A novel gene derived from developing spinal cords, *SCDGF*, is a unique member of the PDGF/VEGF family[1]

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Abstract We isolated a novel gene designated spinal cord-derived growth factor (*SCDGF*). Its expression was increased in chick spinal cords with embryonic development and decreased after hatching. The amino acid sequences of chick and human SCDGFs revealed a putative signal sequence followed by a CUB domain and a region homologous to the members of the platelet-derived growth factor/vascular endothelial growth factor family. Furthermore, human SCDGF secreted from the cells showed a mitogenic activity for 10T1/2 cells in vitro. These results led us to speculate that SCDGF plays an important role in the development of the spinal cord.

Key words: Spinal cord-derived growth factor; Differential display; Chick spinal cord; Mitogenic activity; CUB domain; Platelet-derived growth factor/vascular endothelial growth factor family

1. Introduction

To understand the function of the nervous system in maturity, it is important to determine the mechanism by which the nervous system is formed. During neural development, cells differentiate to specific types of neurons and undergo migration, synapse formation and cell death precisely with respect to time and location [1–5]. Accumulating evidence indicates that morphological differentiation is based on cell–cell interactions through molecules which are located on cell surfaces or secreted from cells [2,3]. Therefore, one of the approaches to reveal the mechanism underlying neural differentiation is to identify molecules, expression of which changes during development.

The development of the spinal cord has been extensively studied morphologically using chick embryos [6,7]. One of the phenomena which occurs during the development is the so-called naturally occurring neuronal cell death: The large ventral horn cells, probably motoneurons, reach their maximum number at embryonic day (E) 5.5 and almost half of them die until E10 in the chick embryo [8]. The trophic dependency hypothesis is now generally accepted as the mechanism of this motoneuronal cell death [9–11].

The present study was conducted to isolate molecules related to differentiation of the spinal cord in chick embryos. To this aim, using the differential display procedure, we isolated several genes, expressions of which changed drastically from E4 to E10. Among them, here we report a novel gene designated spinal cord-derived growth factor (*SCDGF*), the expression of which increased within the limited period of embryonic development of chick spinal cords. The result indicates that SCDGF is secreted from the cells and exhibits a mitogenic activity in vitro. A part of this study was communicated elsewhere [12].

2. Materials and methods

2.1. Animals and cell lines

Fertilized white Leghorn chicken eggs were purchased from a local poulterer (Miyake Eggs). The eggs were incubated at 37°C in a humidified egg chamber. Chinese hamster CHO-K1 cells and mouse 10T1/2 cells obtained from the RIKEN Cell Bank were propagated in Dulbecco's modified Eagle's medium and basal medium Eagle (Gibco BRL), respectively, supplemented with antibiotics (penicillin (10 U/ml, Meiji) and streptomycin (50 μg/ml, Meiji)) and 10% heat-inactivated fetal bovine serum (FBS) (Mitsubishi Kasei).

2.2. RNA isolation, differential display and cDNA cloning

For differential display, 5′-rapid amplification of cDNA ends (RACE) and Northern blotting, total RNAs were extracted from the chick spinal cords and the cultured cells by the LiCl precipitation method. The differential display method was performed using the RNAimage kit (GenHunter) with Ampli Taq Gold (Perkin-Elmer) and [α-^{35}S]dATP (NEN). Samples were resolved in parallel lanes on 6% denaturing polyacrylamide gels. The differentially expressed cDNAs excised from the gels were reamplified, cloned into the pT7-Blue(R) vector (Novagen) and sequenced. To obtain a full-length open reading frame (ORF), 5′-RACE was carried out using the 5′-RACE System, version 2.0 (Gibco BRL). The first strand cDNA was synthesized from the total RNA using a specific primer designed from the sequence of each gene with SUPERSCRIPT II reverse transcriptase (Gibco BRL). Homopolymeric (oligo-dC) tails were added with dCTP to the 3′-ends of the cDNAs using terminal deoxynucleotidyl transferase (Gibco BRL). PCR reactions were performed for amplification of the products using the 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′ primer and a specific primer for each gene. The 5′-RACE products were subcloned into the pT7-Blue(R) vector and sequenced on both strands. Sequencing was performed using a dye-terminator cycle-sequencing ready reaction kit (Perkin-Elmer). Each sample was electrophoresed with an ABI 377 sequencer (Perkin-Elmer).

2.3. Isolation of human SCDGF cDNA

A homology search against the dbEST database of GenBank based

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[1] The nucleotide sequences for chick and human *SCDGF*s have been deposited in the DDBJ/GenBank database with accession numbers AB033829 and AB033831, respectively.

Abbreviations: SCDGF, spinal cord-derived growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; E, embryonic day; TLD, tolloid; BMP1, bone morphogenetic protein 1; NP, neuropilin; 5′-RACE, 5′-rapid amplification of cDNA ends; EST, expression sequence tag; c, chicken; h, human; ORF, open reading frame

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on the amino acid sequence of c*SCDGF* was performed using the TBLASTN program. Two human ESTs (GenBank accession numbers W21436 and AA759138) showed significant similarity to c*SCDGF*. Based on the sequences of these ESTs, two primers (5′-GAAGTTGAGGAACCCAGTGA-3′ and 5′-ACGTCGGTGAGTGATTTGTG-3′) were used for PCR to isolate a cDNA fragment from a human fetal brain cDNA library. Using this fragment as a probe, a full-length h*SCDGF* cDNA was isolated from this cDNA library.

2.4. Mitogenic activity assay using hSCDGF stably transfected cells

A DNA fragment containing a full-length h*SCDGF*, the same fragment followed with the Flag octapeptide coding sequence (h*SCDGF*-Flag), and the h*SCDGF*-Flag lacking the first 10 amino acids of the signal sequence (hNS-*SCDGF*-Flag) were inserted into an expression vector, pcDNA3 (Invitrogen). CHO-K1 cells were stably transfected with the control pcDNA3 vector, pcDNA3 containing h*SCDGF*, h*SCDGF*-Flag or hNS-*SCDGF*-Flag using Lipofectamine™ reagent (Gibco BRL) according to the manufacturer's protocol. Three days after the cells were inoculated at 5×10^5 cells/ml, the supernatant was collected and filtered to remove cell debris. The mitogenic activity of the supernatant was assayed using the mouse 10T1/2 cell line which is one of the mouse embryo fibroblast cell lines. The 10T1/2 cells were seeded at 0.5×10^5 cells/0.5 ml/well in each well of a 24-well plate, and 0.5 ml of the supernatant was added to the wells in triplicate. The number of cells was counted under a phase contrast microscope at indicated days in Fig. 3B.

2.5. Northern blot analysis

Total RNAs (20 μg) were run on a denaturing formaldehyde-agarose gel and transferred to nylon membranes. RNAs blotted onto the membranes were hybridized with a [^{32}P]-labeled full-length c*SCDGF* or h*SCDGF* fragment encoding a full-length ORF as the probe at 65°C in a buffer containing 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA. The membranes were washed twice for 40 min at 65°C by immersing in a solution of 0.2×SSC and 0.1% SDS. The membranes were analyzed with a BAS2000 system (Fuji) and exposed to an X-ray film.

3. Results

3.1. Molecular cloning of cSCDGF and hSCDGF

To identify proteins involved in spinal cord differentiation, we screened genes, expressions of which in chick spinal cords changed from E4 to E10, by the differential display method. Twenty-four cDNA fragments of 4200 examined were differentially expressed (data not shown). Because sequence analyses indicated that most of the fragments did not contain the 5′-regions, we obtained their full-length ORFs using the 5′-RACE method. The nucleotide sequence analysis of one of these cDNAs, the expression of which increased from E4 to E10, revealed that it is a novel gene and has a sequence of 1038 nucleotides encoding a putative protein composed of 345 amino acids (relative molecular mass of 38 938, Fig. 1A). We named this novel gene as spinal cord-derived growth factor (*SCDGF*). To isolate a mammalian homologue, we screened a human fetal brain cDNA library as described in Section 2, and obtained a cDNA clone containing a full-length hSCDGF cDNA encoding a polypeptide (Fig. 1A). The putative amino acid sequences of cSCDGF and hSCDGF were 84.1% identical (Fig. 1A). The hydrophobicity profile of cSCDGF showed that the N-terminal 14 amino acids were highly hydrophobic (Fig. 2) and could encode a signal peptide [13]. Based on a homology search carried out with the BLASTX program using SWISS-PROT, it was revealed that two structural domains already known followed a putative signal sequence: residues 47–163, a region homologous to the CUB domain [14], and residues 234–345, a region homologous to a conserved region in members of the PDGF/VEGF family.

The result of the homology search indicates that the amino acid residues 47–163 of SCDGFs are highly homologous to the CUB domain of the *Xenopus* neuronal recognition molecule, neuropilin (NP) [15]. Fig. 1B illustrates the multiple alignment of cSCDGF and hSCDGF compared with the CUB domains contained in human bone morphogenetic protein 1 (BMP1) [16], *Drosophila* dorso-ventral patterning gene product, tolloid (TLD) [17], human complement subcomponents C1r/C1s [18,19], and NP. SCDGFs showed amino acid identity of about 30% with these CUB domains. Therefore, we considered that the region encoded a CUB domain. Although most of the CUB domains have been reported to contain four cysteines, both cSCDGF and hSCDGF lack the first two cysteines (Fig. 1B).

Only two chicken genes have been reported in the GenBank database as members belonging to the PDGF/VEGF family; cPDGF-A (Accession No. AAF01459) and cVEGF-A (Accession No. P52582). In a conserved region, cSCDGF showed 30.4% and 26.8% amino acid identities with cPDGF-A and cVEGF-A, respectively (Fig. 1C). For human genes belonging to the PDGF/VEGF family, hSCDGF showed 32.1% identity with both hPDGF-A [20] and -B [21], and 29.5%, 25%, 24.1% and 24.1% with hVEGF-D [22], -B [23], -C [24] and -A [25], respectively (Fig. 1C). In addition, cSCDGF and hSCDGF had the eight cysteine residues, which are conserved among members of the PDGF/VEGF family and have been shown to be essential for the correct folding and dimerization by intra- and intermolecular disulfide bonding [20–25], although additional four cysteine residues were also found in this region (Fig. 1C). Furthermore, the amino acid sequence motif PXCVXXXRCXGCC, a hallmark of the PDGF/VEGF family of growth factors, was conserved though not completely. The fourth Val residue in the motif was replaced with Leu, and three amino acids, Asn-Cys-Ala, were inserted before the last two Cys residues in both cSCDGF and hSCDGF.

3.2. The mitogenic activity of hSCDGF in vitro

Members belonging to the PDGF/VEGF family have been

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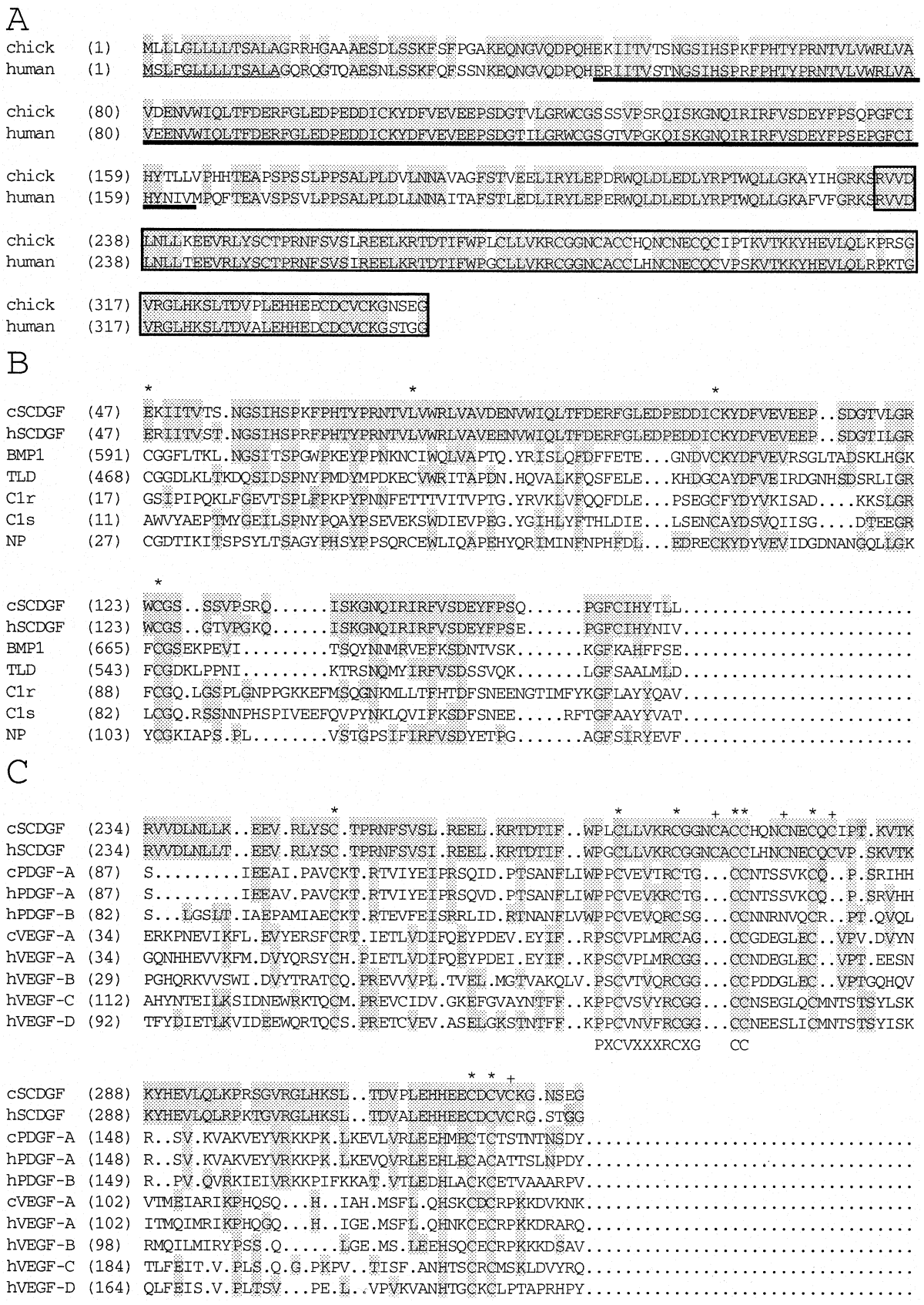
Fig. 1. Deduced amino acid alignments of chick and human SCDGFs. A: Comparison of deduced amino acid sequences of cSCDGF and hSCDGF. A putative secretory signal peptide is underlined. A bold underline indicates a CUB domain, and PDGF/VEGF homologous regions are boxed. Amino acids identical between cSCDGF and hSCDGF are shaded. B: Comparison of CUB domains of cSCDGF and hSCDGF (residues 47–163) with the other CUB domains contained in BMP1 [16], TLD [17], C1s/C1r [18,19] and NP [15]. Amino acids identical to cSCDGF and hSCDGF are shaded. Asterisks indicate the positions of cysteine residues which are characteristic of the CUB domain. Numbers on the left indicate amino acid positions relative to the initiator methionine residue. C: Comparison of conserved regions homologous to the PDGF/VEGF family of cSCDGF and hSCDGF with cPDGF-A chain (GenBank Accession No. AAF01459), hPDGF-A chain [20], hPDGF-B chain [21], cVEGF-A (GenBank Accession No. P52582), hVEGF-A [25], hVEGF-B [24], hVEGF-C [23] and hVEGF-D [22]. Amino acids identical to those of SCDGFs are shaded. Asterisks indicate the cysteine residues which are characteristic of the PDGF/VEGF family, and pluses indicate the additional cysteine residues specific to SCDGF. A conserved sequence motif, PXCVXXXRCXGCC, in all members of the PDGF/VEGF family is denoted under the compared sequences. Numbers on the left indicate amino acid positions relative to the initiator methionine residue.



reported to be growth factors with mitogenic activities in vivo or in vitro [22–28]. Therefore, we examined whether hSCDGF protein is secreted from cells and acts as a growth factor. We generated CHO-K1 cells stably transfected with the mammalian expression vector containing h*SCDGF*, h*SCDGF*-Flag or hNS-*SCDGF*-Flag (see Section 2). The expression of their mRNAs in the transfectants was verified by Northern blot analysis using a probe containing the h*SCDGF* cDNA (Fig.

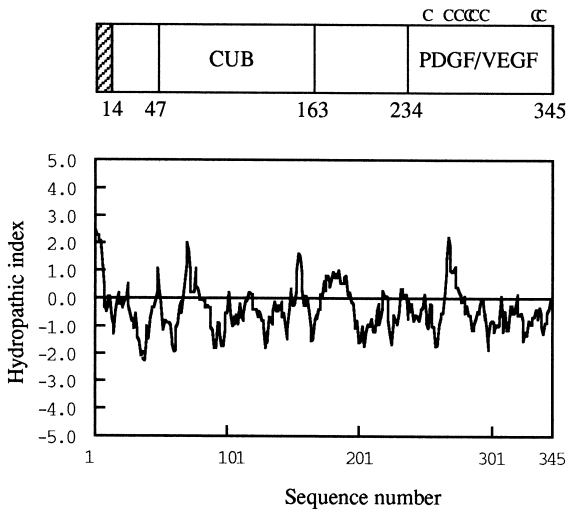


Fig. 2. Schematic structure and hydrophobicity profile of cSCDGF. The Cs indicate the relative positions of the cysteine residues which are characteristic of the PDGF/VEGF family. The shaded box indicates the putative signal peptide. The numbers under the structural map are amino acid positions relative to the initiator methionine residue. CUB, CUB domain; PDGF/VEGF, a region homologous to a conserved region in members of the PDGF/VEGF family. Analysis of hydrophobicity was performed by the DNASIS ver. 3.0 program (Hitachi Software Engineering) using the parameter of Kyte and Doolittle [33].

3A). At the expected size, a single band was detected for each of the transfectants except that for the CHO-K1/vector, although its intensity in CHO-K1/h*SCDGF* cells was about 30-fold stronger than that in CHO-K1/h*SCDGF*-Flag or CHO-K1/hNS-*SCDGF*-Flag cells (data not shown). The mitogenic activities of the culture supernatants of these transfectants were examined by adding them to 10T1/2 cell cultures, which was been shown to respond to PDGF [29]. The supernatants of CHO-K1/h*SCDGF* or CHO-K1/h*SCDGF*-Flag cells stimulated the proliferation of 10T1/2 cells (Fig. 3B). However, no mitogenic activity of the supernatant from the CHO-K1/vector or CHO-K1/hNS-*SCDGF*-Flag was detected (Fig. 3B). The activity of the supernatant of CHO-K1/h*SCDGF* was stronger than that of CHO-K1/h*SCDGF*-Flag.

3.3. Developmental pattern of cSCDGF mRNA expression in chick spinal cords

The developmental changes in c*SCDGF* mRNA expression in chick spinal cords were examined by Northern blot hybridization using the probe containing the c*SCDGF* ORF. A single band of approximately 3.2 kb was detected (Fig. 4). The level of expression of this mRNA gradually increased from E4 to E10. It was highest at E12–16 and rapidly decreased to a low level after hatching.

4. Discussion

In this study, we identified a novel gene, c*SCDGF*, the expression of which increased in chick spinal cords during embryonic development and then decreased after hatching. The putative amino acid sequences of cSCDGF and hSCDGF revealed a signal sequence consisting of 14 hydrophobic amino acids at its N-terminus, followed by a region homologous to the CUB domain and a region homologous to the conserved region of PDGF/VDGF family members.

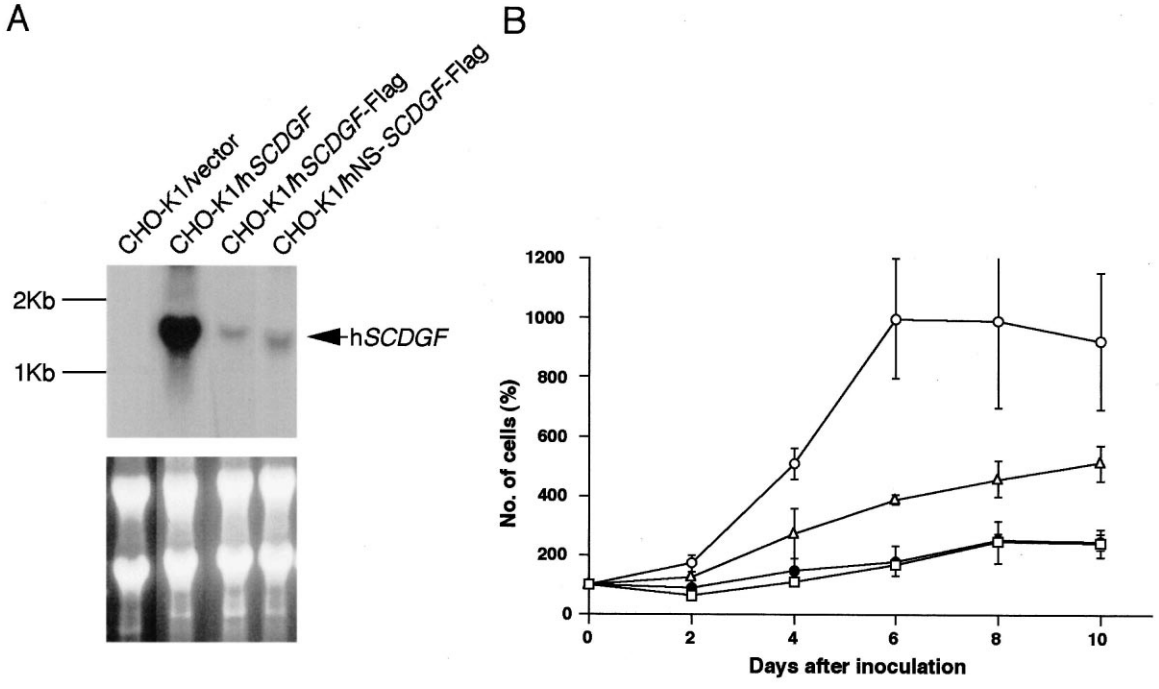


Fig. 3. Mitogenic activity of the supernatants of CHO-K1 cells stably transfected with h*SCGDF*. A: Northern blot analysis of h*SCDGF* mRNA expression in CHO-K1/vector, CHO-K1/h*SCDGF*, CHO-K1/h*SCDGF*-Flag and CHO-K1/hNS-*SCDGF*-Flag cells. The expected position of h*SCDGF* mRNA is indicated by an arrow at the right side. B: Proliferation of 10T1/2 cells incubated with the supernatant from CHO-K1/h*SCDGF* (circles), CHO-K1/h*SCDGF*-Flag (triangles), CHO-K1/NS-h*SCDGF*-Flag (squares) or CHO-K1/vector (solid circles). Results represent the mean ± S.D. of three independent experiments performed in triplicate.

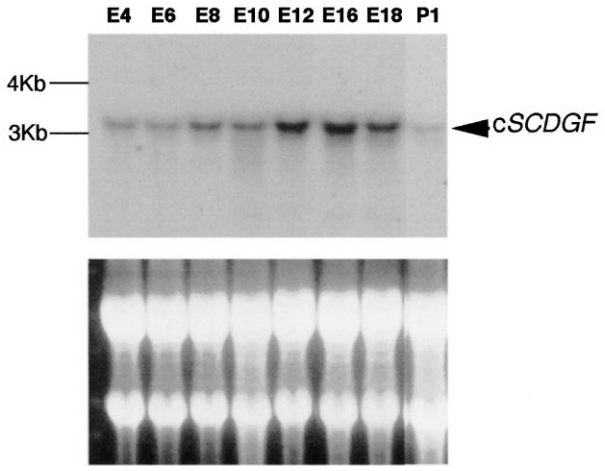


Fig. 4. Northern blot analysis of c*SCDGF* mRNA expression in developing spinal cords. The position of c*SCDGF* mRNA is shown by an arrow on the right. The developmental age (E) is indicated at each lane. P1 indicates one day after hatching. The ethidium bromide staining pattern of the gel showing 18S and 28S rRNAs as control is shown in the lower panel.

The CUB domain was speculated as an extracellular domain of approximately 110 residues and a widespread module found in several proteins. Each of them is functionally different but developmentally regulated [14]. Therefore, we compared the amino acid sequences of CUB domains of cSCDGF and hSCDGF with the CUB domains of other proteins, such as BMP1, TLD, C1r/C1s and NP (Fig. 1B). Notably, although most of the CUB domains reported have four cysteine residues which have been shown to form two disulfide bridges [14], both cSCDGF and hSCDGF lacked the first two cysteines. This is also the case for the first domain of C1r/C1s [18,19], MASP1/2 [30] and the 13th domain of cubilin [31], although the structural and functional significance of the lack of the first two cysteines in CUB domains remains unknown.

VEGFs are thought to be key factors in vasculogenesis and angiogenesis due to their specific mitogenic activity on endothelial cells, and PDGF-A and -B are known to be potent mitogens for smooth muscle cells, glial cells and several other types of cells [22–26]. It was also shown that VEGF-A and PDGFs have a conserved region which expresses mitogenic activity. Based on the structural analysis, cSCDGF and hSCDGF contained a region homologous to the conserved region in PDGF/VEGF family members (Fig. 1C). The most characteristic feature of SCDGF is that it contains a region homologous to the CUB domain, although none of the known members of the PDGF/VEGF family has been reported to possess a CUB domain. These structural characteristics suggest that SCDGF is very similar to members of the PDGF/VEGF family, however, diverged and unique. Therefore, we named this gene as spinal cord-derived growth factor (*SCDGF*).

Because of the structural similarity of SCDGF with the members of PDGF/VEGF family, we examined the mitogenic activity of the SCDGF protein. The supernatants of the CHO-K1/h*SCDGF* and CHO-K1/h*SCDGF*-Flag cells activated proliferation of 10T1/2 cells (Fig. 3B). However, when a part of the putative signal sequence was deleted, no mitogenic activity was detected (Fig. 3B). The results suggest that SCDGF is secreted from the cells, probably with the aid of the N-terminal hydrophobic region as a signal peptide, and acts as a growth factor based on its mitogenic activity on 10T1/2 cells. The mitogenic activity of the supernatants of CHO-K1/h*SCDGF* cells was higher than that of CHO-K1/h*SCDGF*-Flag cells (Fig. 3B). This is probably due to the different expression level of h*SCDGF* mRNA (Fig. 3A) and/or the addition of a Flag tag at the C-terminus.

During the developmental period we examined, the c*SCDGF* mRNA level gradually increased from E4 to E10 and its expression was highest from E12 to E16, and subsequently decreased rapidly after hatching (Fig. 4). Although the function of SCDGF in vivo is not clear, one of the possible functions is its mitogenic action on specific types of spinal cord cells during embryonic development. However, it is known that many kinds of growth factors do not only exhibit mitogenic activities but also perform other functions [26,32]. Therefore, further analysis is necessary to investigate the function of SCDGF during development.

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