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Potential Role of Homer-2a on Cutaneous Vascular Anomaly

Homer protein was identified based on its rapid induction in rat hippocampal granule cell neurons following excitatory synaptic activity. Although the presence of the Homer gene in the peripheral tissues has been observed in previous reports, the physiological function of the Homer protein in these tissues has not been noted. In this experiment, a Homer-2a cDNA fragment was successfully amplified by RT-PCR in the involuting phase of human hemangioma but not in the human vascular malformation and normal vessel. After isolation of full Homer cDNA in a mouse liver cDNA library, E1-deleted recombinant adenovirus expressing the Homer protein (Adv.CMV.mHomer-2a) was constructed to determine its physiological function in peripheral tissues. Adv.CMV.mHomer2a, but not Adv.CMV.LacZ (recombinant adenovirus expressing β-galactosidase), strongly inhibited the growth rate of HUVECs (human umbilical vein endothelial cells) probably via inducing apoptosis determined by acridine orange/ethidium bromide (AO/EB) staining methods. This study suggests that the Homer gene is present in human specimens in the involuting phase of hemangioma, and it might be involved in the growth control.

Key Words : *Homer-2a; Vascular Anomaly; Human Umbilical Vein Endothelial Cells*

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INTRODUCTION

Angiogenesis is essential for sustaining the growth of cancer. However, some benign vascular anomaly in growth phase spontaneously enters the involuting phase, and subsequently results in a complete resolution of the lesion. We hypothesized some genetic components in rapidly growing hemangioma might be implicated in turning to an involuting phase from a growth phase. We used human blood vessels isolated from patients to investigate the genetic switching of benign vascular tumors to the involuting phase, which might provide a new insight into cancer therapy. Of 8 different genes in our preliminary experiments, we observed the Homer-2a gene was specifically expressed in the human involuting phase hemangioma and not in other vascular specimens.

The Homer protein is induced in hippocampal granule cell neurons following excitatory synaptic activity and long-term potentiation (1, 2). It is also induced by drug treatment that alters dopamine signaling in the striatum (3), and by visual stimuli that alter the circardian cycle. The Cupidin and Vesl-2delta1 (4, 5), which are identical to Homer-2a, were independently reported to be expressed in developing mouse cerebellar granule cells during the embryonic period.

N-terminal region of the Homer gene includes a RX5GL GF sequences suggesting a postsynaptic density zone (PDZ) domain (4, 5), which is important for the growth and development in a variety of cells (6). The Homer gene contains a single PDZ-like domain and binds specifically to the carboxy terminus of phosphoinositide-linked metabotropic glutamate receptors. Although most of the studies of the Homer protein have been done on brain development and synaptic activity, the Homer protein has been reported to be present in peripheral tissues during and/or after the development period (7, 8). Therefore, in this study we assessed whether the Homer gene has a role in the growth control of human vascular anomaly.

MATERIALS AND METHODS

Human vascular anomaly specimens

The vascular specimens consisted of those from 7 patients with vascular anomalies including hemangioma in the invo-

Table 1. Patients enrolled in this study

Sex / Age (yr)	Diagnosis
F/51	Venous malformation, lip
M/22	Venous malformation, cheek & temporal area
M/44	Capillary malformation, scalp
F/4	Venous malformation, arm
F/10	Hemangioma (involuting phase), chest wall
F/2	Hemangioma (involuting phase), hand
M/22	Normal vein
M/17	Lymphatic malformation, thigh

luting phase and one normal vessel (Table 1). The diagnosis of each vascular anomaly was confirmed by clinical and histological examinations. The collected specimens from the patients were immediately frozen -80°C in a deep freezer for storage.

Identification and cDNA isolation of the Homer-2a gene

The consensus regions of mouse (124-510 nt. of AF0932 60) and human (191-577 nt. of AF093264) Homer genes were synthesized by RT-PCR using total RNA of human vascular vessel isolated from the patients. The following specific oligonucleotide primers were used: 5′-AGG AAC AGC TAT CGG ATC ATC; 5′-CTT CAG CTT GTC ATT CTC AGA. The PCR product was isolated and subcloned into pGEM-T (Promega, CA, U.S.A.).

Commercially provided Human Multiple Tissue Blot (Clontech, CA, U.S.A.) was used according to the guidance of Clontech for Northern hybridization. The membrane was hybridized with SstI fragment of PCR products of the Homer-2a gene amplified from human vascular anomaly specimens.

The subcloned human Homer-2a cDNA was radiolabeled using a random-labeling kit (Amersham Pharmacia Biotech, U.S.A.) and used for library screening of mouse liver cDNA library constructed in ZAPII (Stratagene, NY, U.S.A.) to obtain the full coding region of Homer-2a.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were grown in Dulbecco's modified Eagles' medium containing 10% fetal bovine serum. The HUVECs (Clontec) were cultured with their reagents according to the recommendations of the manufacturer. Cells between the first and fifth passages were subcultured onto fibronectin-treated 96-well plates (1 μg/well) 24 hr before the start of the experiment. Viable-cell numbers were determined with microscope by the uptake of methylene blue in the viable-cells: the cultured cells were washed with phosphate-buffered saline (PBS), fixed with 10% formalin for 10 min, and incubated with 0.05% methylene blue for 60 min at ambient temperature after washing with PBS.

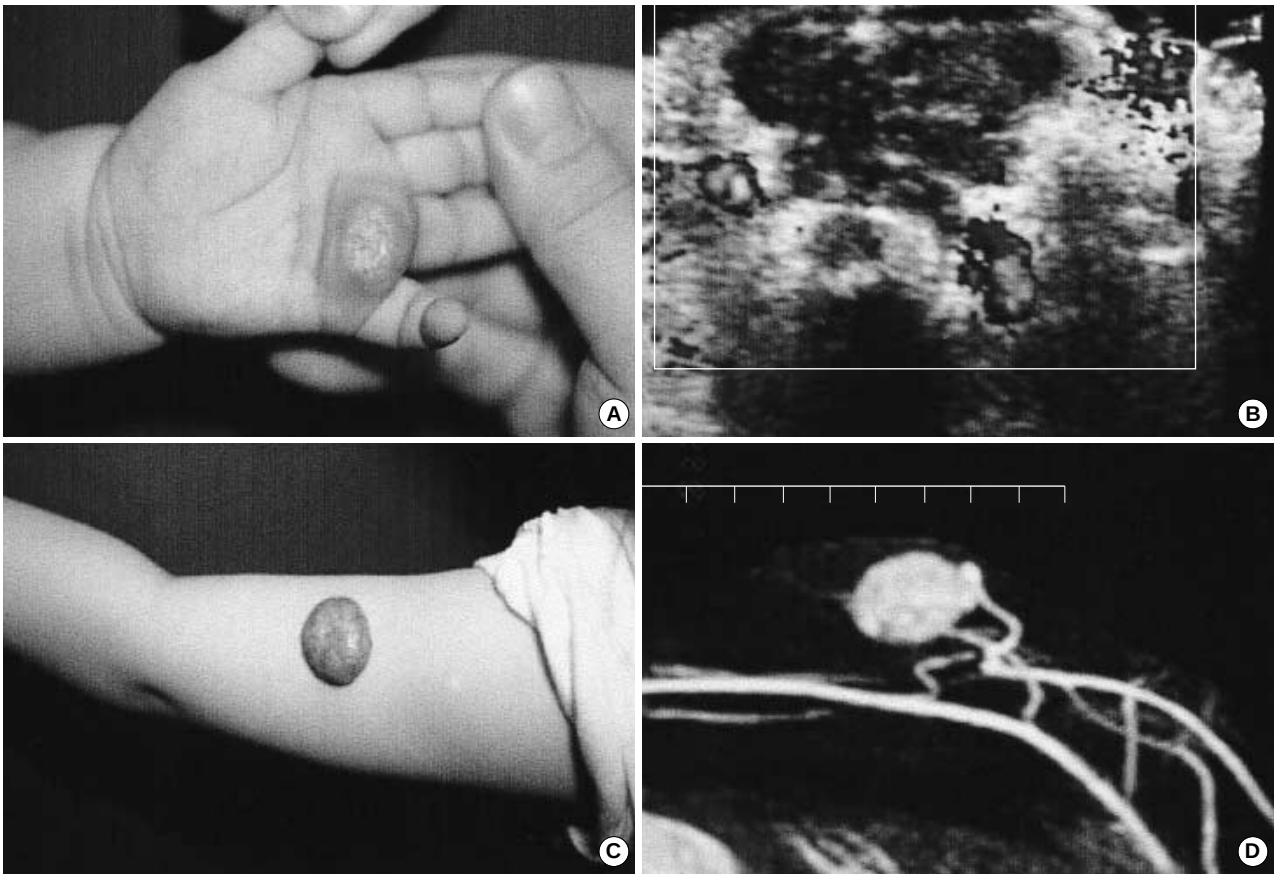

Fig. 1. Representative photographs of the involuting phase hemangioma (A, B) and venous malformation (C, D).
A, B: Involuting hemangioma in a 2-yr-old patient and its color duplex image showing the decreased vascular echo in the center of the mass. C, D: Venous malformation in a 4-yr-old patient and its MR angiographic study showing the draining vessels. The lesion was soft, compressible and extended slowly after birth without any treatment.

The dyes in viable-cells were extracted with 0.33 N HCl, and absorbance at 665 nm was measured with a spectrophotometer.

Functional study of Homer-2a

Adv.CMV.LacZ and Adv.CMVm.Homer-2a are recombinant, replication-defective adenoviruses derived from human adenovirus type 5. They contain a nuclear-targeted beta-galactosidase and human Homer-2a, respectively, both driven by cytomegalovirus (CMV) promoter. Titers of virus stocks were determined by plaque titration on the 293 cell line as described elsewhere, and expressed as plaque-forming units (PFU). The viral particle numbers were measured by optical density at 260 nm.

Acridine orange/ethidium bromide (AO/EB) staining of HUVECs was done to observe the apoptotic pattern induced by Adv.CMV.mHomer-2a. After incubation of the cell suspension (25 μL of 10^6 number cells/mL) with 1 μL AO/EB solution (1 part of 100 μg/mL AO in PBS; 1 part of 100 μg/mL EB in PBS), each sample was mixed just prior to the microscopic examination and quantification. The cell suspension (10 μL) was placed on a microscopic slide, covered with a glass coverslip, and at least 300 cells were examined under a fluorescence microscope using a fluorescein filter and a 60× objective lens.

RESULTS

RT-PCR amplification of the Homer-2a gene (191-577 nt. of AF093264) was done to determine the possibility of its physiological contribution to the growth of vascular anomaly using human vascular tissues. Of 8 human vascular specimens (Fig. 1), the Homer-2a fragment was successfully amplified in 2 cases of involuting hemangioma but not in 5 cases of vascular malformation and a normal vessel specimen (Fig. 2). RT-PCR amplification for alpha-actin was done as positive control in each PCR. The Southern hybridization and sequencing results showed that the expected size of the PCR product was identical to that of the original Homer-2a (data not shown).

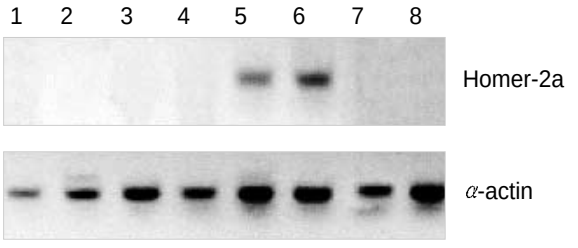

Fig. 2. RT-PCR amplification of Homer-2a cDNA from human vascular specimens.
1. Venous malformation
2. Venous malformation
3. Capillary malformation
4. Venous malformation
5. Hemangioma (involuting phase)
6. Hemangioma (involuting phase)
7. Normal vein
8. Lymphatic malformation

The expression level of Homer mRNA was determined by Northern hybridization of adult human tissues (Fig. 3). The Homer-2a fragment isolated from human tissues was used as a probe in Northern hybridization. Homer-2a mRNA was expressed most abundantly in the pancreas and heart, and slightly in the liver and skeletal muscle. However, Homer-2a mRNA was not detected in the brain tissues. The results of RT-PCR and Northern hybridization suggested that the Homer protein might have physiological functions in adult peripheral vessels.

In order to obtain the Homer cDNA including full coding region, cDNA library screening was done. The RT-PCR

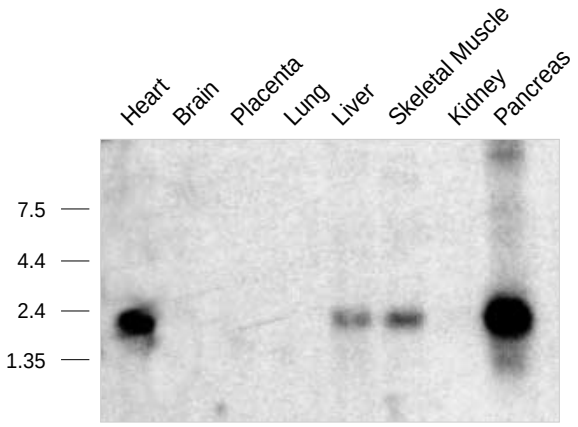

Fig. 3. Northern analysis hybridized with the human Homer-2a cDNA fragment amplified from a human hemangioma specimen (involuting phase) by RT-PCR.

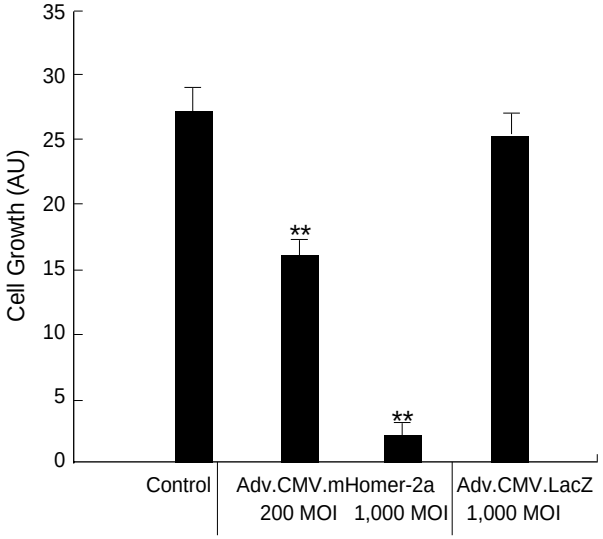

Fig. 4. Effect of Adv.CMV.mHomer-2a on the growth of HUVECs determined by the methylene blue uptake method.
AU; Arbitary Unit, **; $p<0.01$, n=3.

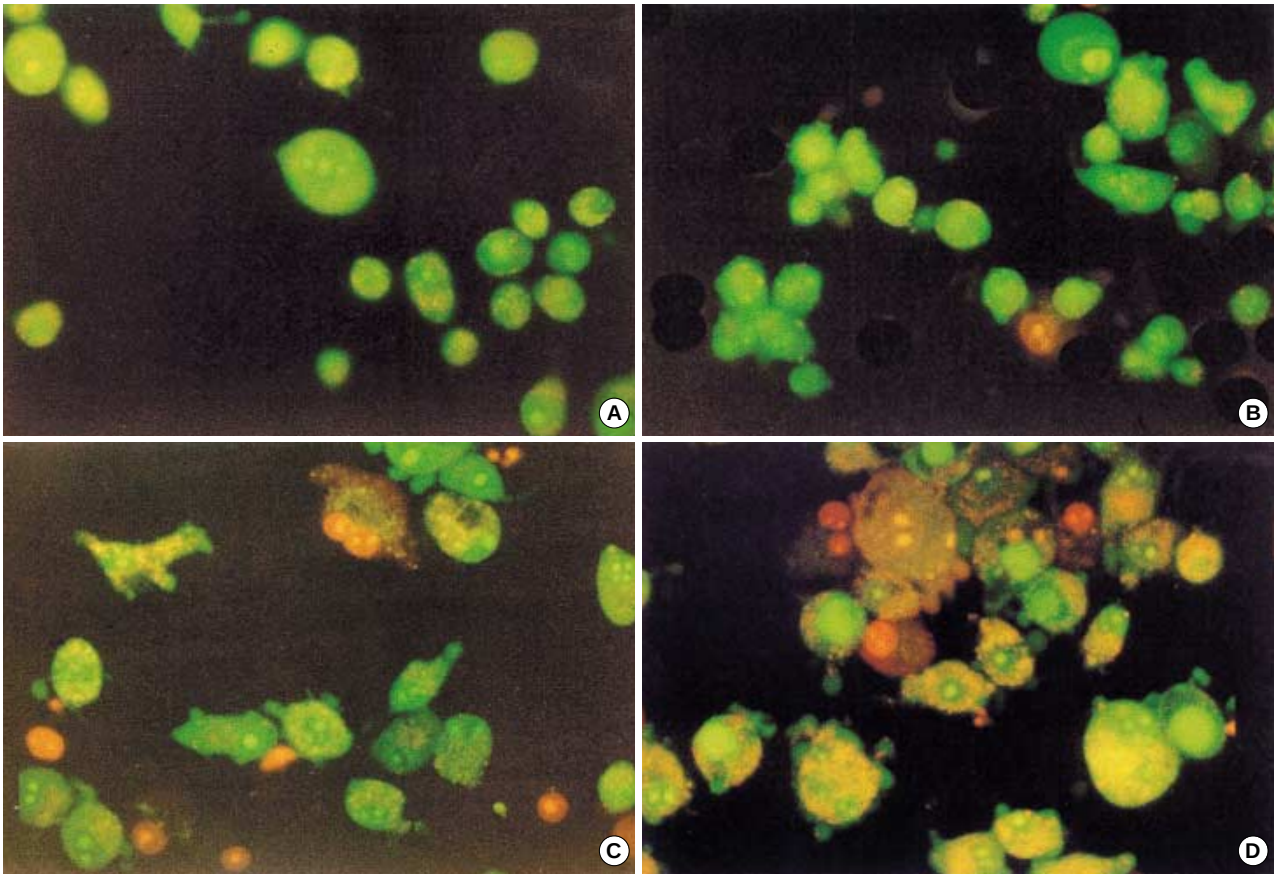

Fig. 5. Acridine orange/ethidium bromide staining of the apoptotic cells (×400).
A: Control, B: Adv.CMV.LacZ 1,000 MOI, C: Adv.CMV.mHomer-2a 200 MOI, D: Adv.CMV.mHomer-2a 1,000 MOI.

fragment of the Homer gene (Homer-2a) was used to screen cDNA libraries prepared from the mouse liver. The search result of the GenBank database identified that one of 6 positive clones had the full coding sequence of the Homer-2a gene except for a slight difference in 5′-UTR (data not shown).

We constructed recombinant defective adenoviral gene including mouse Homer-2a (mHomer-2a) gene to continue the study of its physiological function in peripheral tissues. Adv.CMV.LacZ and Adv.CMV.mHomer-2a are recombinant adenovirus derived from the human adenovirus type 5. They contain a nuclear-targeted beta-galactosidase and mouse Homer2a′, respectively, driven by CMV promoter. Fig. 4 shows the inhibitory effect of Adv.CMV.mHomer2a′ on the rate of growth of HUVECs. After HUVECs were seeded in the fibronectin-treated 96 well plates, Adv.CMV.LacZ (1,000 MOI) or Adv.CMV.mHomer-2a (200 or 1,000 MOI) were applied into the cultured media of each well. Three days later, viable-cell numbers were determined by the uptake of methylene blue. Two hundreds or 1,000 MOI of Adv.CMV.mHomer-2a significantly inhibited the growth of HUVECs (Fig. 4). The growth inhibitory effect by Adv.CMV.mHomer2a in HUVECs showed a dose-dependent pattern. However, 1,000 MOI of Adv.CMV.LacZ did not affect the growth of HUVECs suggesting that the growth inhibition of Adv.CMV.mHomer-2a is not due to the adenoviral products.

To determine the mechanism of growth inhibition in HU VECs induced by Adv.CMV.mHomer-2a, HUVECs were stained with acridine orange/ethidium bromide (AO/EB) and examined under a fluorescence microscope since AO/EB-stained cells clearly show the apoptotic status (Fig. 5). Control cells were stained uniformly green, but apoptotic cells induced by Adv.CMV.mHmoer-2a showed bright green dots. Chromatin condensation and nuclear fragmentation were determined by the presence of orange coloration. In quantitative comparison of the apoptotic cells induced by adenoviral vector including LacZ or Homer-2a, Adv.CMV.mHomer2a but not Adv.CMV.LacZ significantly increased the apoptotic and necrotic cell populations (Fig. 6).

DISCUSSION

The study on Homer-2a/b protein (Homer-2b is an alternative splicing form of Homer-2a, of which the differential functions are not known) has been mostly focused on the brain function and development. However, the Homer gene was

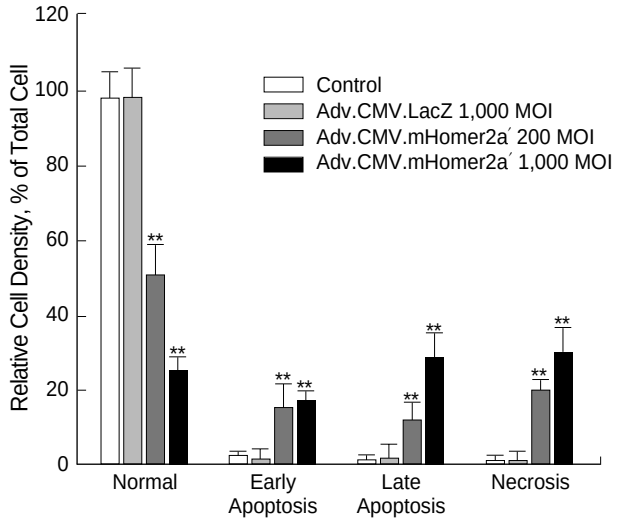

Fig. 6. Quantitative comparison of the apoptotic cells induced by adenoviral vector including LacZ or mHomer-2a (**; $p<0.01$, n=8). The criteria for each components were determined as followings. a. Normal cells: uniformly green stain; b. Early apoptotic cells: stain green and contain bright green dots in the nuclei chromatin condensation & nuclear fragmentation; c. Late apoptotic cells: incorporate ethidium bromide (orange), condensed and fragmented nucleus; d. Necrotic cells: orange stain but with nuclear morphology resembling that of viable cells. Values were calculated from 8 different view fields in LM (× 400) using fluorescein filter.

reported to be present in some adult peripheral tissues such as the heart, liver, and skeletal muscle of the rat in previous reports (2-4). The tissue distribution of the Homer mRNA in this study was similar to that in previous reports. No trace of the Homer mRNA was detected in the brain, in this study which might be plausible considering the regulatory expression pattern of the Homer gene.

All members of the Homer family share a highly conserved N-terminal 120 aa region homologuous to the EVH1 domain [Enabled (Ena)/vasodilator-stimulatied phosphoprotein homology] (31% aa identity) (9), and reminiscent of a minimal PDZ domain (Arg-81 and 87-GlyLeuGlyPhe-90) (10). The functions of the proteins including the PDZ domain (RX5 GLGF) are important in areas as diverse as cell-to-cell interaction, cell differentiation, growth control, ion channels organization, and signal transduction both in CNS and peripheral tissues (3).

Hemangioma is generated by endothelial hyperplasia while vascular malformation is generated by abnormal cell morphogenesis. Therefore, RT-PCR amplification of Homer-2a, specifically from hemangioma but not from other vascular specimens, implicates its role for growth in vascular tissues. The inhibitory effect of Adv.CMV.mHomer-2a on the growth of HUVECs suggests that the Homer family proteins might exert an inhibitory function in the vascular growth. The apoptotic analysis by AO/EB staining suggests that the inhibitory effect of the Homer-2a protein on vascular growth might be via endothelial cell apoptosis.

In this study, the growth status in two hemangioma patients (the only two samples expressing Homer2a) was clinically in the involuting phase (Fig. 2). However, the growth status in all other patients with a vascular malformation showed proliferative. The distinctive expression pattern of Homer-2a in the involuting phase hemangioma, but not in other vascular malformations, is consistent with its inhibitory action on the growth of HUVECs.

This study suggests that the possibility of Homer-2a to induce growing malignant tumor into the involuting phase, and to make a prognosis of proliferating hemangioma.

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