Expression of the Elm1 Gene, a Novel Gene of the CCN (Connective Tissue Growth Factor, Cyr61/Cef10, and Neuroblastoma Overexpressed Gene) Family, Suppresses In Vivo Tumor Growth and Metastasis of K-1735 Murine Melanoma Cells

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Summary

We previously isolated a partial cDNA fragment of a novel gene, Elm1 (expressed in low-metastatic type 1 cells), that is expressed in low-metastatic but not in high-metastatic K-1735 mouse melanoma cells. Here we determined the full-length cDNA structure of Elm1 and investigated the effect of Elm1 expression on growth and metastatic potential of K-1735 cells. The Elm1 gene encodes a predicted protein of 367 amino acids showing ~40% amino acid identity with the CCN (connective tissue growth factor [CTGF], Cyr61/Cef10, neuroblastoma overexpressed gene [Nov]) family proteins, which consist of secreted cysteine-rich proteins with growth regulatory functions. Elm1 is also a cysteine-rich protein and contains a signal peptide and four domains conserved in the CCN family proteins. Elm1 was highly conserved, expressed ubiquitously in diverse organs, and mapped to mouse chromosome 15. High-metastatic K-1735 M-2 cells, which did not express Elm1, were transfected with an Elm1 expression vector, and several stable clones with Elm1 expression were established. The in vivo growth rate of cells expressing a high level of Elm1 were remarkably slower than those of cells expressing a low level of Elm1. Metastatic potential of transfectants was reduced in proportion to the level of Elm1 expression. Thus, Elm1 is a novel gene of CCN family that can suppress the in vivo growth and metastatic potential of K-1735 mouse melanoma cells.

It is now widely accepted that malignant tumors contain heterogeneous populations of cells with regard to metastatic potential (1). The process of cancer metastasis consists of linked sequential steps, including invasion, detachment, intravasation, circulation, adhesion, extravasation, and growth in distant organs (2). Thus, high- and low-metastatic cells in a tumor should be different each other with respect to several biological properties, such as invasiveness, adhesiveness, motility, and proliferation potential. There is much evidence to support the concept that each discrete step of metastasis is regulated by transient or permanent changes at the DNA, messenger RNA (mRNA), and/or protein levels in different genes (3–5). By using the mRNA differential display method, we previously identified a partial 3′ cDNA fragment of a novel gene, Elm1 (for expressed in low-metastatic type 1 cells), that is expressed in low-metastatic K-1735 murine melanoma cells (6). Elm1 was also differentially expressed between high- and low-metastatic B16 murine melanoma cells. A partial Elm1 cDNA fragment of 211 nucleotides showed no significant homology to any recorded sequence in the DDBJ/GenBank/EMBL DNA databases.

Here we determined the full-length cDNA structure of Elm1.
the Elm1 gene. The Elm1 gene encoded a novel type of CCN (connective tissue growth factor [CTGF], Cyr61/ Cef10, and neuroblastoma overexpressed gene [N ov]) family proteins that consisted of secreted cysteine-rich molecules, CTGF, Cyr61/Cef10, and N ov (7–11). CTGF and Cyr61 were induced within minutes of stimulation by serum or growth factors (10, 12) and have a growth regulatory function in fibroblasts or endothelial cells (13, 14). N ov is a protooncogene isolated from myeloblastomatosis-associated virus-induced nephroblastoma (9). Because of the high level of sequence similarities between Elm1 and the other members of the CCN family, it is likely that Elm1 has a function to regulate the growth of normal and/or cancerous cells. Since Elm1 gene expression was high in low-metastatic tumor cells and declined with increasing metastatic potential in two rodent experimental systems, we investigated the biological effects of the Elm1 gene on in vitro growth, in vivo growth, and metastatic potential of K-1735 murine melanoma cells. By introduction of an Elm1 expression vector into high-metastatic K-1735 M-2 cells that did not express endogenous Elm1, it was revealed that expression of Elm1 can inhibit tumor growth in vivo as well as metastasis of K-1735 melanoma cells.

Materials and Methods

Cell Lines. K-1735–derived mouse melanoma cell lines were obtained from I. J. Fidler (University of Texas, Houston, TX; reference 15). Clone 23 (designated C-23) was classified as low metastatic, whereas clone M-2 was high metastatic in syngeneic recipients (16, 17). Serum stimulation was performed using BALB/c 3T3 cells.

Cell Culture and mRNA Isolation. K-1735 cells and BALB/c 3T3 cells were maintained in tissue culture in the DME-10 (DME supplemented with 10% fetal calf serum, sodium bicarbonate solution, l-glutamine, and penicillin-streptomycin). Cells were maintained on plastic and were incubated in 5% CO supersaturated air at 37°C. Cells at 70% confluency were harvested and subjected to mRNA extraction. mRNA was isolated using Fast Track mRNA Isolation Kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's recommendations.

Screening of cDNA Library. To isolate full-length cDNA clones, cDNA libraries from K-1735 C-23 (low-metastatic clone) constructed in lambda ZAP II (Stratagene Corp., La Jolla, CA) using cDNA Synthesis System Plus (Amersham Corp., Arlington Heights, IL) were screened with the cloned cDNA fragment of Elm1 that was previously isolated by an mRNA differential display (6). Isolated clones were sequenced by the A.L.F. DNA Sequencer II with the AutoRead Sequencing Kit (Pharmacia Biotech, Piscataway, N J). DNA sequences were aligned, examined for open reading frames, and compared with DNA sequences in the DDBJ/GenBank/EMBL and amino acid sequence in the SWISS-PROT and PIR protein databases using the FASTA and BLAST programs.

Northern and Southern Blot Analyses. 3 μg of poly(A)+ RNA were size fractionated on a denaturing formaldehyde agarose gel (1.0%) and transferred onto Hybond-N+ membrane (Amersham Corp.). Mouse multiple tissue Northern (MTN) blot, containing 2 μg of poly(A)+ RNA from a variety of tissues, and ZOO BLOT, containing 8 μg of EcoRI-digested genomic DNA from various species, were obtained from Clontech (Palo Alto, CA). Northern blot hybridization was performed at 42°C for 24 h with hybridization buffer containing 50% formamide, 5× NET (750 mM NaCl, 5 mM EDTA, and 75 mM Tris-HCl), 0.5% dry milk, 0.4% SDS, 10% dextran sulfate, and 0.2 mg/ml salmon sperm DNA. Then the membrane was washed with 0.1× SSC and 0.1% SDS at 65°C for 60 min. Southern blot hybridization was performed at 37°C with hybridization buffer described above, and the membrane was washed with 0.1× SSC and 0.1% SDS at 55°C. To confirm the amounts of mRNA loaded in each lane, the blots were hybridized afterwards with a human β-actin probe (Clontech). A DNA probe for the Elm1 gene corresponded to nucleotides 33–2399 of the Elm1 cDNA fragment. A DNA probe corresponding to nucleotides 337–1119 of the Cyr61 mRNA (DDBJ/GenBank/EMBL accession number: M32490) was synthesized by reverse transcription PCR.

Chromosome Mapping of the Elm1 Gene. An interspecific backcross panel formed from (DBA/2J × MSM)F1 × DBA/2J (MSM, M us musculus molossinus) was used for mapping of the Elm1 gene (18). Genotypes of Elm1 for 138 individuals in this panel were determined by RFLP observed in PCR-amplified products. A genomic DNA fragment of 1,193 bp in the Elm1 locus was amplified by primers 5'-CGATATCTTTGCTGACTTG-3' (sense strand) and 5'-CTGAGCTGTAAGAGTCTC-3' (antisense strand), corresponding to nucleotides 1223–1242 and 2396–2415, respectively. The restriction enzyme Sau3AI yielded an easily distinguishable polymorphism between two parental strains, MSM and DBA/2J. The data generated in this study was analyzed (Map Manager v 2.5.6; Roswell Park Cancer Institute, N Y; reference 19).

Serum Stimulation of BALB/c 3T3 Cells. Quiescent BALB/c 3T3 cells were prepared by growth in DMEM-10 to confluence and were incubated in DMEM-0.5% (0.5%) serum for 2 d. For stimulation of quiescent cells, the medium was changed to DMEM-20% (20% serum).

Construction of Expression Vector and DNA Transfection. The pcDNA3 expression vector was purchased from Invitrogen. A cDNA fragment of Elm1 (146–1,309 nucleotides [nt]) consisting of 28 nt of the 5'-untranslated region, 1,101 nt of the coding region, and 35 nt of the 3'-untranslated region was amplified using the primer set Elm1-B (146–165 nt; GTAGCTCCTGT-GCATGGAACTTTACCCTGAG), and ligated to the pcDNA3 expression vector, pcDNA3-Elm1 (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's recommendations.

Expression of Elmarin and DNA Transfection. The pcDNA3 expression vector was purchased from Invitrogen. A cDNA fragment of Elm1 (146–1,309 nucleotides [nt]) consisting of 28 nt of the 5'-untranslated region, 1,101 nt of the coding region, and 35 nt of the 3'-untranslated region was amplified using the primer set Elm1-B (146–165 nt; GTAGCTCCTGT-GCATGGAACTTTACCCTGAG), and ligated to the pcDNA3 vector at the BamHI site (pcDNA3-Aelm1) in the direction of plus strand. K-1735 M-2 cells were transfected with pcDNA3-Aelm1 using LipofectAMINE reagent (GIBCO BRL, Gaithersburg, M D). After 16 h, the medium was changed to DMEM-10. At 38 h of transfection, G418 selection was imposed (800 μg/ml; Geneticin; GIBCO BRL). G418-resistant cells were cloned by using the penicillin cap method and maintained in the medium containing G418. Cell clones resistant to G418 were assayed for the expression of Elm1 mRNA by Northern blotting.

Cell Growth and Tumorigenicity. K-1735 M-2, C-23, and transfectants were seeded at the density of 5 × 104 cells/ml in a 24-well plate. The cells were counted every day from days 1 to 6. 5 wk-old female C3H/HeN mice were obtained from the Animal Production of Japan K urea Corporation (Tokyo, Japan). Tumorigenicity was examined by injecting 106 cells/ml into the subcutis of the mice (n = 5). The length and width of each tumor were recorded three times per week. The tumor volume (V) was calculated by the formula V = 1/2 × length × width2.

Experimental Metastasis Assay. M etastatic potential of the cells was measured by the quantitative lung colony assay as described by Fidler et al. (20). In brief, the cells were injected into the tail vein of 5-wk-old female C3H/HeN mice at the density of 106
cells/0.2 ml (n = 5). Mice were killed when three of five mice with K-1735 M-2 injection were dead, that is, 22 d (Table 2, experiment 1) and 23 d (Table 2, experiment 2) after cell inoculation. The number of lung metastatic colonies > 1 mm in diameter was counted with the aid of magnifying glass.

Statistical Analysis. The in vivo data were analyzed by the Mann-Whitney U test.

DDBJ/GenBank/EMBL Accession Numbers. Cyr61: M32490; mouse Nov (NovM): X96585; fibroblast-inducible secreted protein (Fisp12): M70642.

Results

Isolation and Characterization of the Elm1 Gene. We screened cDNA libraries from low-metastatic K-1735 C-23 cells constructed with oligo dT and random primers using an Elm1 cDNA fragment of 211 bp as a probe. Several clones were obtained, sequenced, and aligned. We have assembled a composite 5,020-bp transcript using the sequences of seven independent clones (Fig. 1). We verified that the assembled sequence was derived from a single gene by reverse transcription PCR. Searches of the DDBJ/GenBank/EMBL nucleotide databases indicated that this sequence has not been reported.

The composite cDNA contained an open reading frame of 1,101 bp with a potential start codon of ATG, which was located 10 bp downstream of an in-frame stop codon. Using this methionine as a translation start site, a peptide of 367 amino acids (40.7 kD) was inferred (Fig. 1). Sequence homology was detected between the predicted amino acid sequence of the Elm1 protein and those of the CCN family proteins. The Elm1 protein showed 38.1, 44.0, and 42.6% identity with Cyr61, Fisp12 (mouse orthologue of CTGF; reference 10), and NovM, respectively. Elm1 has a signal peptide that is conserved in all of the recorded CCN family proteins. The deduced Elm1 amino acid sequence contains a hydrophobic amino terminus with a predicted signal cleavage site between Ala (position 24) and Leu (position 25; reference 21; Fig. 1). CCN family proteins are cysteine rich (10% of all residues) and conserve the four domains: insulin-like growth factor binding protein (IGFBP)-like domain, von Willebrand factor type C repeat (VWC), thrombospondin type 1 repeat (TSP1) domain, and COOH-terminal (CT) domain (7) (Figs. 1 and 2). The deduced Elm1 amino acid sequence contains 38 of the cysteine residues (10.4% of all residues) and showed 40–60% identity with...
Cyr61, Fisp12, NovM in each of the four domains (Fig. 2). Similarities of the amino acid sequence outside the four domains were insignificant for any of the three subclasses. A dendrogram indicates that Elm1 is not an orthologue of other CCN family members (Fig. 2B).

The Elm1 gene is expressed among different species (Fig. 3A), and highly expressed in the kidney and lung, and at lower levels in the heart, brain, spleen, liver, skeletal muscle, and testis (Fig. 3B). The Elm1 locus was mapped to chromosome 15, between the D15Mit17 and D15Mit3 loci, which is not a site of known mouse CCN family genes (22, 23; Fig. 4).

Quiescent BALB/c 3T3 cells were stimulated by serum, and the expression of Elm1 and Cyr61 were examined by Northern blot analysis (Fig. 5). Elm1 was not induced within 30 min, but was induced after 3 h of serum stimulation. Although the amount of β-actin mRNA in lane 1 quantified by densitometric analysis was about half of that in lane 3, the relative level of Elm1 expression in lane 3, that is, the ratio of Elm1 mRNA over β-actin mRNA, was seven times higher than that in lane 1. On the other hand, Cyr61 expression was markedly induced within 30 min (Fig. 5).

Effects of Elm1 Expression on the Growth and Metastasis of Mouse Melanoma Cells

We examined the in vitro growth properties of K-1735 M-2 and transfectants. Transfectants that express large amounts of Elm1, M-2.Elm1.20-3, and M-2.Elm1.23-1 cells showed slightly increased population doubling time and decreased saturation density compared to those of K-1735 M-2 cells (Table 1). Morphological diversities were not observed among the clones in association with the level of Elm1 expression. We next injected the clones subcutaneously into female C3H/HeN mice to evaluate the effect of Elm1 expression on tumorigenicity. All clones were tumorigenic, but the incidence of tumor formation was decreased in the M-2.Elm1.23-1 cells. Moreover, in vivo growth rates of the transfectants became slower in proportion to the increase in the level of Elm1 expression (Fig. 7 and Table 1).

Two independent experiments were performed to evaluate the metastatic ability of transfectants. The numbers of metastatic colonies of the transfectants became smaller in proportion to the increase in the level of Elm1 expression.
Table 1. In Vitro Growth Properties and Tumorigenicity of K-1735 M-2 Cells Transfected with the Elm1 Gene

| Cell line      | Expression of Elm1* | Population doubling time† | Saturation density in media with 10% FCS | Tumorigenicity‡ |
|----------------|---------------------|----------------------------|------------------------------------------|-----------------|
|                |                     |                            |                                          | Expt. 1         | Expt. 2         |
| K-1735 M-2     | 0.0                 | 13.8                       | $1.1 \times 10^6$                       | 5/5 (21)        | 5/5 (22)        |
| M-2.Elm1.18-3  | 0.0                 | 13.1                       | $0.9 \times 10^6$                       | 5/5 (20)        | 5/5 (19)        |
| M-2.Elm1.20-2  | 0.2                 | 13.5                       | $0.8 \times 10^6$                       | 4/5 (27)        | 5/5 (21)        |
| M-2.Elm1.10-1  | 0.6                 | 15.0                       | $0.9 \times 10^6$                       | 3/5 (32)        | 5/5 (24)        |
| M-2.Elm1.6-2   | 1.4                 | 13.3                       | $1.1 \times 10^6$                       | 5/5 (37)        | 5/5 (28)        |
| M-2.Elm1.20-3  | 1.6                 | 16.1                       | $0.5 \times 10^6$                       | 4/5 (>60)       | 5/5 (>60)       |
| M-2.Elm1.23-1  | 6.3                 | 16.0                       | $0.3 \times 10^6$                       | 2/5 (46)        | 1/5 (>60)       |
| K-1735 C-23    | 1.0                 | 13.5                       | $0.9 \times 10^6$                       | 0/5            | 1/5 (49)        |

* The amount of Elm1 and β-actin mRNA was quantified by Northern blot densitometric analysis of the autoradiogram. RNA loading levels were standardized with respect to the amount of β-actin mRNA. Levels of standardized Elm1 mRNA were expressed relative to the standardized level in K-1735 C-23 cells.

† Growth rate was estimated from the logarithmic phase of the growth curve.

‡ Observed for up to 60 d after inoculation of $10^6$ viable cells into 5-wk-old syngeneic mice. Tumorigenicity is expressed by the number of mice with tumors per the number of mice injected. Average days of tumors that reached 1 cm$^3$ in each group are shown in parentheses.
likely that Elm1 itself has growth regulatory function on the NIH3T3 fibroblast. However, it is possible that Elm1 might enhance the mitogenic effect of growth factors other than bFGF.

We found that Elm1 gene transfection resulted in an inhibition of in vivo growth and metastasis formation. Expression of Elm1 had little suppressive effect on in vitro growth, but a marked suppressive effect on in vivo growth. Suppressive effect of Elm1 on metastatic potential was associated with suppressive effect of Elm1 on in vivo growth. In M-2.Elm1.23-1 and M-2.Elm1.20-3 cells that express high levels of Elm1, a reduction in the incidence of subcutaneous tumor formation as well as metastatic formation was observed. The reason why the cells failed to form tumors is presently unknown, but may result from alterations in tumor cell–host interactions, such as angiogenesis and/or responses to stimulatory and suppressive cytokines (25). TSP1 inhibits angiogenesis and modulates endothelial cell adhesion, motility, and growth. The antiproliferative activity of TSP1 is mimicked by a synthetic peptide derived from the type I repeats of TSP1 that antagonizes fibroblast growth factor and induces programmed cell death in bovine aortic endothelial cells (26). Thus, it is possible that an inhibition of angiogenesis by the TSP1 domain of Elm1 leads to the suppression of in vivo growth. Further studies are now in progress on this subject.

Elm1 was isolated as a gene differentially expressed between high- and low-metastatic K-1735 mouse melanoma cells. In K-1735 systems, actin organization, cell adhesion, motility, and a growth rate at a subcutaneous site have been shown to be different between high- and low-metastatic cells (27–29). Elm1 could be a gene that is involved in the growth of K-1735 cells in vivo. It is noted that the Elm1 gene is one of several genes involved in the regulation of metastatic potential in K-1735 cells since it was previously shown that expression of the inducible nitric oxide synthase and nm23 genes suppresses tumorigenicity and metastasis of K-1735 cells (25, 30). These sets of genes would cooperatively affect the metastatic potential of K-1735 cells. A more detailed characterization of the Elm1 gene should allow a critical analysis of the molecular mechanism of metastasis in K-1735 cells.

**Table 2.** Experimental Lung Metastasis of K-1735 M-2 Cells Transfected with the Elm1 Gene

| Cell line* | Incidence | Median | Range |
|------------|-----------|--------|-------|
| Expt. 1    |           |        |       |
| K-1735 M-2 | 5/5       | 126    | 36-162|
| M-2.Elm1.8-3 | 5/5    | 149    | 143-176|
| M-2.Elm1.20-2 | 4/5   | 115    | 0-148 |
| M-2.Elm1.0-1 | 5/5   | 71     | 27-127|
| M-2.Elm1.6-2 | 3/5   | 28     | 0-136 |
| M-2.Elm1.20-3 | 5/5   | 3‡     | 2-7   |
| M-2.Elm1.23-1 | 0/5   | 0‡     | 0     |
| K-1735 C-23  | 0/5     | 0‡     | 0     |
| Expt. 2    |           |        |       |
| K-1735 M-2 | 5/5       | 94     | 10-153|
| M-2.Elm1.8-3 | 5/5    | 123    | 34-156|
| M-2.Elm1.20-2 | 4/5   | 66     | 0-142 |
| M-2.Elm1.0-1 | 3/5   | 10     | 0-100 |
| M-2.Elm1.6-2 | 5/5   | 5      | 3-116 |
| M-2.Elm1.20-3 | 3/5   | 8      | 0-11  |
| M-2.Elm1.23-1 | 3/5   | 1‡     | 0-7   |
| K-1735 C-23  | 0/5     | 0‡     | 0     |

* Syngeneic mice were injected intravenously with $10^5$ viable cells and killed after 22 (experiment 1) and 23 d (experiment 2) of injection. The number of lung colonies was determined using a magnifying glass.
‡ The number of lung colonies of these cells were significantly different from that of K-1735 M-2 cells (Mann-Whitney U test, $P < 0.01$).
Figure 8  Metastatic potential of K-1735 M-2 cells transfected with the Elm1 gene. Syngeneic mice were injected intravenously with $10^5$ cells and killed 23 d after injection. A, K-1735 M-2; B, M-2.Elm1.8-3; C, M-2.Elm1.20-3; D, M-2.Elm1.23-1.

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