Expression of Thrombin-Cleaved Osteopontin and Integrin $\alpha 9$ and $\beta 1$ Signaling Pathway Molecules in Chronic Subdural Hematomas

Koji Osuka (✉ kosuka@aichi-med-u.ac.jp)

Aichi Medical University <https://orcid.org/0000-0002-6301-3189>

Yusuke Ohmichi

Kanazawa Medical University: Kanazawa Ika Daigaku

Mika Ohmichi

Kanazawa Medical University: Kanazawa Ika Daigaku

Chiharu Suzuki

Aichi Medical University: Aichi Ika Daigaku

Masahiro Aoyama

Aichi Medical University: Aichi Ika Daigaku

Kenichiro Iwami

Aichi Medical University: Aichi Ika Daigaku

Yasuo Watanabe

Showa Pharmaceutical University: Showa Yakka Daigaku

Shigeru Miyachi

Aichi Medical University: Aichi Ika Daigaku

Research Article

Keywords: angiogenesis, integrin $\alpha 9$, integrin $\beta 1$, N-half osteopontin

Posted Date: December 28th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1181122/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Chronic subdural hematoma (CSDH) is considered to be an inflammatory and angiogenic disease. Osteopontin is an extracellular matrix protein. Osteopontin is cleaved by thrombin, resulting in N-half osteopontin, which is more prominent in integrin signal transduction. We examined the expression of N-half osteopontin in the CSDH fluid and the expression of integrin $\alpha 9$ and $\beta 1$ and the downstream components of the angiogenic signaling pathways in the outer membrane of CSDHs. Twenty samples of CSDH fluid and 8 samples of CSDH outer membrane were included. The concentrations of N-half osteopontin in the CSDH fluid were measured using ELISA kits. The expression of integrin $\alpha 9$ and $\beta 1$, vinculin, talin-1, focal adhesion kinase (FAK), paxillin, α -actin, Src and β -actin was examined by western blot analysis. The expression of integrin $\alpha 9$ and $\beta 1$, FAK and paxillin was also examined by immunohistochemistry. We investigated whether CSDH fluid could activate FAK in cultured endothelial cells *in vitro*. The concentration of N-half osteopontin in CSDH fluid was significantly higher than that in the serum. Western blot analysis revealed above-mentioned molecules. In addition, integrin $\alpha 9$ and $\beta 1$, FAK and paxillin were localized in the endothelial cells of vessels within the CSDH outer membrane. FAK was significantly phosphorylated immediately after treatment with CSDH fluid. Our data suggest that N-half osteopontin in CSDH fluid promotes neovascularization in endothelial cells through integrins $\alpha 9$ and $\beta 1$. The N-half osteopontin and integrin signaling pathway might be a useful therapeutic target for treating the growth of refractory CSDH.

Introduction

Chronic subdural hematoma (CSDH) is a neovascularized and inflammatory disease. High concentrations of growth factors and inflammatory mediators in CSDH fluids have been reported and are involved in angiogenesis within the outer membrane. Vascular endothelial growth factor (VEGF) and angiopoietin are some of these growth factors [1, 2], while inflammatory mediators include interleukin-6 and high-mobility group box 1 (HMGB1) [3, 4]. These factors in CSDH fluid activate the nuclear factor κB (NF- κB) [5] and Ras/MEK/ERK pathways [6], phosphoinositide 3-kinase (PI3-kinase)/protein kinase B (Akt) pathway [7] and Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling pathway [8] in endothelial cells, resulting in angiogenesis and inducing the growth of the CSDH outer membrane.

Osteopontin is an extracellular matrix (ECM) protein that was first identified in osteoblasts. Osteopontin has been implicated as an important factor in bone remodeling. Osteopontin plays critical roles in physiological and pathological processes, including inflammation through integrin receptors. After cerebral ischemia, osteopontin plays a role in matrix remodeling, which renovates new matrix-cell interactions [9]. Osteopontin extends astrocyte process and repairs blood-brain barrier dysfunction after ischemic stroke [10]. Thrombin-cleaved osteopontin (N-half osteopontin) plays a more effective protection than full-length osteopontin after focal cerebral ischemia in mice [11] and is useful as a blood indicator of acute atherothrombotic cerebral ischemia [12]. These previous data suggested that N-half osteopontin is also closely involved in inflammatory central nervous diseases.

Therefore, we explored whether N-half osteopontin exists in CSDH fluid and investigated the expression of integrin and subsequent signaling pathway molecules in the outer membrane of CSDH using western blotting and immunohistological analyses.

Material And Methods

All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise specified.

Patients

This study included twenty patients who underwent surgical trepanation surgery for CSDH at Aichi Medical University Hospital. These patients, thirteen men and seven women, ranged in age from 41 to 83 years (mean age of 65 years). The Certified Clinical Research Review Board of Aichi Medical University Hospital approved this study (17-H047). Informed consent was obtained from each patient or the patient's family.

Analysis of thrombin-cleaved osteopontin in CSDH fluid

Fluids from 20 consecutive CSDHs were obtained during trepanation surgery. As a control, serum samples were obtained from 5 patients suffering from CSDH. After collection, all samples were immediately centrifuged, and the supernatant was stored at -80°C until analysis.

We measured the concentration of N-half osteopontin using enzyme-linked immunosorbent assay (ELISA) kits (IBL, Gunma, Japan) according to the manufacturer's instructions. The mean minimum detectable dose of these assays was 8.3 pg/mL for N-half osteopontin.

Western blotting analysis

Eight samples of the outer membrane of CSDHs obtained during surgery were included. The membranes were homogenized in 75 μ L of homogenization buffer containing 50 mM Tris base/HCl (pH 7.5), 0.1 mM dithiothreitol, 0.2 mM ethylenediaminetetraacetate, 0.2 mM ethylene glycol bis(aminoethyl ether)tetraacetate, 0.2 mM phenylmethylsulfonyl fluoride, 1.25 μ g/mL pepstatin A, 0.2 μ g/mL aprotinin, 1 mM sodium orthovanadate, 50 nM sodium fluoride, 2 mM sodium pyrophosphate and 1% Nonidet P-40. The homogenates were centrifuged at 12,000 $\times g$ for 10 min at 4°C. The protein concentrations of the supernatants were separated using 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

The proteins in the gel were transferred to polyvinylidene difluoride membranes and incubated with primary polyclonal antibodies against integrin β 1 (Cell Signaling Technology, Danvers, MA), vinculin (Cell Signaling Technology), talin-1 (Cell Signaling Technology), focal adhesion kinase (FAK, Cell Signaling Technology), paxillin (Gene Tex, Irvine, CA), α -actinin (Cell Signaling Technology), Src (Cell Signaling Technology) and β -actin (Sigma) and with primary monoclonal antibody against integrin α 9 (R&D Systems, Minneapolis, MN). All antibodies were used at a 1:750 dilution and incubated overnight at 4°C.

After washing away unbound antibodies, the membranes were incubated in secondary antibodies with horseradish peroxidase (Sigma) at a 1:3000 dilution for 30 min at room temperature. The reactions were developed with ECL Plus (GE Healthcare, Buckinghamshire, UK). Positive controls were RAW264.7 cell lysate (Cell Signaling Technology), rat liver lysate (BD Bioscience, Franklin Lakes, NJ) and A431 cell lysate (Santa Cruz Biotechnology, Dallas, TX).

Histological examinations

For the analysis of the cellular expression of integrin $\alpha 9$, integrin $\beta 1$, FAK and paxillin, immunohistochemical staining was performed on samples from three patients at room temperature using the avidin-biotinylated peroxidase complex (ABC) technique. To preserve the outer membrane of the CSDH samples, the membranes were incubated in 10 mL of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h and then embedded in paraffin.

In this study, 10- μ m-thick sections were prepared on a microtome and mounted onto MAS-coated glass slides (Matsunami Glass, Kishiwada Japan). The sections were deparaffinized in xylene, immersed in decreasing concentrations of ethanol, and rehydrated in water. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 100% methanol for 20 min. All sections for immunostaining were processed for microwave-enhanced antigen retrieval. Slide-mounted sections immersed in 0.01 M sodium citrate buffer (pH 6.0) were placed for 15 min in a 700-W microwave oven at maximum power.

Nonspecific immunoreactivity was blocked by incubation with goat or donkey serum for 30 min, depending on the primary antibody. The samples were treated with primary antibodies against integrin $\alpha 9$ (R&D Systems) at a dilution of 1:150, integrin $\beta 1$ (Cell Signaling Technology) at a dilution of 1:100, paxillin (Gene Tex) at a dilution of 1:500 and FAK (Cell Signaling Technology) at a dilution of 1:500 over 2 nights at 4°C. After several rinses in PBS, the samples were incubated with secondary biotinylated antibodies (anti-goat IgG 1:200, anti-rabbit IgG 1:200; Santa Cruz Biotechnology) at room temperature for 2 h. After several more rinses in PBS, they were incubated with Vectastain ABC reagent (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) for 1 h. After several more rinses in PBS, the bound peroxidase was visualized by incubating the sections with a solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Aldrich) and 0.01% H_2O_2 in 0.05 M Tris-HCl (pH 7.4) for 10 min. After several rinses in water, the immunostained sections were dehydrated and cover-slipped with Entellan new (Merck, Kenilworth, NJ).

Cultured vascular endothelial cells

Endothelial cells of mouse brain (b.End3) were obtained from the HPA Cultured Collection (London, United Kingdom). The endothelial cells were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) that contained 10% fetal bovine serum under the circumstances at 37°C and 5% CO_2 .

Effect of CSDH fluid on FAK

We centrifuged CSDH fluids to remove debris and collected together. The b.End3 cells were incubated with serum-containing media that contained CSDH fluid obtained during trepanation surgery. The volumes of the media and CSDH fluid were 7.5 mL and 2.5 mL per culture dish, respectively. Protein lysates were prepared from the harvested cells at 5 min, 15 min, and 60 min (n = 3 per group). We used b.End3 cells treated with media alone as the control (n = 3). Total cell lysates were subjected to western blotting analysis using antibodies against *p*-FAK at Tyr³⁹⁷ (Thermo Fisher Scientific, Tokyo, Japan), FAK (Cell Signaling Technology) and β -actin (Sigma) as discussed above. All antibodies were used at a 1:750 dilution. The band intensities were quantitated using densitometry with the ImageQuant software (GE Healthcare).

Statistical analysis

Data are expressed as the mean $\pm$ standard error. The Mann-Whitney U test was used for the analysis between two groups. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by the Fisher's post hoc test, as appropriate. Significance was indicated when $p < 0.05$.

Results

Concentration of N-half osteopontin in CSDH fluid

The concentration of N-half osteopontin in CSDH fluid ($29,451.5 \pm 8,146.5$ pg/ml) was significantly higher than that in serum (365.8 ± 73.2 pmol/L) according to the Mann-Whitney U test ($p < 0.01$, Fig. 1).

Western blotting analysis of integrins and the angiogenic signaling pathway

Fig. 2 shows the results of western blotting analyses of integrin $\beta 1$ and $\alpha 9$ and the downstream signaling pathway components. Nearly constant β -actin levels were detected in CSDH outer membrane samples. Integrins $\beta 1$ and $\alpha 9$, vinculin, talin-1, FAK, paxillin, α -actinin and Src were detected in almost all samples; however, in some cases, the signals were weak. Positive controls revealed that these molecules had been correctly detected.

Histological observations

Integrin $\alpha 9$ (Figs. 3A and B), integrin $\beta 1$ (Figs. 3C and D), FAK (Figs. 3E and F) and paxillin (Figs. 3G and H) were localized in the endothelial cells of vessels within the outer membrane. Note that higher magnifications clearly demonstrate that these molecules are expressed in the endothelium (Figs. 3B, D, F and H). In the negative controls examined without primary antibodies, the endothelial cells were consistently negative for the markers listed above (Fig. 3I).

Activation of FAK in endothelial cells by CSDH fluid

To investigate the role of CSDH fluid, we examined phosphorylation of FAK in endothelial cells following treatment with subdural hematoma fluid (Fig. 4). The addition of CSDH fluid to cultured vascular

endothelial cells resulted in significantly higher phosphorylated FAK levels compared with the controls 5 min after treatment ($p < 0.05$). In contrast, the FAK and b-actin levels were constant.

Discussion

The expression of N-half osteopontin in CSDH fluid was significantly higher than that in serum. Molecules of integrins and the subsequent angiogenic pathway intermediates were detected in the outer membrane of CSDH. Integrin $\alpha 9$ and $\beta 1$, FAK and paxillin were expressed in the endothelium of vessels of the CSDH outer membrane. CSDH fluid activated FAK in the endothelial cells immediately after treatment.

The extracellular matrix protein osteopontin is a glycoprotein and is involved in physiological and pathological events during inflammatory processes. The concentrations of thrombin-cleaved osteopontin in synovial fluid correlate well with the severity of knee osteoarthritis [13]. Compared with full-length osteopontin, N-half osteopontin induced markedly greater cell attachment through integrin receptors [14]. Disease activity in lupus nephritis correlates with urine N-half osteopontin instead of full-length osteopontin, suggesting that N-half osteopontin is an indicator of inflammation of the kidney [15]. A previous study revealed that excessive coagulation, generation of thrombin and increased fibrinolysis occur within CSDH fluids [16]. Considering previous studies and our data together, osteopontin is cleaved by thrombin within CSDH fluids, and N-half osteopontin plays a role in inflammatory reactions in CSDH outer membranes. To the best of our knowledge, this study is the first to demonstrate the existence of N-half osteopontin in CSDH fluids.

Integrins are α/β heterodimeric cell surface receptors that mediate cell-cell and cell-ECM interactions and orchestrate cell attachment, movement, growth, differentiation and survival. Integrin $\alpha 9$ is widely expressed in a variety of cell types, including epithelium, *in vivo* [17]. Integrin $\beta 1$ is the main β subunit for $\alpha 9$ in these cells [17]. The $\beta 1$ class of integrins takes part in many aspects of vascular biology, especially angiogenesis [18]. $\beta 1$ integrins play an important role in endothelial cell adhesion, migration, survival during angiogenesis and vascular remodeling [19, 20]. A deficit of endothelial $\beta 1$ integrins prohibited endothelial cell maturation, migration and sprouting and induced endothelial cell apoptosis [21]. Thrombin-cleaved osteopontin can attach to the integrin $\alpha 9$ and $\beta 1$ via the sequence SVVYGLR, which is located between the arginine-glycine-aspartic acid (RGD) sequence and the thrombin cleavage site [22]. From our data, there might be a possibility that thrombin-cleaved osteopontin, i.e., N-half osteopontin, induces angiogenesis through this integrin $\alpha 9$ and $\beta 1$ in the endothelium of the outer membrane.

After these integrin receptors combine with extracellular matrix, the formation of complex multiprotein structures occurs. FAK is a regulator of signals from the ECM to the cytoplasmic actin cytoskeleton (Fig. 5) [23]. Angiogenesis is mandatory for tumor development. FAK participates in endothelial cell proliferation, which has been revealed to control tumor angiogenesis in many cancers [24] and promotes angiogenesis in overexpressed transgenic mice [25]. FAK induces tumor angiogenesis in a dose-dependent manner [26]. Both FAK and Src form a dual kinase complex, which play an important role in promoting VEGF-associated tumor angiogenesis [27]. α -Actin is a highly conserved protein and a member

of the actin cross-linking protein family. α -Actin is phosphorylated on tyrosine residue by FAK and binds to actin [28]. These molecules regulate the flow of signals from the extracellular matrix to the actin cytoskeleton and induce angiogenesis (Fig. 5).

Paxillin is an adaptor protein located at the interface between the actin cytoskeleton and the plasma membrane [29] and is one of the key components of integrin signaling (Fig. 5). The FAK/Src complex phosphorylates tyrosine and serine residues of paxillin and promotes cell migration and regulates adhesion turnover at the cell front through paxillin [30]. Netrin-1 is a laminin-like secreted protein that is thought to be an axon guidance molecule during neural development. Netrin-1 activates the FAK/Src/paxillin pathway and modulates angiogenesis, which is accompanied by the upregulation of VEGF [31]. Both talin and vinculin also play an important role in cell growth, morphogenesis, and cell migration during the development. Marked defects in focal adhesions and embryonic death occur in case of loss of either talin or vinculin in mice [32, 33]. Talin is also the key regulator of the link between the cytoskeleton and integrins, having multiple interaction sites for other adhesion components (Fig. 5) [34]. Talin-1 is essential for endothelial proliferation and postnatal angiogenesis [35]. Furthermore, vinculin is a key regulator of cell adhesion by direct interactions with talin and actin (Fig. 5) [36, 37]. Our data revealed that all these molecules were confirmed by western blot analysis and were located in the endothelium of CSDH outer membranes by immunohistochemistry. Moreover, FAK in the endothelial cells was activated by CSDH fluid. N-half osteopontin activated by thrombin signals through integrin $\alpha 9$ and $\beta 1$ located on the cell surface to the actin cytoskeleton and induces angiogenesis within the CSDH outer membrane.

Angiogenesis is a complex process regulated by numerous receptors, growth factors, ECM-cell interactions and so on. The concentration of VEGF in hematoma fluid has been reported to be a highly important mechanistic factor in the pathophysiological progression and growth of CSDH and angiogenesis [2, 38]. The angiogenic effect of VEGF depends on the presence of integrin $\beta 1$ [39]. FAK and Src coordinate together for angiogenesis induced by VEGF *in vitro* [40]. We have revealed that activation of mitogen-activated protein kinases (MAPKs) occurs in CSDH outer membranes by VEGF and plays a critical role in the angiogenesis of CSDHs [41, 6]. Phosphorylated c-Jun N-terminal kinase (JNK) is expressed in the vascular endothelium of the CSDH outer membrane. FAK activates JNK through an extraordinary mechanism involving the recruitment of paxillin to the plasma membrane [42]. Taken together, we have to consider targeted therapy against not only growth factors but also the osteopontin/integrin pathway (Fig. 5).

In the present study, we have to remark several limitations. First, from our limited number of patients, we could not detect a correlation between the concentrations of N-half osteopontin in CSDH fluids, the data from western blot analyses and the growth stage of CSDH. Further studies, including more patients, will be necessary to clarify this point. Second, we only found out the presence of integrin $\alpha 9$ and $\beta 1$ and subsequent angiogenic signaling molecules in the outer membrane of CSDHs. We have to find out these signaling molecules are activated during the development of CSDH.

In the present study, we detected the expression of N-half osteopontin in CSDH fluids and integrin $\alpha 9$ and $\beta 1$, FAK, paxillin, vinculin and the subsequent angiogenic signaling pathway in the CSDH outer membrane for the first time. Significantly high concentrations of N-half osteopontin in CSDH fluid might play an important role in angiogenesis and inflammation in CSDH, resulting in the growth of the hematoma. This angiogenic signaling pathway through integrin $\alpha 9$ and $\beta 1$ might be a therapeutically alternative target for the treatment of refractory CSDH.

Declarations

Acknowledgements

None.

Authors' Contribution

KO and YO equally designed the study with significant instruction from YW and SH. MO, CS, MA and KI contributed to the data collection and elaboration of the article. CS and YW contributed valuable contributions to graphical designs. All authors reviewed and edited the manuscript and are responsible for its content. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

Funding

This work was supported in part by a Japanese Grant-in-Aid for Scientific Research (C), Grant Number 17K10853 (KO).

Data Availability

All data generated during this study are included in this published article.

Ethics Approval

Approval was obtained from the ethics committee of Aichi Medical University (17-H047).

Conflict of interest

The authors have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

Consent to participate

Written informed consent was obtained from the patients or the patient's families.

Consent to publish

Patients signed informed consent regarding publishing their data.

Code Availability

Not applicable.

References

1. Isaji T, Osuka K, Ohmichi Y, Ohmichi M, Naito M, Nakano T, Iwami K, Miyachi S (2020) Expression of Angiopoietins and Angiogenic Signaling Pathway Molecules in Chronic Subdural Hematomas. *J Neurotrauma* 37(23):2493–2498. doi:10.1089/neu.2020.7042
2. Weigel R, Hohenstein A, Schilling L (2014) Vascular endothelial growth factor concentration in chronic subdural hematoma fluid is related to computed tomography appearance and exudation rate. *J Neurotrauma* 31(7):670–673. doi:10.1089/neu.2013.2884
3. Frati A, Salvati M, Mainiero F, Ippoliti F, Rocchi G, Raco A, Caroli E, Cantore G, Delfini R (2004) Inflammation markers and risk factors for recurrence in 35 patients with a posttraumatic chronic subdural hematoma: a prospective study. *J Neurosurg* 100(1):24–32. doi:10.3171/jns.2004.100.1.0024
4. Osuka K, Watanabe Y, Usuda N, Iwami K, Miyachi S, Takayasu M (2020) Expression of high mobility group B1 and toll-like receptor-nuclear factor κ B signaling pathway in chronic subdural hematomas. *PLoS ONE* 15(6):e0233643. doi:10.1371/journal.pone.0233643
5. Osuka K, Watanabe Y, Usuda N, Aoyama M, Kawaguchi R, Takeuchi M, Takayasu M (2017) Activation of Nuclear Factor-kappa B in Endothelial Cells of Chronic Subdural Hematoma Outer Membranes. *Neurosurgery* 80(4):571–578. doi:10.1093/neuros/nyw100
6. Osuka K, Watanabe Y, Usuda N, Atsuzawa K, Aoyama M, Niwa A, Nakura T, Takayasu M (2012) Activation of Ras/MEK/ERK signaling in chronic subdural hematoma outer membranes. *Brain Res* 1489:98–103. doi:10.1016/j.brainres.2012.10.013
7. Funai M, Osuka K, Usuda N, Atsuzawa K, Inukai T, Yasuda M, Watanabe Y, Takayasu M (2011) Activation of PI3 kinase/Akt signaling in chronic subdural hematoma outer membranes. *J Neurotrauma* 28(6):1127–1131. doi:10.1089/neu.2010.1498
8. Osuka K, Watanabe Y, Usuda N, Aoyama M, Kawaguchi R, Watabe T, Takayasu M (2016) Activation of signal transducer and activator of transcription 3 in endothelial cells of chronic subdural hematoma outer membranes. *World Neurosurg* 91:376–382. doi:10.1016/j.wneu.2016.04.025
9. Wang X, Loudon C, Yue TL, Ellison JA, Barone FC, Solleveld HA, Feuerstein GZ (1998) Delayed expression of osteopontin after focal stroke in the rat. *J Neurosci* 18(6):2075–2083. doi:10.1523/jneurosci.18-06-02075.1998
10. Gliem M, Krammes K, Liaw L, van Rooijen N, Hartung HP, Jander S (2015) Macrophage-derived osteopontin induces reactive astrocyte polarization and promotes re-establishment of the blood brain barrier after ischemic stroke. *Glia* 63(12):2198–2207. doi:10.1002/glia.22885

11. Doyle KP, Yang T, Lessov NS, Ciesielski TM, Stevens SL, Simon RP, King JS, Stenzel-Poore MP (2008) Nasal administration of osteopontin peptide mimetics confers neuroprotection in stroke. *J Cereb Blood Flow Metab* 28(6):1235–1248. doi:10.1038/jcbfm.2008.17
12. Ozaki S, Kurata M, Kumon Y, Matsumoto S, Tagawa M, Watanabe H, Ohue S, Higaki J, Ohnishi T (2017) Plasma thrombin-cleaved osteopontin as a potential biomarker of acute atherothrombotic ischemic stroke. *Hypertens Res* 40(1):61–66. doi:10.1038/hr.2016.110
13. Hasegawa M, Segawa T, Maeda M, Yoshida T, Sudo A (2011) Thrombin-cleaved osteopontin levels in synovial fluid correlate with disease severity of knee osteoarthritis. *J Rheumatol* 38(1):129–134. doi:10.3899/jrheum.100637
14. Senger DR, Perruzzi CA, Papadopoulos-Sergiou A, Van de Water L (1994) Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. *Mol Biol Cell* 5(5):565–574. doi:10.1091/mbc.5.5.565
15. Kitagori K, Yoshifuji H, Oku T, Sasaki C, Miyata H, Mori KP, Nakajima T, Ohmura K, Kawabata D, Yukawa N, Imura Y, Murakami K, Nakashima R, Usui T, Fujii T, Sakai K, Yanagita M, Hirayama Y, Mimori T (2016) Cleaved Form of Osteopontin in Urine as a Clinical Marker of Lupus Nephritis. *PLoS ONE* 11(12):e0167141. doi:10.1371/journal.pone.0167141
16. Kawakami Y, Chikama M, Tamiya T, Shimamura Y (1989) Coagulation and fibrinolysis in chronic subdural hematoma. *Neurosurgery* 25(1):25–29. doi:10.1097/00006123-198907000-00005
17. Palmer EL, Ruegg C, Ferrando R, Pytela R, Sheppard D (1993) Sequence and tissue distribution of the integrin alpha 9 subunit, a novel partner of beta 1 that is widely distributed in epithelia and muscle. *J Cell Biol* 123(5):1289–1297. doi:10.1083/jcb.123.5.1289
18. Mettouchi A, Meneguzzi G (2006) Distinct roles of beta1 integrins during angiogenesis. *Eur J Cell Biol* 85(3–4):243–247. doi:10.1016/j.ejcb.2005.09.010
19. Carlson TR, Hu H, Braren R, Kim YH, Wang RA (2008) Cell-autonomous requirement for beta1 integrin in endothelial cell adhesion, migration and survival during angiogenesis in mice. *Development* 135(12):2193–2202. doi:10.1242/dev.016378
20. Lei L, Liu D, Huang Y, Jovin I, Shai SY, Kyriakides T, Ross RS, Giordano FJ (2008) Endothelial expression of beta1 integrin is required for embryonic vascular patterning and postnatal vascular remodeling. *Mol Cell Biol* 28(2):794–802. doi:10.1128/mcb.00443-07
21. Malan D, Wenzel D, Schmidt A, Geisen C, Raible A, Bölck B, Fleischmann BK, Bloch W (2010) Endothelial beta1 integrins regulate sprouting and network formation during vascular development. *Development* 137(6):993–1002. doi:10.1242/dev.045377
22. Yokosaki Y, Matsuura N, Sasaki T, Murakami I, Schneider H, Higashiyama S, Saitoh Y, Yamakido M, Taooka Y, Sheppard D (1999) The integrin alpha(9)beta(1) binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. *J Biol Chem* 274(51):36328–36334. doi:10.1074/jbc.274.51.36328
23. Parsons JT, Martin KH, Slack JK, Taylor JM, Weed SA (2000) Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* 19(49):5606–5613.

24. Lechertier T, Hodivala-Dilke K (2012) Focal adhesion kinase and tumour angiogenesis. *J Pathol* 226(2):404–412. doi:10.1002/path.3018
25. Peng X, Ueda H, Zhou H, Stokol T, Shen TL, Alcaraz A, Nagy T, Vassalli JD, Guan JL (2004) Overexpression of focal adhesion kinase in vascular endothelial cells promotes angiogenesis in transgenic mice. *Cardiovasc Res* 64(3):421–430. doi:10.1016/j.cardiores.2004.07.012
26. Kostourou V, Lechertier T, Reynolds LE, Lees DM, Baker M, Jones DT, Tavora B, Ramjaun AR, Birdsey GM, Robinson SD, Parsons M, Randi AM, Hart IR, Hodivala-Dilke K (2013) FAK-heterozygous mice display enhanced tumour angiogenesis. *Nat Commun* 4:2020. doi:10.1038/ncomms3020
27. Mitra SK, Schlaepfer DD (2006) Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol* 18(5):516–523. doi:10.1016/j.ceb.2006.08.011
28. Izaguirre G, Aguirre L, Hu YP, Lee HY, Schlaepfer DD, Aneskievich BJ, Haimovich B (2001) The cytoskeletal/non-muscle isoform of alpha-actinin is phosphorylated on its actin-binding domain by the focal adhesion kinase. *J Biol Chem* 276(31):28676–28685. doi:10.1074/jbc.M101678200
29. Turner CE (2000) Paxillin and focal adhesion signalling. *Nat Cell Biol* 2(12):E231–236. doi:10.1038/35046659
30. Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol* 6(2):154–161. doi:10.1038/ncb1094
31. Yang X, Li S, Zhong J, Zhang W, Hua X, Li B, Sun H (2017) CD151 mediates netrin-1-induced angiogenesis through the Src-FAK-Paxillin pathway. *J Cell Mol Med* 21(1):72–80. doi:10.1111/jcmm.12939
32. Priddle H, Hemmings L, Monkley S, Woods A, Patel B, Sutton D, Dunn GA, Zicha D, Critchley DR (1998) Disruption of the talin gene compromises focal adhesion assembly in undifferentiated but not differentiated embryonic stem cells. *J Cell Biol* 142(4):1121–1133. doi:10.1083/jcb.142.4.1121
33. Xu W, Baribault H, Adamson ED (1998) Vinculin knockout results in heart and brain defects during embryonic development. *Development* 125(2):327–337
34. Klapholz B, Brown NH (2017) Talin - the master of integrin adhesions. *J Cell Sci* 130(15):2435–2446. doi:10.1242/jcs.190991
35. Pulous FE, Carnevale JC, Al-Yafeai Z, Pearson BH, Hamilton JAG, Henry CJ, Orr AW, Petrich BG (2021) Talin-dependent integrin activation is required for endothelial proliferation and postnatal angiogenesis. *Angiogenesis* 24(1):177–190. doi:10.1007/s10456-020-09756-4
36. Humphries JD, Wang P, Streuli C, Geiger B, Humphries MJ, Ballestrem C (2007) Vinculin controls focal adhesion formation by direct interactions with talin and actin. *J Cell Biol* 179(5):1043–1057. doi:10.1083/jcb.200703036
37. Izard T, Vorrhein C (2004) Structural basis for amplifying vinculin activation by talin. *J Biol Chem* 279(26):27667–27678. doi:10.1074/jbc.M403076200

38. Hua C, Zhao G, Feng Y, Yuan H, Song H, Bie L (2016) Role of Matrix Metalloproteinase-2, Matrix Metalloproteinase-9, and Vascular Endothelial Growth Factor in the Development of Chronic Subdural Hematoma. *J Neurotrauma* 33(1):65–70. doi:10.1089/neu.2014.3724
39. Bloch W, Forsberg E, Lentini S, Brakebusch C, Martin K, Krell HW, Weidle UH, Addicks K, Fässler R (1997) Beta 1 integrin is essential for teratoma growth and angiogenesis. *J Cell Biol* 139(1):265–278. doi:10.1083/jcb.139.1.265
40. Eliceiri BP, Puente XS, Hood JD, Stupack DG, Schlaepfer DD, Huang XZ, Sheppard D, Cheresch DA (2002) Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. *J Cell Biol* 157(1):149–160. doi:10.1083/jcb.200109079
41. Aoyama M, Osuka K, Usuda N, Watanabe Y, Kawaguchi R, Nakura T, Takayasu M (2015) Expression of Mitogen-Activated Protein Kinases in Chronic Subdural Hematoma Outer Membranes. *J Neurotrauma* 32(14):1064–1070. doi:10.1089/neu.2014.3594
42. Igishi T, Fukuhara S, Patel V, Katz BZ, Yamada KM, Gutkind JS (1999) Divergent signaling pathways link focal adhesion kinase to mitogen-activated protein kinase cascades. Evidence for a role of paxillin in c-Jun NH(2)-terminal kinase activation. *J Biol Chem* 274(43):30738–30746. doi:10.1074/jbc.274.43.30738

Figures

Figure 1

Concentrations of thrombin-cleaved osteopontin (N-half osteopontin) in serum (serum, n = 5) and chronic subdural hematoma (CSDH, n = 20). The concentration of N-half osteopontin in CSDH fluid was

significantly higher than that in serum by the Mann-Whitney U-test. Data represent the median values and 25th and 75th percentiles with maximum/minimum whiskers. **p* < 0.05 by the Mann-Whitney U-test.

Figure 2

Western blots showing the expression of integrin α9 and β1 and the subsequent angiogenic pathway molecules in the outer membrane of chronic subdural hematomas from eight patients. Integrins β1 and α9, vinculin, talin-1, focal adhesion kinase (FAK), paxillin, a-actinin and Src were detected in almost all cases. Positive controls are shown in the right three lanes and suggest that these molecules were correctly detected. RAW 264.7, murine leukemia macrophage cell line lysate; Rat liver, rat liver whole cell lysate; A431 cell lysate, epidermoid carcinoma cell lysate.

Figure 3

Ten-micrometer consecutive slices were immunostained with polyclonal antibodies against integrin $\alpha 9$ (A and B), integrin $\beta 1$ (C and D) focal adhesion kinase (FAK, E and F) and paxillin (G and H) using the ABC method. The areas within the rectangle, labeled A, C, E and G, are shown at a higher magnification in panels B, D, F and H, respectively. Note that these molecules were expressed in endothelial cells (B, D, F and H). Slices immunostained without primary antibodies are shown in (I). Scale bars = 100 μm .

Figure 4

Cultured b.End3 cells were incubated with chronic subdural hematoma fluid for 5, 15 and 60 min. b.End3 cells treated with media alone were used as the control. Cell lysates were subjected to Western blotting with anti-phosphorylated focal adhesion kinase at Tyr³⁹⁷ (α-p-FAK), anti-FAK (α-FAK) and anti-β-actin (α-β-actin) antibodies. The histograms show the amount of p-FAK relative to total FAK. Phosphorylation of p-FAK significantly increased in the cultured b.End3 cells compared with the controls 5 min after treatment. The mean ± SE values from the data of 3 series are shown. *p < 0.05 vs the control (1-way ANOVA followed by Fisher's PLSD)

chronic subdural hematoma fluids

Figure 5

The signal transduction of VEGF and N-half osteopontin in the outer membrane of chronic subdural hematoma (CSDH). N-half osteopontin binds to the integrin α9 and β1 receptor on the cell surface. Vinculin, talin, FAK, paxillin, and Src are the main regulators that transduce the signal from the integrin located on the cell surface to the actin cytoskeleton. VEGF is also involved in angiogenesis, activating MAPK in the CSDH outer membrane. These pathways collaborate with each other and induce rigid angiogenesis. References are presented in encircled numbers. †This study revealed for the first time that N-half osteopontin exists within CSDH fluids.

ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor.