Induction of tumor growth and lung metastasis by a cDNA cloned from an aflatoxin B₁-induced rat liver cancer cell line

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Summary

Previously we isolated a cancer cell line AFB-1 from a hepatocellular carcinoma that had been induced by aflatoxin B₁ in a male Fischer 344 rat. In the present study, we constructed the subtracted cDNA libraries between mRNAs from AFB-1 and rat normal liver cell line BL9, and identified a 650 bp cDNA which hybridized with mRNA of AFB-1 but not with that of BL9 in Northern blot analysis. We transfected the 650 bp cDNA/vector recombinant into AFB-1 and BL9, and then inoculated either transfected cells or parental cells at 1×10^7 cells/rat subcutaneously at the dorsal region of syngenic rats. The transfection of AFB-1 with the 650 bp cDNA/vector recombinant increased the incidence of subcutaneous tumor growth (14 of 14 in male rats inoculated with transfected AFB-1 *versus* 8 of 13 in those with parental AFB-1, $P < 0.02$) and that of lung metastasis (9 of 14 in male rats inoculated with transfected AFB-1 *versus* 0 of 13 in those with parental AFB-1, $P < 0.001$). The transfection of the 650 bp cDNA/vector recombinant transformed normal liver cell line BL9 into cancer cells i.e. the transfected BL9 formed subcutaneous tumor nodules in 9 of 10 male rats and in all 10 female rats and formed metastatic foci in the lungs in 6 of 10 female rats. This is the first instance where a cDNA from a subtracted cDNA library not only enhanced subcutaneous tumor growth and lung metastasis of cancer cells but also induced those activities in normal cells.

Key words : aflatoxin, cDNA, metastasis, hepatocellular carcinoma, rat

(Received: June 7, 2001, Received & Accepted: July 24, 2001)

Introduction

Aflatoxins are produced by some toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*, contaminate human foods and animal feeds, and have been inferred to be one of the etiological factors for human liver cancers¹⁻⁵⁾. Aflatoxin B₁ is an extremely potent hepatocarcinogen for many species of animals and has been extensively studied in the rat to elucidate the modes of toxic

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action and neoplastic induction^{1,6,7}. Previously we isolated a cancer cell line AFB-1 from a hepatocellular carcinoma which had been induced by aflatoxin B₁ in a male Fischer 344 rat^{8,9}. The present study was carried out to identify the gene expression that was specific to AFB-1. We constructed the subtracted libraries between mRNAs from AFB-1 and normal liver cell line BL9, and identified a 650 bp cDNA of which ³²P-labeled antisense cRNA probe gave a signal in Northern hybridization with mRNA of AFB-1 but not with that of BL9. The DNA sequences of more than 95% homology to the 650 bp cDNA are present in human chromosome 5 and *E. coli* genomic DNA but the function of the DNA sequences is not known. Therefore we transfected the 650 bp cDNA/vector recombinant into AFB-1 and BL9 and examined the tumorigenic activities of the transfected cells in syngenic rats. Here, we present the first evidence that a cDNA from the subtracted cDNA library not only enhanced subcutaneous tumor growth and lung metastasis of cancer cells but also induced those activities in normal cells.

Materials and Methods

Cells and animals The cancer cell line AFB-1 was isolated by the authors from a hepatocellular carcinoma that had been induced by aflatoxin B₁ in a male Fischer 344 rat⁸. Normal liver cell line BL9 was established by Dr. G. E. Neal and his colleagues, Medical Research Council Laboratories, United Kingdom^{6,10,11}, and kindly supplied by them. Cells were cultured in Williams' E medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum (Grand Island Biological Co.), 2 mM glutamine and 50 µg/ml gentamycin at 37 °C in humidified air containing 5% CO₂ in a CO₂ incubator. Animals used were Fischer 344/JCI rats (CLEA Japan Inc., Tokyo), housed in an air-conditioned room kept at 23 °C and 60% relative humidity on a 12 hr light/dark cycle, and maintained on a basal diet CE-2 (CLEA Japan Inc.) and water *ad libitum*. The animals were killed under ether anaesthesia. Tumors and lung tissues for microscopic examination were fixed in 20% buffered formalin, embedded in paraffin, sectioned at 4 µm thickness, and stained with H&E. All animal protocols were approved by the Laboratory Animal Welfare Committee and conducted with strict compliance to guidelines established by the Laboratory Animal Center Committee, School of Medicine, Chiba University.

Construction of cDNA and subtracted libraries Construction of cDNA libraries and subtracted libraries was made according to a textbook of Molecular Cloning¹² and the manufacturers' instructions. Total cellular RNA was extracted with guanidium thiocyanate and isolated by centrifugation in cesium chloride solutions¹². mRNA was isolated on oligo(dT)-cellulose column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and cDNA library was constructed using Riboclone cDNA synthesis system, *Xba* I primer adaptor 5'-GTGACTCTAGA(T)₁₅-3', Riboclone EcoRI adaptor ligation system, *Eco*RI/*Xba* I GEM-2 arms, and Packagene extract system (Promega Co., Madison, WI). The cDNA libraries of mRNAs of BL9 and AFB-1 had titres of 5 × 10⁴ ~ 5 × 10⁵ pfu/ µg GEM-2 arms and contained cDNA inserts ranging 350 ~ 1,500 bp in length. Constructions of the subtracted libraries, the subtracted BL9 minus AFB-1 library and the subtracted AFB-1 minus BL9 library, were made using a large amount of sense cRNA. Briefly, GEM-2 of a cDNA library was digested with *Xba* I and then cRNA was prepared by transcription of the digested GEM-2 with T7

RNA polymerase. Antisense cDNA was prepared by transcription of cRNA with avian myeloblastosis virus reverse transcriptase. BL9 antisense cDNA was hybridized with approximately 10-fold amount of AFB-1 sense cRNA and the subtracted BL9 minus AFB-1 antisense cDNA was isolated by hydroxylapatite chromatography. The subtracted BL9 minus AFB-1 antisense cDNA was further purified by a repeat hybridization with approximately 100-fold amount of AFB-1 cRNA followed by hydroxylapatite chromatography. The subtracted AFB-1 minus BL9 antisense cDNA was prepared in the same way. Then the subtracted antisense cDNA was annealed with T7 spacer primer, 5'-GGGAGACCGGAATTC-3', and double-stranded by Klenow DNA polymerase. The subtracted double-stranded cDNA was digested with *EcoRI* and *Xba* I, ligated to *EcoRI/Xba* I GEM-2 arms, and mixed with packaging extract system.

PCR amplification of cDNA and DNA sequencing Amplifications of cDNAs in the subtracted libraries were carried out using a PCR system, Zymoreactor (Atto Co., Ltd., Tokyo), in a final volume of 100 μ l containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM dNTPs, 50 pmoles of a pair of primers, T7 promotor primer 5'-TAATACGACTCACTATAGGG-3' and SP6 promotor primer 5'-GATTTAGGTGACACTATAG-3' (Promega Co.), 2.5 units of Taq DNA polymerase (Nippon Gene Co., Ltd., Tokyo), and 200 ng of GEM-2/cDNA recombinant of a subtracted library. After preincubation for 0.3 min at 72 °C, 30 amplification cycles were performed, each consisting of denaturation for 2.5 min at 94 °C, annealing for 2.0 min at 55 °C, and extension at 72 °C for 2.5 min. The PCR was completed by a final extension for 5 min at 72 °C. Amplified DNAs were electrophoresed on 2 % agarose gel (Nu Sieve GTG, FMC Bioproducts, Rockland, ME) and divided into H fraction (1,350 ~ 600 bp DNAs) and L fraction (600 ~ 200 bp DNAs). The DNAs in H and L fractions were further amplified using the second pair of primers, *EcoRI* site primer 5'-CTATAGGGAGACCGGAATTC-3' and *Xba* I site primer 5'-GGCTGCAGGTGCGACTCTAGA-3' (Rikaken Co., Ltd., Tokyo, Japan). The amplified DNAs were electrophoresed, and the DNA bands specific to either subtracted library were isolated. The isolated DNAs were digested with *EcoRI* and *Xba* I, and the cDNAs were subcloned into the plasmid pUC118. Dye terminator cycle sequencing method was used to sequence cDNA in the plasmid. Both M13 primer M4 5'-GTTTTCCAGTCACGAC-3' and M13 primer RV 5'-CAGGAAACAGCTATGAC-3' were used to sequence each cDNA in both the 5' and 3' directions using the dideoxy terminator reaction chemistry in an automated ABI 377 sequencing system (Applied Biosystems-Prism-Perkin Elmer, Foster City, CA).

Northern hybridization Northern hybridization was carried out according to a textbook of Molecular Cloning¹²⁾. Approximately 5 μ g of mRNA was denatured by glyoxal and DMSO, loaded onto 1% agarose gel containing 10 mM NaPO₄ (pH 7.0) and 10 mM sodium iodoacetate, and electrophoresed at 3.5 V/cm in 10 mM NaPO₄ (pH 7.0) under recirculation of buffer. The electrophoresis was stopped when bromophenol blue moved 6.5 cm. The lanes containing DNA and RNA molecular weight markers (Promega Co.) were cut and stained with ethidium bromide at a concentration of 0.5 μ g/ml in 1 × MOPS (20 mM 4-morpholinepropanesulfonic acid/5 mM NaOAc/1 mM EDTA). The lanes containing mRNAs were placed in contact with Hybond-N+ filter (Amersham International plc, Amersham, UK) and the mRNAs were transferred to the filter in an ascending flow of 20 × SSC (3M NaCl/0.3M sodium citrate). The mRNA was fixed to the filter by baking at 80 °C in a vacuum oven. The filter was prehybridized and hybridized at 42 °C in a formamide solution (50%

formamide/5 × SSPE/5 × Denhardt's reagent/0.5% sodium dodecylsulfate/0.01% fragmented salmon sperm DNA). The probes used for hybridization were ³²P-labeled antisense cRNAs that were prepared according to a textbook of Molecular Cloning¹²⁾. Briefly, the cDNA/pUC118 recombinants were digested with *Eco*RI and *Xba* I, and cDNAs were subcloned into the plasmid pGEM-3Z containing SP6 and T7 promoters (Promega Co.). Each cDNA/pGEM-3Z recombinant was digested with *Eco*RI to prepare the template DNA for the synthesis of an antisense cRNA. Each ³²P-labeled antisense cRNA probe was prepared by transcription of the template DNA by SP6 RNA polymerase in the reaction mixture (40 μl) containing 1 × transcription buffer (40 mM Tris-HCl, pH 7.5/6 mM MgCl₂/2 mM spermidine/10 mM NaCl), 10 mM DTT, 40 units of RNase inhibitor, 0.5 mM each of ATP, CTP, and UTP, 1 pmole of template DNA, 7.4 MBq of [γ-³²P]GTP (specific activity: 29.6 TBq/mmol, New England Nuclear, Boston, MA), and 40 units of SP6 RNA polymerase. More than 60% of ³²P-GTP was incorporated into the probes and the specific activities of all the probes were approximately 10⁹ cpm/μg anti-sense cRNA. Hybridized bands were visualized by autoradiography on Fuji Bioimage Analyzer 2500 (Fuji Film Co., Ltd., Tokyo).

Transfection of the 650 bp cDNA into BL9 and AFB-1 The 650 bp cDNA, a cDNA found in the AFB-1 minus BL9 subtraction library, was cloned in an expression vector pCI-neo (Promega Co.) that contained the neomycin phosphotransferase gene to confer the mammalian cells resistance to aminoglycoside antibiotics such as neomycin and geneticin sulfate (G418). Either pCI-neo vector or the 650 bp cDNA/pCI-neo recombinant was mixed with Tfx-50 [N,N,N',N'-tetramethyl-N,N'-bis(2-hydroxyethyl)-2,3-dioleoyloxy-1,4-butanediammonium iodide and L-dioleoyl phosphatidylethanolamine] (Promega Co.) at a 1:3 charge ratio and introduced into BL9 or AFB-1 according to the manufacturer's protocol. The cells were then passaged and selected with the selection medium containing 1,000 μg/ml of G418 (Wako Pure Chemicals Ind., Tokyo). The selected cells showed a stable resistance to G418 and did even after recovery from frozen storage at -80 °C for more than 1 year. All the parental AFB-1 and BL9 died by the exposure to 400 μg/ml of G418.

Statistical analysis The Fischer's exact probability test was performed to compare incidences of subcutaneous tumor growth and lung metastasis. Statistical significance level was *P* < 0.02 or below.

Results

cDNAs from subtracted libraries The cDNAs in the subtracted libraries were amplified by PCR as described in Materials and Methods and the electrophoregram of cDNAs at the second amplification is shown in Fig.1. The BL9 minus AFB-1 subtracted library released a specific DNA band at approximately 300 bp (249 bp) position, and the AFB-1 minus BL9 subtracted library released specific DNA bands at approximately 700 bp (650 bp), 650 bp (604 bp), and other 3 positions. The numbers in the parentheses are the exact numbers of base pairs of cDNAs at the cloning sites that were determined by DNA sequencing.

Northern hybridization and sequence of 650 bp cDNA Northern hybridization was carried out on mRNAs of BL9 and AFB-1 using ³²P-labeled antisense cRNAs of 249 bp, 604 bp and 650 bp cDNAs as a probe. ³²P-labeled antisense cRNA of the 650 bp cDNA showed a hybridization signal at approximately 4 mm from the slot in the lane for mRNA of AFB-1 but none in the lane for that of BL9

Fig. 1. Electrophoregram of cDNAs amplified by PCR using a pair of *Eco*RI site primer and *Xba* I site primer.

cDNAs in the subtracted BL9 minus AFB-1 library and those in the AFB-1 minus BL9 library were first amplified using a pair of SP6 promoter primer and T7 promotor primer. The amplified cDNAs were electrophoresed on 2% agarose gel and divided into 2 fractions, H fraction (1,350 ~ 600 bp DNAs) and L fraction (600 ~ 200 bp DNAs). The cDNAs of H and L fractions were secondly amplified using the second pair of *Eco*RI site primer and *Xba* I site primer. The amplified cDNAs were electrophoresed on 2% agarose gel and cDNA bands were visualized by staining with ethidium bromide. DNA molecular weight markers used were *X174/Hae* digest and 100 base-pair ladder.

Fig. 2. Northern blot analysis on mRNA of AFB-1 and that of BL9 probed to 32 P-labeled antisense cRNA of 650 bp cDNA. The upper part of the figure is the autoradiogram showing a hybridization signal in the lane of mRNA of AFB-1 but none in the lane for that of BL9. The lower part of the figure is the electrophoregram showing DNA molecular weight markers (*λ* /*Hind* III digest) and a scale of 0 to 9 cm. The positions of agarose gel slots are on the line at zero point of the scale and the positions of RNA molecular weight markers are shown by bars (left to right; 6,583, 4,881, 3,638, 2,604, 1,908, 1,383, 955, 623, 281 base).

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TCYGGGTTAT TGGATAGGC AATGCCCTAA TTTTATTNGG GATGACTTAA AATGCTGAT 60
ATGATANGGA GAGATGGAT TATGACTCG CTGTCATAT AGCTCGAAT TACTAGTGG 120
TETKATGA TATAGTGAC AATTTTATC TTGGGTTC TTAAGTTGG TNGATAACA 180
TTGTGTTTC AGGATNCG GAATCTAAC AGCTGGGCG AAAATNCG CGCTTTTGG 240
GCGANGTGA AGCGGCACT GCAACTATC TTGTGAGAG ACTCTACNG GATTGGTTT 300
AAAGTATAC ATAGATAGA CTTTCTCT GAGAAGAA AATATCANG TGATNCGC 360
TATATGCCCT TGATGGGAG GAATGATCT GATGGGACTG AGTGATCGA ATACTGAAA 420
AATCAGAC AGGCTGTC CAGAACGCG CTGGATGG CTGCTNATT TTTGATNTG 480
GTGATATTG AATGATTTC AGCGTCTCT GATTATNCG ATAAATCAT GAAAAACTA 540
CTCTCTCT GGGGTTTT TTGGGATAT ATAGGGACA GAAATNGGC CCAGATGAC 600
ACTGAGATG AATTGGATG CTTTATAAA AATGCACT ATCTGAAAA 650

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Fig. 3. Sequence of 650 bp cDNA in the AFB-1 minus BL9 subtracted library.

(Fig. 2). This finding indicated that the 650 bp cDNA was derived from an mRNA specific to the cancer cell line AFB-1 and that the pertinent RNA was a large mRNA or a heterogeneous nuclear RNA. On the other hand, the 32 P-labeled 604 bases and 249 bases antisense cRNAs showed no hybridization signals in both lanes of mRNAs of AFB-1 and BL9 (data not shown). The sequence of 650 bp cDNA is shown in Fig. 3 (the sequences of 249 bp and 604 bp cDNAs are not shown).

Effect of Transfection with the 650 bp cDNA on Subcutaneous Tumor Growth and Lung Metastasis of AFB-1 and BL9 The cells of AFB-1, BL9, or either transfected with pCI-neo vector or the 650 bp cDNA/pCI-neo recombinant were inoculated at 1×10^7 cells/rat into the subcutaneous tissue at the dorsal region of syngenic male and female rats, 4-5 weeks of age. A total of 6 groups of animals were maintained on basal diet for 12 weeks and examined on the incidences of subcutaneous tumor growth and lung metastasis (Table 1). The parental AFB-1 formed tumor nodules at the subcutaneous site of inoculation in 8 of 13 male rats and in 5 of 10 female rats. Metastatic tumor nodules in the lungs were found in none of 13 male rats but in 2 of 10 female rats. The transfection of AFB-1 with the 650 bp cDNA/pCI-neo recombinant markedly increased the incidences of subcutaneous tumor growth and lung metastasis in male rats; the AFB-1 transfected with the 650 cDNA/pCI-neo recombinant formed subcutaneous tumor nodules in all 14 animals and metastatic tumor nodules in the lungs in 9 of 14 animals, respectively (significant at $P < 0.02$ and 0.001, respectively, as compared with the parental AFB-1). The transfection of AFB-1 with pCI-neo vector slightly increased the incidences of subcutaneous tumor growth and lung metastasis in male rats, but these increases were statistically no significant. The parental BL9 and the BL9 transfected with pCI-neo vector formed no tumor nodules over the period of 12 weeks. On the contrary, the BL9 transfected

Table 1. Effect of Transfection of the 650 bp cDNA on Subcutaneous Tumor Growth and Lung Metastasis of Rat Liver Cancer Cell Line AFB-1 and Normal Liver Cell Line BL9

Cell lines	Transfected DNA	No. and sex of rats	Incidences	
			Subcutaneous tumor growth	Lung metastasis
AFB-1	None	13 M	8	0
		10 F	5	2
	pCI-neo	14 M	11	4
		10 F	3	2
	650 bp cDNA/pCI-neo	14 M	14 ^a	9 ^d
		10 F	5	5
BL9	None	10 M	0	0
		10 F	0	0
	pCI-neo	10 M	0	0
		10 F	0	0
	650 bp cDNA/pCI-neo	10 M	9 ^b	0
		10 F	10 ^c	6 ^e

^{a-e} Significant at $P < 0.02$ (a), $P < 0.0001$ (b), $P < 0.00001$ (c), $P < 0.001$ (d) and $P < 0.01$ (e) by Fischer's exact probability test as compared with the incidence of subcutaneous tumor growth or that of lung metastasis in a group of rats of the same sex inoculated with parental AFB-1 or BL9 (None).

with the 650 bp cDNA/pCI-neo recombinant formed tumor nodules at the subcutaneous site of inoculation in 9 of 10 male rats ($P<0.0001$) and in all 10 female rats ($P<0.00001$). Metastatic tumor nodules were also found in the lungs of 6 of 10 female animals ($P<0.01$). These findings indicated that the transfection of the 650 bp cDNA/pCI-neo recombinant transformed normal cell line BL9 into cancer cells.

The parental AFB-1 formed soybean- to pigeon's egg-size (1.5 to 4 cm in diameter) tumor nodules in the subcutaneous tissue at 12 weeks after inoculation (Fig. 4A). The growth of the AFB-1 transfected with the 650 bp cDNA/pCI-neo recombinant was so rapid that the AFB-1 transfected with the recombinant formed palpable millet-size (1 to 2 mm in diameter) nodules in the subcutaneous tissue within 2 weeks, pigeon egg-size nodules at 4 weeks, and hen's egg-size (4.5 to 6 cm in diameter) nodules at 8 weeks. When the nodules grew up to hen's egg-size or larger, necrotic changes in the internal parts of the nodules became macroscopically evident and proceeded to liquefactive necrosis and bloody nodules (Fig. 4C). Milky-white and translucent tumor nodules distributed over the lung lobules (Fig. 4E). All the tumor nodules consisted of epithelial cells which varied in size and had a large size ratio of nucleus/cytoplasm and showed abnormal mitotic figures (Fig. 4, B, D and F). The tumor cells showed solid invasive growth and did not constitute ductal structures. The BL9 transfected with the 650 bp cDNA/pCI-neo recombinant formed a soybean-size tumor nodule which was clearly demarcated from the surrounding subcutaneous tissue (Fig. 5A) and millet-size metastatic tumor nodules which distributed over the lung lobules (Fig. 5C). All the tumor nodules consisted of undifferentiated spindle-shaped cells showing a marked variation in size and a large size ratio of nucleus/cytoplasm and looked like fibrosarcoma rather than hepatocellular carcinoma (Fig. 5, B and D).

Discussion

AFB-1 had a potent metastatic activity when it had just been isolated and cloned⁸⁾. This cancer cell line preferred to move from the site of inoculation into the abdominal and thoracic cavities to form multiple metastatic nodules rather than form tumor nodules at the subcutaneous site of inoculation⁸⁾. In order to demonstrate the gene expression that was specific to this cancer cell line, we constructed the subtracted libraries between mRNAs from cancer cell line AFB-1 and normal liver cell line BL9 and consequently identified a 650 bp cDNA of which ³²P-labeled antisense cRNA probe gave a signal in Northern hybridization with mRNA of AFB-1 but not with that of BL9. In the course of this study, AFB-1 ceased to express the mRNA that was hybridizable with the 650 bp cDNA and lost its original metastatic activities. In order to demonstrate the activity of the 650 bp cDNA, we cloned the 650 bp cDNA in an expression vector pCI-neo, transfected AFB-1 with the 650 bp cDNA/pCI-neo recombinant, and inoculated the transfected AFB-1 into the subcutaneous tissue of the syngenic rats. It was found that the transfected AFB-1 exhibited a marked activity of metastasizing into the lung in male rats (Table 1). The metastasis induced by the transfection of the 650 bp cDNA/pCI-neo recombinant occurred exclusively in the lung and was quite different from the original one that metastasized also into abdominal cavity to form many tumor nodules throughout serous membrane and metastatic foci in the kidney and pancreas.⁸⁾ The metastatic activity induced by

Fig. 4. Subcutaneous tumor growth and lung metastasis in male rats inoculated with either parental AFB-1 or AFB-1 transfected with the 650 bp cDNA/pCI-neo recombinant. A and B: Subcutaneous tumor of the parental AFB-1. (A) The tumor, approximately 3 cm in diameter, consists of several nodular aggregates (arrows). (B) A nodule is an assembly of small colonies, being divided by connective tissues. An extensive infiltration of lymphocytes is noted in the connective tissues. H&E, $\times 300$. C and D: Subcutaneous tumor of the AFB-1 transfected with the 650 bp cDNA/pCI-neo recombinant. (C) Huge tumor nodules develop under the subcutaneous tissue at the dorsal region of the animal. The major part of the nodules is necrotic and a milky-white layer of tumor growth is seen at the periphery of the nodules (arrows). (D) This micrograph shows the growth of tumor cells around blood vessels and abnormal mitotic figures. Degenerative to necrotic changes are noted in the area that is distant from the blood vessels. H&E, $\times 500$. E and F: Lung metastasis of the AFB-1 transfected with the 650 bp cDNA/pCI-neo recombinant. (E) Metastatic tumor nodules, several millimeters in diameter, are seen in the lung lobules (arrows). (F) This micrograph shows an expanding growth of a tumor nodule and the infiltration of tumor cells into the parenchyma of lung (arrows). H&E, $\times 150$.

Fig. 5. Subcutaneous tumor growth and lung metastasis in female rats inoculated with the BL9 transfected with the 650 bp cDNA/pCI-neo recombinant. A and B: Subcutaneous tumor. (A) The tumor forms an oval or global nodule, approximately 1.5 cm in diameter, in the derma. (B) The tumor nodule consists of spindle-shaped cells showing a large size ratio of nucleus/cytoplasm. Mitotic figures and collagen fibers are noted. This tumor looks like a fibrosarcoma. H&E, $\times 300$. C and D: Lung metastasis. (C) Small metastatic nodules, 1-2 mm in diameter, are noted on the lung lobules (arrows). (D) The tumor nodule consists of spindle-shaped cells similar to those of the subcutaneous tumor and shows an expanding growth of tumor cells in the parenchyma of lung. A moderate infiltration of lymphocytes is noted. H&E, $\times 250$.

the transfection of the 650 bp cDNA/pCI-neo recombinant was closely associated with the increased incidence of subcutaneous tumor growth and might be consistent with the growth dominance model of metastatic cancer cells¹³.

BL9 is a normal liver cell line that had been isolated from normal rat liver^{6,10,11} and exhibits epithelial cell structures as a hepatocyte^{6,9-11}. The parental BL9 and the BL9 transfected with pCI-neo vector did not form any tumor nodules by the transplantation in the rats (Table 1). Interestingly, the present study showed that the BL9 transfected with the 650 bp cDNA/pCI-neo recombinant not only formed tumor nodules at the subcutaneous site of inoculation but also metastasized into the lungs, indicating that the transfection of the 650 bp cDNA/pCI-neo recombinant transformed normal liver cells into cancer cells. We speculate that an initiation step of carcinogenesis might have already occurred in BL9 and that the transfection of the 650 bp cDNA might constitute the second step for conversion of BL9 cells into cancer cells^{14,15}. The transformed BL9 cells were spindle-shaped¹⁵, constructed nodules which looked like fibrosarcoma rather than hepatocellular carcinoma, and

exhibited a sex difference in the activity of metastasizing into lung tissue preferably in the female animals. It is difficult for us to explain the mechanisms underlying these phenomena.

Analysis on the expression of the 650 cDNA in the transfected AFB-1 and BL9 cells by dot blot and Northern blot detected no clones which produced the mRNA that hybridized with ³²P-labeled antisense RNA of the 650 bp cDNA. The homology analysis on GenBank databases indicates that the DNA sequences of more than 95 % identity to the 650 bp cDNA are present in human chromosome 5 (Accession: AC022123) and *E. coli* genomic DNA (Accession: D90789). However, the sequence of the 650 bp cDNA contains no open reading frame and no homologous sequences in the swissprot and pir protein databases. Therefore, the 650 bp cDNA is unlikely to code a protein sequence. The Northern blot of Fig. 2 indicates that the transcript of the 650 bp cDNA is the component of a large RNA molecule, possibly a heterogeneous nuclear RNA. We speculate that this DNA sequence contributes to malignant transformation not as a protein coding sequence but as an enhancer or a functional intervening sequence. Further studies are necessary to clarify the function of the DNA sequence in the genome that is homologous to the 650 bp cDNA. To our knowledge, this is the first instance where a cDNA from a subtracted cDNA library not only enhanced subcutaneous tumor growth and lung metastasis of cancer cells but also induced those activities in normal cells.

We are very grateful to Dr. Gordon E. Neal, Medical Research Council Laboratories, United Kingdom for kindly donating BL9 and related cell lines. This work was supported in part by Cooperative Research Program of Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University.

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アフラトキシ B₁誘発ラット肝癌細胞株より分離した cDNAによる腫瘍形成並びに肺転移の誘発

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先に，我々はアフラトキシ B₁をフィッシャー 344系雄ラットに投与して肝細胞癌を誘発し，肝癌細胞株 AFB-1を分離した．本研究において，我々はAFB-1のmRNAとラット正常肝細胞株BL9のmRNAについてサブトラクションcDNAライブラリーを作製し，ノーザン・ブロット解析によりAFB-1のmRNAとハイブリッドを形成するがBL9のmRNAとはハイブリッドを形成しない650 bp cDNAを検出した．我々は，650 bp cDNA/ベクター組換え体DNAをAFB-1とBL9に導入し，次いで，元の細胞または組換え体DNA導入した細胞 1×10^7 個を同系ラットの背部皮下に移植した．650 bp cDNA/ベクター組換え体DNAの導入はAFB-1の皮下腫瘍形成率を増大させ（元のAFB-1を移植した雄ラットにおける形成率が13例中9例に対し，組換え体DNA導入AFB-1を移植した雄ラットにおける形成率は14例中14例， $P < 0.02$ ），また，肺転移率も増大させた（元のAFB-1を移植した雄ラットにおける転移率は13例中0例に対し，組換え体DNA導入AFB-1を移植した雄ラットにおける転移率は14例中9例， $P < 0.001$ ）．650 bp cDNA/ベクター組換え体DNAの導入は正常肝細胞株BL9を癌細胞に転換した，即ち，組換え体DNAを導入したBL9は雄ラット10例中9例，雌ラット10例全例に皮下腫瘍を形成し，また，雌ラット10例中6例に肺転移巣を形成した．本研究は，サブトラクションcDNAライブラリーから分離したcDNAが癌細胞の腫瘍形成並びに肺転移を促進すると共に，正常細胞に腫瘍形成能並びに肺転移能を誘発することを初めて示したものである．

キーワード：アフラトキシ B₁，cDNA，転移，肝細胞癌，ラット