together with growth factors was added to each well. After incubation for 48 h, hepatocytes were labeled with 1 μCi/well [methyl-^{3}H]-thymidine for 24 h, and then radioactivity was measured.

SDS-PAGE and immunoblot analysis Solvents were removed from biologically active fractions from reversed-phase HPLC in a Speed Vac concentrator (Savant, Farmingdale, NY). These fractions were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by structural characterization. Dried samples were redissolved in Laemmli's sample buffer.[20] After heating at 100°C for 5 min, the whole mixture was loaded onto 15% polyacrylamide gel and electrophoresis was carried out using a buffer consisting of 25 m*M* Tris, 192 m*M* glycine and

0.1% SDS. The gel was then fixed and silver-stained using 2D-Silver Stain·II (Daiichi Pure Chemicals Co., Ltd., Tokyo) or subjected to electroblotting onto a polyvinylidene difluoride (PVDF) membrane (Nihon Millipore Kogyo, Yonezawa). The resulting PVDF membrane was treated for 3 h with a blocking buffer consisting of 5% skim milk (Difco, Detroit, MI), 1% BSA and 0.01% Antifoam A (Sigma) in PBS, and then incubated overnight at 4°C with rabbit antiserum against recombinant human bFGF (rbFGF)[21,22] diluted 1/200 with the blocking buffer. The membrane was washed with 0.1% Tween-20 in PBS, and with PBS at intervals of 5 min, three times, respectively, followed by brief rinsing with the blocking buffer. Finally, it was incubated with ^{125}I-labeled protein A (Amersham) at 37°C for 1 h, washed as above and exposed to XAR films (Eastman Kodak Co., Rochester, NY) at −80°C with intensifying screens.

Determination of N-terminal sequences Another PVDF membrane was subjected to N-terminal sequence analysis as reported previously.[23] Briefly, after the proteins on the membrane had been stained with Coomassie Blue, visualized bands were cut out. The N-terminal amino acid sequence of the protein in each band was then determined using a 470A protein sequencer (Applied Biosystems, Foster City, CA).

RNA blot analysis To investigate the expression of bFGF and acidic FGF (aFGF) in hepatocellular carcinoma, poly(A)$^+$RNAs were isolated and subjected to RNA blotting as described previously.[24] Probes used in this study were cleaved from plasmids pTB669[21] and pTB917,[25] which carry cDNAs encoding human bFGF and aFGF, by digestion with *Eco*RI-*Bgl*II and *Eco*RI-*Bam*HI, respectively.

RESULTS

Purification of growth factor produced by C-Li21 cells Since preliminary experiments had disclosed that the growth factor produced by C-Li21 cells had strong affinity for heparin, we used heparin-affinity chromatography procedures for the first purification step. As shown in Fig. 1, almost all of the mitogenic activity was eluted at 1.1–1.3 *M* NaCl from a Heparin-5PW column. Bioactive fractions from the several Heparin-5 PW affinity chromatography steps were combined, concentrated and subjected to reversed-phase HPLC. The elution profile of a representative reversed-phase HPLC is shown in Fig. 2. Two major peaks of DNA synthesis were obtained, coinciding with two peaks in the absorption profile indicated by asterisks in Fig. 2. Fractions 40, 41 and 44 of the reversed-phased HPLC showing marked mitogenic activities (Fig. 2) were further analyzed by SDS-PAGE and successive silver-staining (Fig. 3A). Both fractions 40 and 41 showed triplet bands with relative molecular masses of about 27, 24 and 23 kilodaltons (kD), and fraction 44 showed a single band representing a protein with a relative molecular mass of about 19 kD. The amounts of the 27-, 24-, 23- and 19-kD polypeptides in these three fractions were estimated to be about 16, 6, 26 and 20 μg, respectively. These amounts were equivalent to about 6.6×10^9 cells as a starting mate-

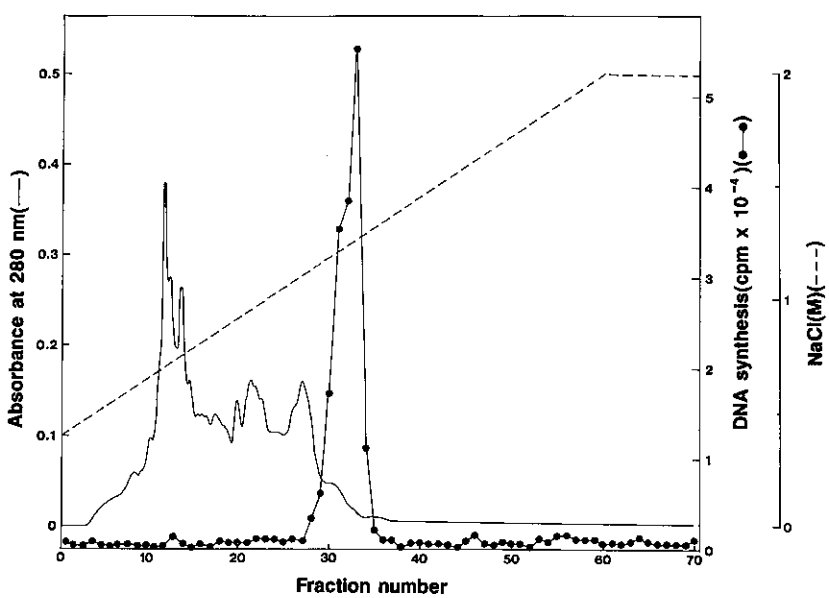

Fig. 1. Heparin-5PW affinity chromatography of eluate from Affi-Gel heparin equivalent to about 2.5×10^9 C-Li21 cells as a starting material. An aliquot of each fraction was diluted 1/100 with 0.1% BSA/PBS, and 2 μl of the diluted fraction was assayed for mitogenic activity toward BALB/c3T3 cells. Individual points indicating DNA synthesis are averages from triplicate determinations.

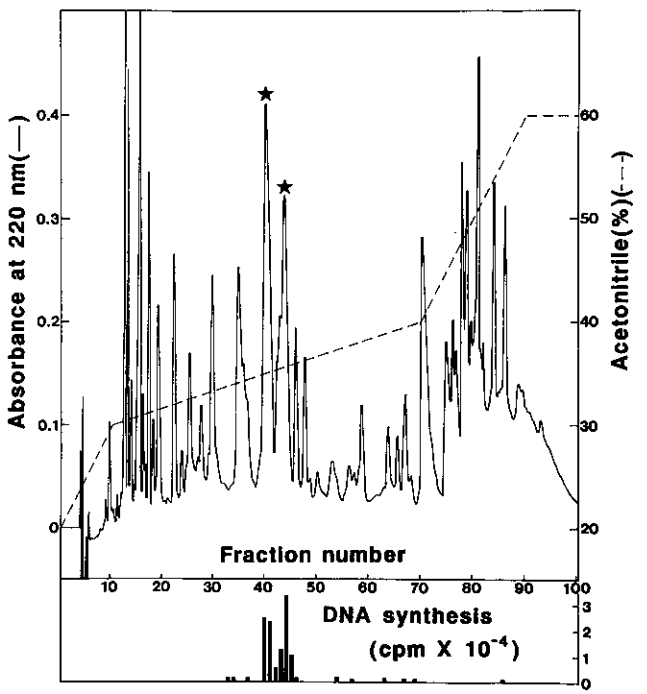

Fig. 2. Reversed-phase HPLC of bioactive fractions of Heparin-5PW affinity chromatography equivalent to about 6.6×10^9 C-Li21 cells as a starting material. An aliquot of each fraction was immediately diluted 1/1000 with 0.1% BSA/PBS, and 2 μl of the diluted fraction was assayed for mitogenic activity toward BALB/c3T3 cells. Individual bars indicating DNA synthesis are averages from triplicate determinations. Two peaks in the absorption profile coinciding with two major peaks of DNA synthesis are indicated by asterisks.

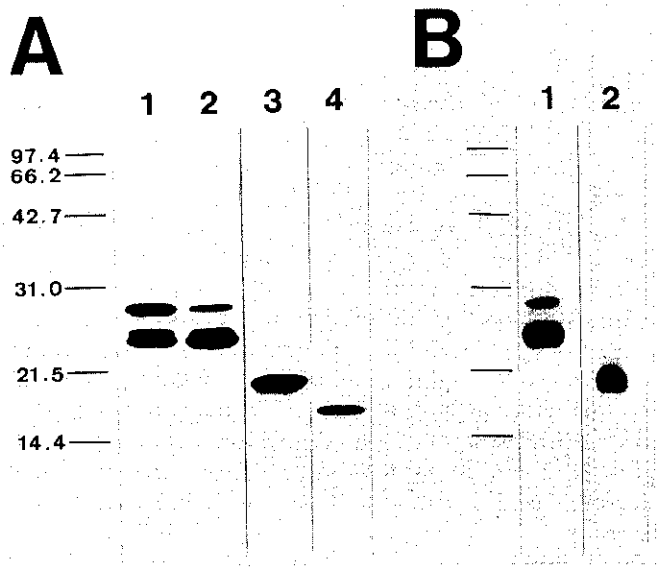

Fig. 3. (A) SDS-PAGE analysis of fractions 40, 41 and 44 (lanes 1, 2 and 3, respectively) from reversed-phase HPLC shown in Fig. 2. Each fraction was dried, and the residue was redissolved in Laemmli's sample buffer and separated on 15% polyacrylamide gel by SDS-PAGE. The gel was subsequently fixed and silver-stained. Lane 4 shows 200 ng of rbFGF[21] loaded on the same gel. (B) Immunoblot analysis of fractions 41 and 44 (lanes 1 and 2, respectively). In lane 1, the bands of the 24- and 23-kD polypeptides are fused. Bars indicate mobilities of molecular mass markers in kD.

rial. In addition, immunoblotting showed that anti-bFGF antiserum recognized all four polypeptides (Fig. 3B).

N-Terminal sequence analysis Whereas analyses of the 27- and 24-kD polypeptides gave no amino acid peaks, the 23- and 19-kD polypeptides were found to have the N-terminal amino acid sequences X-X-X-Gly-X-Gly-Arg-Ala-Pro-Glu-Arg-Val-Gly-Gly and X-X-Arg-Leu-Tyr-X-Lys-Asn-X-Gly-Phe-Phe, respectively. However, the recovery of phenylthiohydantoin amino acid at each step was very low in both analyses. Comparison of these sequences with the established sequences of growth factors indicated that they coincided with Gly^{-40}-Gly^{-27} and Pro^{29}-Phe^{40} of human bFGF,[14, 15, 26] respectively.

Expression of basic and acidic FGF in HCC RNA blot analyses were performed to examine the expression of basic and acidic FGF in HCC cell lines and surgically resected cancerous and non-cancerous livers. As shown in Fig. 4, all of the examined HCCs expressed bFGF at detectable levels, whereas transcripts of bFGF were hardly detectable in non-cancerous livers after the same exposure period. This was especially obvious when expression of bFGF in surgically resected HCCs was compared with that in the corresponding surrounding non-cancerous liver (Fig. 4, lanes 7–10). Among the examined HCCs, C-Li21 cells expressed bFGF at a particularly high level with at least 5.9-, 3.6-, 3.2-, 2.0-and 1.2-kilobase transcripts.

Expression of aFGF in the same specimens was also investigated with a human aFGF probe. As shown in Fig. 5, all of the established cell lines expressed several species of aFGF-specific mRNAs, but no transcripts were detectable in surgically resected liver tissues, including two cases of HCC. With regard to the expression of basic and acidic FGF in C-Li21 cells, expression of aFGF might be almost negligible in comparison with that of bFGF. This corresponded to the findings that no peak attributable to aFGF, which has a lower affinity for heparin than bFGF,[4] was observed upon Heparin-5PW affinity chromatography (Fig. 1).

Mitogenic activity toward hepatocytes Mitogenic activities toward hepatocytes of bioactive fractions from reversed-phase HPLC (Fig. 2) were examined by [^{3}H]-thymidine incorporation assay using primary-cultured rat hepatocytes in comparison with those of recombinant

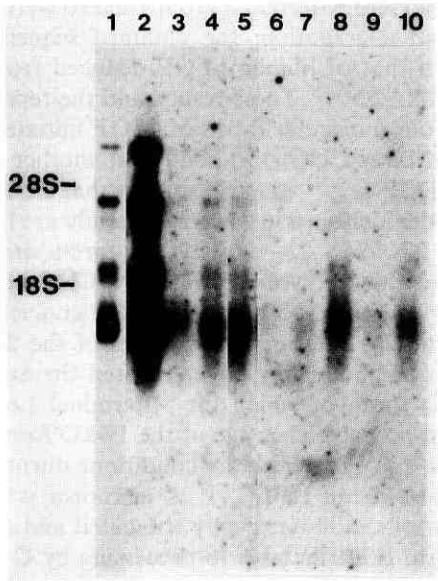

Fig. 4. RNA blot analysis of bFGF. Poly(A)$^+$RNAs (5 μg/lane) isolated from HCC cell lines, C-Li21 (lanes 1 and 2), C-Li22 (lane 3), C-Li23 (lane 4) and C-Li24 (lane 5), and surgically resected normal liver (lane 6), non-cancerous livers (lanes 7 and 9) and HCCs (lanes 8 and 10) were analyzed with a human bFGF probe. Normal liver shown in lane 6 was obtained by resection for a metastasis of colon cancer. Non-cancerous livers shown in lanes 7 and 9 were obtained by resection for HCCs shown in lanes 8 and 10, respectively, and diagnosed as chronic hepatitis on the basis of histopathological examination. HCCs shown in lanes 8 and 10 had not been treated by procedures such as transarterial embolization or injection before hepatectomy and were diagnosed as Edmondson Grades[27] II and III, respectively; the cells were 100% viable by histopathological examination. Exposure periods for lane 1 and 2–10 were 8 and 240 h, respectively. Positions of 28S and 18S ribosomal RNAs are marked.

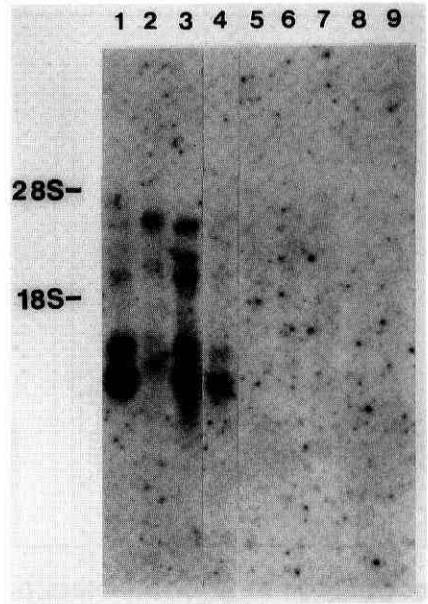

Fig. 5. RNA blot analysis of aFGF. Poly(A)$^+$RNAs (5 μg/lane) isolated from C-Li21 (lane 1), C-Li22 (lane 2), C-Li23 (lane 3), C-Li24 (lane 4), a normal liver (lane 5), non-cancerous livers (lanes 6 and 8) and HCCs (lanes 7 and 9) were analyzed with a human aFGF probe. Lanes 1–9 in this figure correspond to lanes 2–10 in Fig. 4 and were exposed for 240 h. Positions of 28S and 18S ribosomal RNAs are marked.

human bFGF initiated at Pro[10] and composed of 146 amino acids,[21] and mouse epidermal growth factor (EGF; Toyobo Co., Ltd., Osaka). The results are presented in Fig. 6. EGF showed prominent mitogenic activity toward hepatocytes as described previously.[19] The growth factor fractions from the reversed-phase HPLC and rbFGF also stimulated DNA synthesis by hepatocytes, although at lower levels than EGF. All the factors stimulated maximal DNA synthesis at 10 ng/ml. At this concentration, the levels of stimulation induced by fractions 40 and 44, rbFGF and EGF were 4.7, 4.3, 2.4 and 15.7 times the background level, respectively. At any concentration, the mitogenic activities of fractions 40 and 44 were higher than that of rbFGF, even though they had been exposed to the acidic conditions of reversed-phase HPLC for more than 1 h.

Table I. Stability of Growth Factor Activity[a]

Treatment	Conditions	Mitogenic activity
Heat	60°C, 1 min	24[b]
	60°C, 3 min	0
	80°C, 1 min	0
Heparin	1 μg/ml	115
	10 μg/ml	120
	100 μg/ml	132
Acid	pH 2, 1 h, 20°C	57 (17)[c]
	pH 2, 3 h, 20°C	51 (6)

a) Effects of treatments were assessed by [^{3}H]thymidine incorporation assay using BALB/c3T3 cells. Combined active fractions from Heparin-5PW affinity chromatography (Fig. 1) were added to quiescent BALB/c3T3 cells at a concentration inducing half-maximal stimulation in the case of no treatment.
b) Values are means of five determinations and are given as percentages of the control.
c) Values in parentheses present data for rbFGF.

Stability of mitogenic activity The effects of some physicochemical treatments on the mitogenic activities of growth factor(s) produced by C-Li21 cells were in-

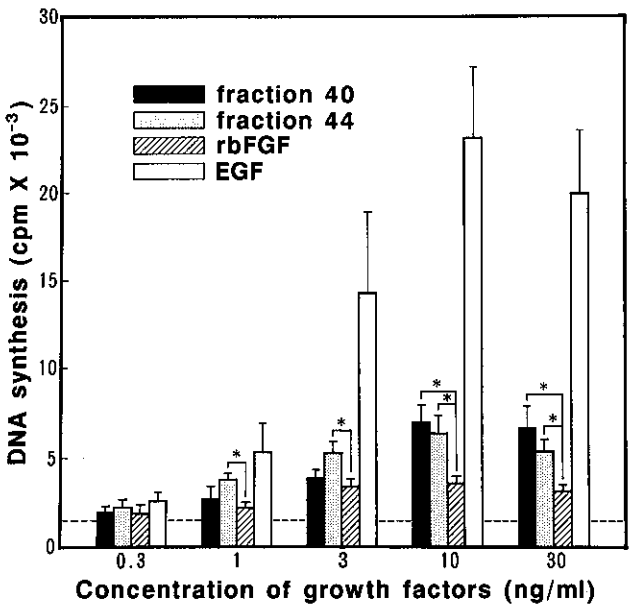

Fig. 6. Mitogenic activities of fractions 40 and 44 from the reversed-phase HPLC shown in Fig. 2, rbFGF and EGF toward primary-cultured rat hepatocytes. Concentrations of fractions 40 and 44 were determined by SDS-PAGE using rbFGF as standards. Bars indicate mean±standard deviation of five determinations. The dotted line indicates the background level of the assay. Statistically significant differences between DNA synthesis of HPLC fractions and rbFGF by Student's *t* test are indicated as * ($P<0.01$).

vestigated using combined bioactive fractions from Heparin-5PW affinity chromatography (Fig. 1) and [^{3}H]thymidine incorporation assay with BALB/c3T3 cells. Representative results are summarized in Table I. The growth factor(s) activity was completely destroyed by heat treatment at 60°C for 3 min or at 80°C for 1 min. Addition of heparin enhanced the activity slightly. Even after treatment at pH 2 for 3 h at 20°C, the growth factor(s) retained almost half of its activity, whereas rbFGF lost nearly all of its activity under the same conditions.

DISCUSSION

Growth factors of four different molecular sizes, with strong mitogenic effects on BALB/c3T3 cells, were purified from C-Li21 cells. Immunoblot analysis using anti-bFGF antiserum showed that all the molecules were structurally related to bFGF. N-Terminal sequence analyses of these molecules suggested that most of the four polypeptides were N-terminally blocked. However, small proportions of the 23-kD and 19-kD polypeptide samples had free N-terminal groups and so were available for Edman degradation; the obtained sequences coincided with that of human bFGF deduced from human bFGF cDNA.[14, 15, 26] These results and the reported existence of four molecular forms of bFGF initiated with an AUG and three CUG start codons in another HCC cell line, SK-HEP-1,[14, 15] strongly suggest that all of the four polypeptides identified in the present study are bFGF and that the 27-, 24-, 23- and 19-kD forms are possibly initiated at the first, second and third CUG codons and the AUG codon, respectively. It is also conceivable that the determined N-terminal sequences of the 23-kD and 19-kD polypeptides may have reflected the existence of the 23-kD form, of which the N-terminal Leu is truncated, and probable cleavage of the 19-kD form between Asp[28] and Pro[29] under acidic conditions during or after the reversed-phase HPLC. It is unknown whether the formation of the N-terminally truncated and unblocked 23-kD form is attributable to processing by C-Li21 cells or to the purification procedures. Following the present study, we isolated some cDNA clones considered to encode the above polypeptides from a C-Li21 cDNA library and performed nucleotide sequence analyses of some parts of them (unpublished data). The nucleotide sequences determined in the study completely coincided with that of human bFGF,[14, 26] confirming that the four polypeptides identified in the present study were bFGF.

RNA blot analysis disclosed that all of the four HCC cell lines established in our laboratory expressed bFGF, and that surgically resected HCCs also expressed it at higher levels than the corresponding adjacent noncancerous liver tissue. These results suggested some general implications of bFGF in hepatocarcinogenesis. It is known that hypervascularity is one of the most common characteristics of large HCC.[28] However, recent angiographic studies of small HCC have revealed that the smaller the HCC, the lower its degree of hypervascularity, and that adenomatous hyperplastic lesions containing tiny HCC can not be detected by angiography.[13, 29] Thus it may be speculated that bFGF begins to be expressed in HCC at a certain stage and subsequently promotes neovascularization of tumor vessels and successive tumor growth.

All C-Li21-24 cells can proliferate in serum-free medium, and C-Li21 cells in particular, which express bFGF at the highest level (Fig. 4), can proliferate in RPMI1640 medium without any supplements (unpublished data). These findings prompted us to investigate the involvement of bFGF in the autocrine growth of HCC cells. Besides the data described here, we also examined the effect of anti-bFGF neutralizing antibodies[22] on C-Li21 cells, but no obvious growth inhibition of C-Li21 cells could be observed (unpublished data). We then examined the effect of bFGF on normal

rat hepatocytes and demonstrated for the first time that bFGF has a mitogenic effect on hepatocytes, similarly to aFGF.[19] Moreover, the high-molecular-mass forms of bFGF are more mitogenic toward these cells than rbFGF, which is almost identical[21] to the originally identified shorter form initiated at Pro[10] and composed of 146 amino acids.[30–32] These findings suggest the possibilities that bFGF promotes the proliferation of HCC cells and that amino acid extensions upstream from the Pro may enhance the biological activity of bFGF toward hepatocytes. However, before it can be concluded whether bFGF is involved in autocrine growth of HCC cells, a more detailed examination will be necessary.

Besides mitogenic activity toward normal rat hepatocytes, the high-molecular-mass forms of bFGF had another property distinct from the low-molecular-mass form. Although it is known that bFGF is rapidly inactivated upon exposure to acidic conditions,[33] we noticed in this study that the bioactivity of bFGF produced by C-Li21 cells was well retained even after reversed-phase HPLC with TFA (Fig. 2). We then examined the effect of acid treatment on the mitogenic activities of the high-molecular-mass forms produced by C-Li21 cells as well as rbFGF. As shown in Table I, the high-molecular-mass forms showed marked tolerance to acidity in comparison with rbFGF, the low-molecular-mass form. Thus it may be considered that addition of amino acid sequences at the N-terminus alters the tertiary structure of bFGF, conferring tolerance to acidity. For this reason, the high-molecular-mass forms may act more effectively than the low-molecular-mass form under acidic conditions *in vivo*.

ACKNOWLEDGMENTS

BALB/c3T3 clone A31-1-1 cells were obtained from the Japanese Cancer Research Resources Bank. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan for the Comprehensive 10-Year Strategy for Cancer Control. Y. Shimoyama is an awardee of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research, Japan.

(Received May 14, 1991/Accepted August 1, 1991)

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