表1 Kernohanのグリオーマ分類

Kernohanのgrading分類	他の組織分類とKernohan gradingとの関係	
	組織分類	Kernohan grading
astrocytoma(grade 1-4)	astrocytoma	grade 1
	anaplastic astrocytoma	grade 3
	astroblastoma	grade 2-3
	polar spongioblastoma	grade 1
	glioblastoma multiforme	grade 4
ependymoma(grade 1-4)	ependymoma	grade 1
	ependymoblastoma	grade 2-4
	medulloepithelioma	grade 4
oligodendroglioma(grade 1-4)	oligodendroglioma	grade 1
	oligodendroblastoma	grade 2-4
neuro-astrocytoma(grade 1-4)	neurocytoma	
	ganglioneuroma	grade 1
	gangliocytoma	
	ganglioglioma	
	neuroblastoma	
	spongioneuroblastoma	grade 2-4
	glioneuroblastoma	
medulloblastoma	medulloblastoma	

類が確立されていく中で，分子生物学の進歩により分子レベルでの腫瘍病態が次々と解明され始め，従来の形態学に裏打ちされた脳腫瘍病理学は変革を迫られることになった．そこで，WHOの国際癌研究機関(International Agency for Research on Cancer: IARC)は1997年，'Pathology and Genetics of Tumours of the Nervous System'を発刊し，分子生物学的知見をふんだんに取り入れ，腫瘍の概念から病因・病態・予後を含めた脳腫瘍病理の最新情報を提供した[6]．この刊行物は2000年に改訂され，その中に脳腫瘍の新しいWHO分類が記載され，'脳腫瘍WHO分類2000'と呼ばれている(表2)[7]．この新分類では腫瘍を組織発生別に，神経上皮組織腫瘍，末梢神経腫瘍，髄膜腫瘍，リンパ腫・造血器系腫瘍，胚細胞性腫瘍，トルコ鞍部腫瘍，転移性腫瘍の7群に大別しており，tanycytic ependymomaやchordoid glioma of the 3rd ventricleなど新しい腫瘍が追加されたが，cysts and tumour-like lesionsやpituitary adenoma，chordomaなど臨床上重要な腫瘍が削除されており，まだ完全版とはいえない．

3. 今後の課題と展望

脳腫瘍の組織型を知ることはその腫瘍の外科的切除の程度や補助療法を決定するうえで重要であるだけでなく，患者の予後を予見するためにも必要不可欠である．したがって，腫瘍の組織分類は臨床病態と高い相関があって初めて意味をなすが，病理組織像がその腫瘍のすべてを表してくれるわけではない．例えば，oligodendrogliomaの中には病理組織学的にはastrocytomaと識別が困難なものもあり，遺伝子診断を行い，1番染色体短腕の欠失(1pLOH)または19番染色体長腕の欠失(19qLOH)の有無を知ることによって治療方針の決定や予後判定を正確に行うことができる．特に1pLOHがあればその腫瘍には化学療法が奏効する[8]ことから，遺伝子診断をも含めた正確な組織診断は適切な治療を行うためにも極めて重要である．glioblastomaにおいても，EGFR増幅を示すglioblastoma(primary)とTP53の異常を有するglioblastoma(secondary)とでは，その発生過程における遺伝子異常の違いから，今後，新たな薬剤の

表2 神経系腫瘍のWHO分類(2000)

TUMOURS OF NEUROEPITHELIAL TISSUE
(神経上皮組織腫瘍)

Astrocytic tumours
Diffuse astrocytoma
  Fibrillary astrocytoma
  Protoplasmic astrocytoma
  Gemistocytic astrocytoma
Anaplastic astrocytoma
Glioblastoma
  Giant cell glioblastoma
  Gliosarcoma
Pilocytic astrocytoma
Pleomorphic xanthoastrocytoma
Subependymal giant cell astrocytoma

Mixed gliomas
Oligoastrocytoma
Anaplastic oligoastrocytoma

Ependymal tumours
Ependymoma
  Cellular
  Papillary
  Clear cell
  Tanycytic
Anaplastic ependymoma
Myxopapillary ependymoma
Subependymoma

Choroid plexus tumours
Choroid plexus papilloma
Choroid plexus carcinoma

Glial tumours of uncertain origin
Astroblastoma
Gliomatosis cerebri
Chordoid glioma of the 3rd ventricle

Neuronal and mixed neuronal-glial tumours
Gangliocytoma
Dysplastic gangliocytoma of cerebellum (Lhermitte-Duclos)
Desmoplastic infantile astrocytoma/ganlioglioma
Dysembryoplastic neuroepithelial tumour
Ganglioglioma
Anaplastic ganglioglioma
Central neurocytoma
Cerebellar liponeurocytoma
Paraganglioma of the filum terminale

Neuroblastic tumours
Olfactory neuroblastoma (Esthesioneuroblastoma)
Olfactory neuroepithelioma
Neuroblastomas of the adrenal gland and sympathetic nervous system

Pineal parenchymal tumours
Pineocytoma
Pineoblastoma
Pineal parenchymal tumour of intermediate differentiation

Embryonal tumours
Medulloepithelioma
Ependymoblastoma
Medulloblastoma
  Desmoplastic medulloblastoma
  Large cell medulloblastoma
  Medullomyoblastoma
  Melanotic medulloblastoma
Supratentrial primitive neuroectodermal tumour (PNET)
  Neuroblastoma
  Ganglioneuroblastoma
Atypical teratoid/rhabdoid tumour

TUMOURS OF PERIPHERAL NERVES
(末梢神経腫瘍)

Schwannoma
(neurilemmoma, Neurinoma)
  Celluar
  Plexiform
  Melanotic

Neurofibroma
  Plexiform

Perineurioma
  Intraneural perineurioma
  Soft tissue perineurioma

Malignant peripheral nerve sheath tumour (MPNST)
  Epithelioid
  MPNST with divergent mesenchymal and/or epithelial differentiation
  Melanotic
  Melanotic psammomatous

TUMOURS OF THE MENINGES
(髄膜腫瘍)

Tumours of meningothelial cells
Meningioma
  Meningothelial
  Fibrous (fibroblastic)
  Transitional (mixed)

（表2つづき）

Psammomatous
Angiomatous
Microcystic
Secretory
Lymphoplasmacyte-rich
Metaplastic
Clear cell
Chordoid
Atypical
Papillary
Rhabdoid
Anaplastic

Mesenchymal, non-meningothelial tumours
Lipoma
Angiolipoma
Hibernoma
Liposarcoma (intracranial)
Solitary fibrous tumour
Fibrosarcoma
Malignant fibrous histiocytoma
Leimyoma
Leiomyosarcoma
Rhabdomyoma
Rhabdomyosarcoma
Chondroma
Chondrosarcoma
Osteoma
Osteosarcoma
Osteochondroma
Haemangioma
Epitelioid haemangioendothelioma
Haemagiopericytoma
Angiosarcoma
Kaposi sarcoma

Primary melanocytic lesions
Diffuse melanocytosis
Melanocytoma
Malignant melanoma
Meningeal melanomatosis

Tumours of uncertain histogenesis
Haemangioblastoma

LYMPHOMAS AND HAEMOPOIETIC NEOPLASMS
（リンパ腫・造血器系腫瘍）
Malignant lymphomas
Plasmacytoma
Granulocytic sarcoma

GERM CELL TUMOURS
（胚細胞性腫瘍）
Germinoma
Embryonal carcinoma
Yolk sac tumour
Choriocarcinoma
Teratoma
  Mature
  Immature
  Teratoma with malignant transformation
Mixed germ cell tumours

TUMOURS OF THE SELLAR REGION
（トルコ鞍部腫瘍）
Craniopharyngioma
  Admantinomatous
  Papillary
Granular cell tumour

METASTATIC TUMOURS
（転移性腫瘍）

登場や遺伝子治療の応用により，治療反応性や予後における差が出てくる可能性がある．

近年，遺伝子診断に有力な解析法が登場してきた．それは，一度に数千から数万個の遺伝子の増幅や欠失を定量的に調べることのできるマイクロアレイ法である[9]．本法を用いて，個々の脳腫瘍の遺伝子発現あるいは欠失などを解析し，腫瘍の生物学的特性や臨床病態との関連を加味した新しい腫瘍の分類法が将来可能になるかもしれない．

一方，腫瘍の発生母細胞として神経幹細胞の存在もクローズアップされてきた．神経幹細胞に特異的に発現するMusashi 1が一部のglioblastomaでも発現しており[10]，腫瘍発生における新しい展開が今後進展して行くものと思われる．20世紀初頭に提唱されたBailey-Cushingの組織分類が，その分子生物学的裏付けをもって証明されるときが近い将来にやってくるかもしれない．

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総説

中枢神経系原発悪性リンパ腫の治療

大西丘倫

Treatment of Primary Central Nervous System Lymphoma

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Recent clinical studies for defining a standard treatment of primary central nervous system lymphomas (PCNSL) have demonstrated that high-dose methotrexate (MTX)-based chemotherapy that precedes whole brain irradiation is most effective for achieving longer survival rates for the patients. But the high-dose MTX chemotherapy not infrequently induces delayed neurotoxicity, which produces progressive dementia, particularly in elderly patients older than 60 years old. Furthermore, there are some MTX-resistant tumors and patients with severe renal disfunction in whom high-dose MTX cannot be employed. To reduce severe neurotoxicity, PCNSL patients were treated by chemotherapy alone with radiotherapy deferred as long as possible, but the prognostic outcome was worse than that of the combination therapy of high-dose MTX and radiation. In the future, determination of the optimum dose of MTX, selection of additional effective chemotherapeutic agents and possible reduction of radiation dose should all be solved in order to establish a standard treatment for PCNSL.

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Key words : CNS lymphoma, high-dose methotrexate, chemotherapy, tretament
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はじめに

中枢神経系原発悪性リンパ腫（PCNSL）は，きわめて悪性の脳腫瘍であり，治療がなされなければ急速に進行し，多くは診断から約1カ月以内に死に至る劇症型の腫瘍である．他の脳腫瘍と同様に，PCNSLもその治療の第一選択として外科的摘出術が1980年代前半までは行われてきた．しかし，予想に反して，手術単独の治療ではたかだか3～5カ月の生存しか得られず，また多くのPCNSLが脳深部に発生することもあり，手術を行うことによりむしろ神経症状の悪化をきたした．一方，放射線治療が有効であることが示されてから，PCNSLに対する基本的治療は，腫瘍亜全摘出を行った後，全脳放射線照射を行うことであった[20)21)]．このように手術に放射線を組み合わせても，平均生存期間は約1年半程度であった．

PCNSLという疾患が一般には比較的稀であったこともあり，この当時のデータは大部分retrospectiveな臨床研究から得られたものであったが，この20年の間にPCNSLの患者が著増しており，また症例数の増加に伴って，prospective studyが欧米を中心に積極的になされてきた．その結果，大量のmethotrexate（MTX）をベースにした化学療法を放射線治療前に行うことによってPCNSL患者の生命予後を大幅に延長できることが示さ

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Table 1 Results of chemotherapy with NHL regimens in PCNSL

Treatment	MST (mo)
MACOP-B+WBRT[6]	14
WBRT+VEPA[33]	25.5
CHOP+WBRT[27]	9.6
CHOD+WBRT[32]	16.1
Randomized trial[24]	
WBRT alone	26
WBRT+CHOP	14
WBRT+PCV regimen[7]	41

NHL：non-Hodgkin's lymphoma；MST：median survival time；MACOP-B：methotrexate, doxorubicin, cyclophosphamide, vincristine, predonisone, bleomycin；WBRT：whole brain radiotherapy；VEPA：vincristine, doxorubicin, cyclophosphamide, prednisolone；CHOP (D)：cyclophosphamide, doxorubicin, vincristine, prednisone (dexamethasone)；PCV：procarbazine, lomustine, vincristine

れてきた．本稿では，各治療法の概説を行うとともに，PCNSL 治療の標準化を目指した治療の現状と課題について述べたい．

ステロイドホルモン

グルココルチコイドが，PCNSL 患者の症状を劇的に改善するだけでなく，腫瘍縮小効果があることは以前より知られており，しばしば診断的治療の目的でも使用されてきた．グルココルチコイドによる抗腫瘍作用の分子メカニズムとして，グルココルチコイドがリンパ腫細胞の細胞内受容体と結合し，核に移行して細胞死（アポトーシス）へのシグナルを誘発させることが考えられている[36]．このとき，p53 の正常機能やカスパーゼの活性化は必要でなく，BCL-2 蛋白により細胞死は抑制される．

コルチコステロイドホルモン単独の投与により，PCNSL 患者の約 40%で画像上でも部分，あるいは完全緩解を示す[19]．しかし，その効果の多くは一時的で，投与中止後，数週から数カ月以内に再発をきたす．時にステロイドホルモンの治療は，その中断によりリバウンド現象を起こし，急激な腫瘍の増大のため通常の治療を行えないまま死に至るケースもある．

以上のことから，ステロイドホルモンの投与は，PCNSL の確定診断が得られるまでは，原則として避けるべきである．

手　術

PCNSL の多くは，多発性であったり，視床や基底核，脳梁などの脳内深部に発生しており，広汎な腫瘍切除を行う機会は少ないが，たとえ腫瘍全摘出を行っても，手術単独治療での平均生存期間は 3〜5 カ月でしかない[15)28]．最近の多変量解析の報告では，部分摘出はむしろ独立した予後不良因子となっている[2]．以上より，手術は，脳ヘルニアをきたしそうな巨大な腫瘤の減圧を除いて，組織診断のための生検術が主目的となる．

放射線治療

PCNSL は，解剖学的には全身性非ホジキン病の病期ステージで 1E に相当する．ステージ 1 の全身性非ホジキン病，diffuse large cell に対する放射線単独治療の 5 年生存率は 94%ときわめて良好な治療成績であるのに対して[35]，PCNSL への放射線単独治療における 5 年生存率はたった 4%しかない[9)25]．これまでの PCNSL に対する放射線単独治療の retrospective study においても，50〜60 Gy の全脳照射による奏効率は 79%と比較的良好であるが，生存期間中央値（MST）は 21.5 カ月であった[19]．米国の Radiation Therapy Oncology Group（RTOG）による prospective phase II study でも，40 Gy の全脳照射に 20 Gy の局所 boost を行った結果，奏効率は 81%と良好であったが，MST は 12.2 カ月であった[25]．また，再発した患者の 88%が，boost を行った部位の局所再発で，放射線線量の増量は腫瘍制御には有効ではなかったことが示された．他の放射線単独治療の臨床研究でも，45 Gy 以上の放射線線量の増大は生存期間を延長するには至らなかったと報告している[8]．最近，PCNSL 単発性病変に対して局所照射による治療の試みがなされたが，照射野を拡げた拡大照射のほうが腫瘍制御率および生存期間の延長に有効であった[34]．さらに，PCNSL 患者の剖検の結果，すべての患者で画像上正常と思われる領域にも広汎に腫瘍浸潤がみられ[22]，たとえ画像上単一病変であっても，PCNSL に対する放射線治療法として全脳に 40〜45 Gy 照射し，局所 boost は行わないことが現在の標準治療である．

化学療法

後述するように現在，大量 MTX 療法は PCNSL の標準的な化学療法と考えられているが，これは，全身性非ホジキン病に著効を示した代表的な多剤併用化学療法であ

る CHOP（cyclophosphamide, doxorubicin, vincristine, prednisone）が PCNSL には有効ではなかったためである．

これまでの多施設第 2 相臨床試験[27]で，CHOP 治療 2 コース後，全脳照射（50.4 Gy）を行った結果，CHOP に対する奏効率は 63%であったが，MST はたった 9.6 カ月であった（Table 1）．同様な結果が RTOG 多施設 prospective study でも得られた[32]．そこでは CHOD（prednisone の代わりに dexamethasone を使用）の後，全脳照射がなされたが，MST は 16.1 カ月，2 年生存率は 42%であった．また，放射線単独治療と CHOP 後，放射線照射を行った治療との無作為試験では，放射線単独群の MST が 26 カ月，3 年生存率 29%であったのに対し，CHOP＋放射線治療群の MST は 14 カ月，3 年生存率 28%であった[24]．

現時点で CHOP ならびに CHOP に準ずる他の標準的化学療法で，PCNSL に有効性を示すエビデンスは得られていない．その最大の理由として，いずれの薬剤も正常の脳血液関門（BBB）を透過し得ないことが挙げられる．悪性グリオーマと同様，PCNSL でも腫瘍本体周囲の正常脳内にび漫性の腫瘍浸潤がみられ，この領域までは多くの薬剤が到達しない．したがって，PCNSL に対する化学療法の有効性の鍵の一つは，正常 BBB 透過性薬剤の選択にある．しかし，BBB を通過しえるニトロソウレア系薬剤も，CHOP 同様，PCNSL に対して有効性は示されていない．ただ，この中で，補助化学療法として放射線照射後に PCV（procarbazine, lomustine, vincristine）療法を行った Chamberlain らの報告[7]では，MST が 41 カ月と比較的良好な結果を示している．Procarbazine（PCB）は thiotepa や cytarabine（Ara-C）と同様，リンパ腫細胞に対して細胞毒性のある薬剤であり，化学療法有効性のもう一つの鍵は，リンパ腫細胞に対して高い感受性を有する薬剤か否かにある．

大量 MTX（HD-MTX）療法

MTX は，通常用量では BBB を通過しないが，1 g/m^2 以上の大用量では正常 BBB を透過可能で，さらに中枢神経系のリンパ腫細胞に感受性のある薬剤である．

DeAngelis ら[10]は，31 例の PCNSL 患者に対して，1 g/m^2 の MTX と MTX の髄腔内投与を全脳照射前に行い，放射線治療終了後，大量 Ara-C（3 g/m^2）療法を 2 コース行う第 2 相試験を行った．その結果，MTX に対する奏効率は 64%で，27 名が治療終了後完全緩解を示し，MST は 42.5 カ月であった．長期フォローでは，5 年生存率が 22.3%で，55%が再発を示したが，5 年以上再発がなければそれ以降，再発はみられなかった．主要な副作用は，長期生存例に発症する遅発性白質脳症であった．特に 60 歳以上の高齢者に発生しやすく，進行性の痴呆と血管障害をきたし，半数以上がそれが原因で死亡した．

この報告以後も大量 MTX 療法の有効性を追試する数多くの第 2 相試験や多施設トライアル[1)11)14)18)]が行われる（Table 2）とともに，retrospective にも大量 MTX を含む治療に関する review がなされた[2)5)12)29)]（Table 3）．その結果，PCNSL に対して，大量 MTX は唯一有効な薬剤であり，その後の放射線治療との併用療法が現在，長期生存において最も優れた治療成績を示すことが明らかとなった．

一方，本治療法では，晩発性に比較的高頻度（15～30%）で神経毒性（白質脳症）が発症する副作用も指摘された（Fig. 1）．特に高齢者ではその頻度が高く，大きな課題を残すこととなった．

有効な治療法を求めて

PCNSL 治療に対する大量 MTX の有効性を維持しながら「大量 MTX 療法＋放射線治療」における神経毒性を回避するために，採られた方策は放射線照射を行わないか，または遅らすことであった．Freilich ら[13]は，放射線照射を行わず，大量 MTX をベースとした化学療法のみを行い，奏効率が 92%，MST が 30.5 カ月という成績を得た．その中で，13 例のうち 1 例のみが MTX によると思われる認知障害をきたした．一方，8 g/m^2 というきわめて大用量の MTX のみを用いた全国多施設試験（NABTT 96-07）がなされたが，完全緩解 52%を含む奏効率は 74%で，再発までの生存期間中央値は 12.8 カ月，MST は 23 カ月いう成績しか得られなかった[3]．同様な大容量（8 g/m^2）の MTX 単独治療が prospective multicenter trial（NOA-03）としてなされたが，成績がきわめて不良のため（完全緩解はたかだか 29.7%で，37.8%が増大を示した），早期に本試験は終了することとなった[17]．これらの多施設試験で，神経毒性はみられていないことは評価しうるが，たとえ大用量の MTX を用いてもその効果には限界があることが示された．

McAllister ら[23]は，74 例の PCNSL 患者に対して cyclophosphamide の全身投与後，マンニトールによる BBB の opening を行い，MTX（2.5 g）の動脈内投与と PCB，dexamethasone の全身投与を行うというプロトコールを月 1 回，1 年間継続して行った治療成績（放射線治療は再発まで施行しない）を報告した．完全緩解 65%を含む

Table 2 Phase II trials and a multicenter trial of high-dose MTX-based chemotherapy and radiotherapy in PCNSL

Studies	Treatment	MST (mo)	RR	DNT
DeAngelis (1992)[10]	HD-MTX (1 g/m^2) + WBRT + HD-Ara-C	42.5	64%	9.7%
Glass (1994)[14]	HD-MTX (3.5 g/m^2) + WBRT	33	88%	8%
Hiraga (1999)[18]	HD-MTX (100 mg/kg) + WBRT	39.3	79%	17%
Abrey (2000)[1]	HD-MTX (3.5 g/m^2) + PCB + VCR + WBRT + HD-Ara-C	60	90%	25%
DeAngelis (2002)[11] (RTOG 93-10)*	HD-MTX (2.5 g/m^2) + PCB + VCR + WBRT + HD-Ara-C	37	94%	15%

MST; median survival time, RR; response rate, DNT; delayed neurotoxicity, HD-MTX; high-dose methotrexate, WBRT; whole brain radiotherapy, PCB; procarbazine, Ara-C; cytarabine, VCR; vincristine
*A multicenter trial of the Radiation Therapy Oncology Group

Table 3 Retrospective reviews of the treatment of PCNSL: Independent prognostic factors for longer survival

Reference	No. of patients	Prognostic factor
Reni, et al[29] (*Ann Oncol*, 1997)	1,180	Age: ≦60 yrs, WBRT: >40 Gy HD-MTX, it-CHT
Blay, et al[5] (*J Clin Oncol*, 1998)	226	HD-MTX-based CHT
Bataille, et al[2] (*J Neurosurg*, 2000)	248	Age: <60 yrs, WBRT, CHT with HD-MTX and ATC
Ferreri, et al[12] (*Neurology*, 2002)	370	CHT + RT > RT alone, HD-MTX, HD-MTX + HD-Ara-C

WBRT; whole brain radiotherpy, HD-MTX; high-dose methotrexate, it-CHT; intrathecal chemotherapy, ATC; anthracycline, RT; radiotherapy

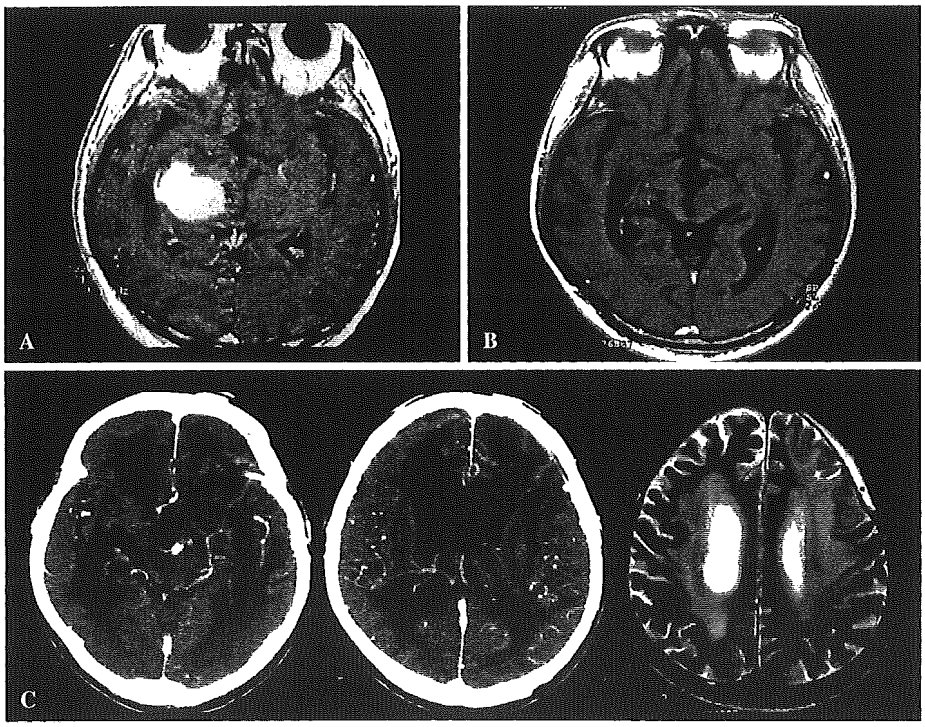


Fig. 1 MRI and CT showing the progress after treatment with high-dose MTX and whole brain radiotherapy (40 Gy). Gd-enhanced MRI before the treatment (**A**), Gd-enhanced MRI at a month after the treatment (**B**), enhanced-CT scans (2 pictures on the left) showing complete remission of the tumor and ventricular dilatation, and T2-weighted MRI (right) demonstrating periventricular white matter hyperintensity consistent with leukoencephalopathy at 3.5 years after the treatment (**C**). The patient became demented about 3 years after the PCNSL treatment.

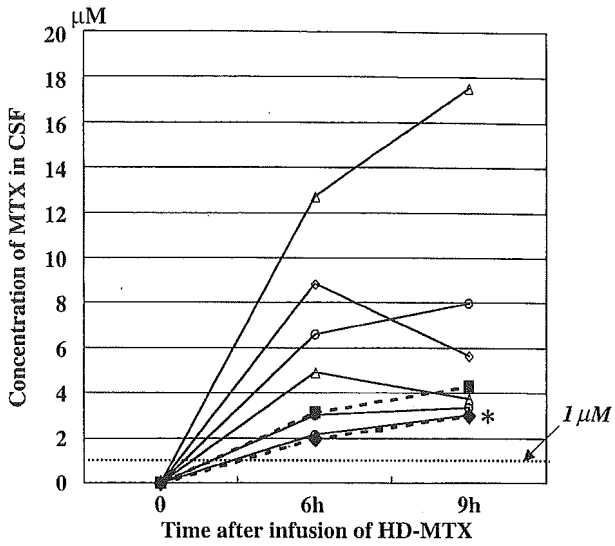
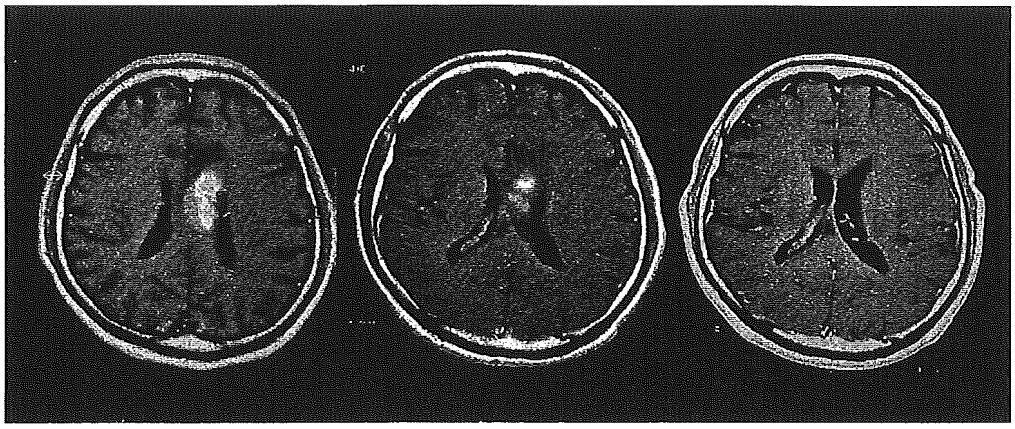


Fig. 2 Time course of CSF concentration of MTX in 8 PCNSL patients who received rapid intravenous infusion of high-dose MTX of 3.5 g/m^2 (6 solid lines) or 2.5 g/m^2 (2 dashed lines). One μM of MTX is the minimum dose for the cytotoxic effect.



before HD-MTX **after 1st course** **after 3rd course**

Fig. 3 Gd-enhanced MRI of the patient before and after receiving a dose of 2.5 g/m^2 MTX. The CSF concentration of MTX in this patient is shown in **Fig. 2** (dashed line with *). The tumor completely disappeared after the third course of high-dose MTX chemotherapy.

奏効率は84%で，MSTは40.7カ月，放射線治療を要した患者は全体で21名(28%)であった．ただ，BBB openingの手技は煩雑で，さまざまな副作用が起こりえること，また，治療期間が1年と非常に長いこと，晩発性の神経毒性の発症については不明で，高濃度MTXによる白質脳症発生のリスクが高いことなどの理由から，本治療法は一般には広まっていない．

PCNSL治療に対する今後の展望

本邦では，現在PCNSLに対する治療として，「大量MTX（3.5 g/m^2，点滴静注）化学療法3コース終了後，全脳放射線治療（30〜40 Gy）」が標準であり，大量MTX療法の導入以来，新たな進展はない．本治療法の問題点をもう一度整理してみると，以下の4点に集約できる．①副作用として晩発性の神経毒性がある．②生存率の向上はみられたが依然MSTは30〜40カ月，5年生存率は約30%と，全身性非ホジキン病と比べ，治療成績は不良である．③MTXに感受性を示さない腫瘍が10%程度ある．④腎障害の患者には使用できない．

問題点①に対しては，MTXの濃度を下げること，全脳への放射線線量を減量，あるいは再発まで放射線照射を延期することが挙げられる．MTXそのものが白質脳症に対する責任薬剤であり，放射線照射の有無にかかわらず神経毒性を示す．これまでわれわれはMTX投与後の髄液中のMTX濃度を測定してきた．3.5 g/m^{2}の3時間急速静脈内投与では，従来報告したと同様[18]，投与開始後6時間で髄液内濃度はピーク値を示した（**Fig. 2**）．一

方，2.5 g/m²の濃度でも，MTXの治療有効濃度の1 μMを上回っており，腫瘍も完全緩解を示した（Fig. 3）．Ferreriらの PCNSL 患者370例の多施設試験の結果[12]でも，MTX濃度と治療効果に関しては1～2.9 g/m²と3 g/m²以上の濃度間に有意な差はなかったと報告している．今後，MTXの副作用を軽減するためにもMTXの至適用量の検討が必要である．

問題点②，③に対しては，MTXに他の薬剤（正常BBBを通過しえ，リンパ腫細胞毒性を持つもの）を併用すること，抗CD20モノクローナル抗体や腫瘍ワクチンの応用が考えられる[4)31)]．全身性の非ホジキンリンパ腫に化学療法が奏効したのは，doxorubicinに加え複数の抗癌剤を併用して用いたからである．Omuroら[26]は，比較的大量MTX（1 g/m²）にBBB透過性のあるthiotepa（30 mg/m²）とPCB（75 mg/m²）を併用することにより，高い奏効率（82%）と12年以上の生存率が12%と，大量MTX（3.5 g/m²）単剤に匹敵する効果があることを報告している．また，Ferreriら[12]は，大量MTXに大量Ara-Cを併用したほうがより生存効果が高いことを報告している．今後，これらの薬剤の併用についても検討が必要である．

④に対しては，PCV療法や今後temozolomideの使用が期待される．PCV療法については，放射線照射後の補助療法[7]や，再発例や進行例でのsecond-line治療[16]として比較的良好な反応が示されており，MTXが投与できない症例には今後有用であるかもしれない．また，現時点では本邦では保険適用にはないが，temozolomideのPCNSLでの有効性が報告されており[30]，MTXとの併用以外にMTX使用不可の場合に選択可能な薬剤となるかもしれない．

おわりに

PCNSLの標準治療法として，現時点では大量MTX（3.5 g/m²）化学療法の後，全脳放射線照射（30～40 Gy）が行われている．しかし，その効果はまだ十分とはいえず，晩発性神経障害，MTX耐性，腎障害患者に対する課題はもちろんのこと，急速に進行する病勢の中でMTX療法の実践が可能か，ステロイド治療は不可避といった臨床の場で直面する問題も多い．確かにPCNSL患者の急増がみられるものの，依然，一施設当たりの患者数は年間数例程度であり，本疾患の未解決な病態を明らかにし，将来有効な治療法を確立していくには，本邦においても多施設の共同研究が必須と思われる．今後の治療の動向に関する指針を得るため，現在，当施設ではMTXの濃度を2.5 g/m²とし，併用薬剤としてthiotepaおよびPCBを用いる大量MTX多剤併用化学療法の有効性に関する臨床研究を開始している．

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要　旨

中枢神経系原発悪性リンパ腫の治療

大西　丘倫

中枢神経系原発悪性リンパ腫（PCNSL）の標準治療として，現在，大量の methotrexate（MTX）をベースにした化学療法を，放射線照射に先行して行うことが推奨されている．この治療法は，従来の放射線単独治療や全身性非ホジキン病に著効を示した CHOP 療法より優れた奏効率と生存期間の延長を招来し，現時点で PCNSL 初発例治療の第一選択となっている．しかし，晩発性に発生する進行性の白質脳症が 15～30%の頻度で起こること，MTX 抵抗性を示す腫瘍の存在，高度腎障害患者には使用できないなど，幾つかの問題も未解決のままである．特に重篤な神経毒性を軽減するために，大量 MTX を含む化学療法単独治療の大規模試験がなされたが，結果は「大量 MTX＋全脳照射」を超えるものではなかった．今後の課題として，MTX の適正濃度の検討，有効な併用薬剤の選択，放射線線量の低減が挙げられる．

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Tumorigenesis and Neoplastic Progression

Up-Regulation of Angiopoietin-2, Matrix Metalloprotease-2, Membrane Type 1 Metalloprotease, and Laminin 5 γ 2 Correlates with the Invasiveness of Human Glioma

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Diffuse infiltration of malignant human glioma cells into surrounding brain structures occurs through the activation of multigenic programs. We recently showed that angiopoietin-2 (Ang2) induces glioma invasion through the activation of matrix metalloprotease-2 (MMP-2). Here, we report that up-regulation of Ang2, MMP-2, membrane type 1-MMP (MT1-MMP), and laminin 5 γ 2 (LN 5 γ 2) in tumor cells correlates with glioma invasion. Analyses of 57 clinical human glioma biopsies of World Health Organization grade I to IV tumors displaying a distinct invasive edge and 39 glioma specimens that only contain the central region of the tumor showed that Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 were co-overexpressed in invasive areas but not in the central regions of the glioma tissues. Statistical analyses revealed a significant link between the preferential expression of these molecules and invasiveness. Protein analyses of microdissected primary glioma tissue showed up-regulation and activation of MT1-MMP and LN 5 γ 2 at the invasive edge of the tumors, supporting this observation. Concordantly, in human U87MG glioma xenografts engineered to express Ang2, increased expression of MT1-MMP and LN 5 γ 2, along with MMP-2 up-regulation, in actively invading glioma cells was also evident. In cell culture, stimulation of glioma cells by overexpressing Ang2 or exposure to exogenous Ang2 promoted the expression and activation of MMP-2, MT1-MMP, and LN 5 γ 2. These results suggest that up-regulation of Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 is associated with the invasiveness displayed by human gliomas and that induction of these molecules by Ang2 may be essential for glioma invasion. *(Am J Pathol 2005, 166:877–890)*

A hallmark of highly malignant human gliomas is their rapid invasive growth into surrounding brain parenchyma. The infiltration by glioma cells throughout the brain renders these tumors incurable even by the combined approaches of surgery, radiotherapy, chemotherapy, and immunotherapy.[1–3] Studies from both *in vitro* and *in vivo* tumor model systems have suggested a link between increased expression and activation of several matrix metalloproteases (MMPs) such as MMP-2 and membrane type 1 (MT1)-MMP and malignant glioma invasiveness.[4] These MMPs produced by tumor, endothelial, and/or stromal cells degrade extracellular matrix at the invasive fronts of the glioma cells, thus removing the extracellular matrix barrier allowing subsequent tumor cell migration into newly created, more permissive spaces in the adjacent brain structures.[5,6] Additionally, increased expression and activation of laminin (LN) 5 γ 2, a basement membrane protein, has been linked to the invasiveness of malignant human cancers such as breast, lung, pancreatic, colon, esophageal, and cervical cancers.[7] Accumulating evidence demonstrates that interaction between MMP-2 and MT1-MMP leads to the activation of MMP-2[5] and interactions between MMP-2,

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MT1-MMP, and LN 5 γ 2 results in the activation of LN 5 γ 2.[8,9] However, the involvement and mechanisms of these proteins in the initiation and maintenance of human glioma cell invasion are not well understood.

Angiopoietin-2 (Ang2) is an angiogenic factor that plays critical roles in angiogenesis and tumor progression. During angiogenesis, Ang2 antagonizes Ang1 activity by competitively inhibiting the binding of Ang1 to their cognate endothelial receptor, Tie2, causing destablization of the vasculature. Ang2 also acts in concert with vascular endothelial growth factor to regulate vessel growth.[10] In human cancers, increased expression of Ang2 in tumor cells is closely correlated to the progression, invasiveness, and metastases of lung, gastric, colon, and breast cancers.[11–15] Stable overexpression of Ang2 by human tumor cells promotes tumor growth, angiogenesis, and metastases in animals.[12,16] We recently have shown that Ang2 induces human glioma cell invasion through the activation of MMP-2 both *in vivo* and *in vitro*. Up-regulated expression of Ang2 and MMP-2 were found in the invasive regions of primary human glioma specimens and in intracranial xenografts of U87MG glioma cells that stably overexpress Ang2.[17] Here, we report that co-expression of Ang2, MT1-MMP, and LN 5 γ 2 were found in the invasive areas, but not in the central regions of primary human glioma specimens. Furthermore, Ang2 induces the expression of MT1-MMP and LN 5 γ 2 in invasive glioma xenografts formed by human U87MG Ang2-expressing glioma cells in the murine brain and in cell culture. These data strongly suggest that Ang2 induces glioma cell invasion through the stimulation of MMP-2, MT-1 MMP, and LN 5 γ 2 in Tie2-deficient glioma cells.

Materials and Methods

Cell Lines and Reagents

Human U87MG glioma cells were obtained from American Type Culture Collection (Rockville, MD) and their culture was previously described.[18] Fetal bovine serum was from HyClone Inc., Salt Lake City, UT. The following reagents were used in this study: anti-Myc-tag antibody (1 μg/ml) from Medical & Biological Laboratories, Ltd., Nagoya, Japan; anti-Ang2 (C-19, 1:50) and anti-LN 5 γ 2 antibodies (C-20, 1:100 or 1 μg/ml) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; anti-mouse CD31 antibody (MEC 13.3, 1:1000) from BD PharMingen, San Diego, CA; anti-MMP-2 (AB809, 1:100), anti-MT1-MMP antibodies (AB8012, 1:500; AB805, 1:500; and AB815, 1 μg/ml), anti-TIMP-2 antibody (mAb 3310, 1:200) from Chemicon Int., Temecula, CA; anti-MMP-9 antibody (1:1000) from CalBiochem., San Diego, CA; recombinant human Ang2 protein (623-AN-025), anti-Ang2 antibody (AF623, 1:200) from R&D Systems, Minneapolis, MN; and anti-von Willebrand factor (vWF) antibody (1:1000) from DAKO, Carpinteria, CA. The secondary and tertiary antibodies were from Vector Laboratories (Burlingame, CA) or Jackson ImmunoResearch Laboratories (West Grove, PA). A DAB elite kit was from DAKO, Aqua block was from East Coast Biologics, Inc. (North Berwick, ME). Cell culture media and other reagents were from Invitrogen/BRL, Grand Island, New York; Sigma Chemicals, St. Louis, MO; or Fisher Scientific, Hanover Park, IL.

Immunohistochemical (IHC) Analyses of Primary Human Glioma Specimens

Of the 96 human glioma specimens investigated (Table 1 and data not shown), there were 8 pilocytic astrocytomas (PA) [World Health Organization (WHO) grade I], 8 diffuse astrocytomas (DA) (WHO grade II), 3 oligoastrocytomas (OA) (WHO grade II), 7 oligodendrogliomas (OD) (WHO grade II), 10 anaplastic astrocytomas (AA) (WHO grade III), 6 anaplastic oligodendrogliomas (AOD) (WHO grade III), 3 anaplastic oligoastrocytomas (AOA) (WHO grade III), and 51 glioblastoma multiforme (GBM) (WHO grade IV). Among these grade I to IV glioma specimens, 57 samples display an invasive edge and 39 samples contain only the central area of the tumors identified by hematoxylin and eosin (H&E) staining. In addition, four normal human brain specimens were also included as normal controls. All of the tumor samples were surgical specimens obtained during the last 7 years at the Department of Neurosurgery, Saitama Medical School, Saitama, Japan. The normal brain samples were from the cerebral hemisphere or cortex of the brain and obtained through autopsies from patients who did not have any brain lesions. The specimens were fixed in 10% formaldehyde and embedded in paraffin. The WHO glioma grade and the presence of invasive areas in the primary glioma biopsies were verified using H&E-stained tissue sections by Dr. Takanori Hirose, a neuropathologist at the Department of Pathology, Saitama Medical School, Saitama, Japan. The diagnosis of the WHO tumor grade and invasiveness was further confirmed by Dr. Ronald Hamilton, a neuropathologist at the Department of Pathology, University of Pittsburgh, Pittsburgh, PA. The 5-μm sections were deparaffinized in prewarmed xylene (55°C) for 5 minutes followed by dehydration. After washing with Tris-buffered saline, the antigen was retrieved by boiling the sections in a citrate buffer (pH 6.0) twice for 5 minutes. The IHC analyses using diaminobenzidine as a chromogen were performed using anti-Ang2 (C-19), anti-MMP-2 (AB809), anti-MT1-MMP (AB8012), anti-LN 5 γ 2 (C-20), and anti-vWF antibodies as previously described.[17] Quantitative analyses of blood vessel densities were performed on serial-cut human glioma specimens that were stained with the anti-vWF antibody as previously described.[19]

Statistical Analyses

A χ^2 test for trend was performed to examine the association between positive staining for Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 and each area of the glioma specimens (center area, border area, and invasive area) using the StatView Version 5.0 software (SAS Institute Inc., Cary, NC). A two-sided P value was calculated on the basis of the χ^2 test. P values less than 0.05 were considered significant.

Table 1. Immunohistochemical Staining for Ang2, MMP-2, MT1-MMP, and LN5γ2

Case	Histology (WHO grade)	Ang2			MMP-2			MT1-MMP			LN5γ2		
		Center	Border	Invasion	Center	Border	Invasion	Center	Border	Invasion	Center	Border	Invasion
J140#	Normal	±			−			−			−		
J141#	Normal	±			−			−			−		
J142#	Normal	1+			−			−			−		
J143#	Normal	±			−			−			−		
J39	P.A. (I)	±	2+	1+	2+	2+	2+	−	1+	1+	1+	2+	1+
J40*	P.A. (I)	2+	2+	2+	±	1+	1+	±	1+	±	−	2+	2+
J42	P.A. (I)	−	2+	1+	−	1+	1+	−	1+	1+	−	1+	1+
J43	P.A. (I)	1+	2+	1+	2+	2+	2+	−	1+	1+	1+	2+	2+
J49	P.A. (I)	−	1+	±	±	3+	3+	−	−	−	−	1+	±
J51	P.A. (I)	±	1+	2+	±	1+	2+	−	1+	1+	−	1+	±
J182	P.A. (I)	−	±	±	1+	2+	2+	1+	2+	2+	1+	2+	1+
J13	D.A. (II)	−	3+	1+	−	3+	2+	−	3+	2+	−	3+	1+
J63	D.A. (II)	1+	1+	2+	−	1+	1+	1+	2+	2+	−	1+	1+
J173	D.A. (II)	1+	2+	1+	±	2+	2+	1+	2+	1+	2+	3+	2+
J178	O.A. (II)	1+	2+	1+	±	2+	1+	1+	2+	1+	2+	3+	2+
J19	O.D. (II)	1+	2+	2+	±	2+	1+	±	1+	1+	±	±	±
J48	O.D. (II)	1+	1+	1+	±	1+	2+	±	1+	1+	−	1+	1+
J181	O.D. (II)	±	1+	1+	1+	2+	1+	1+	1+	1+	1+	2+	2+
J10	A.A. (III)	1+	3+	2+	−	1+	1+	±	2+	2+	1+	3+	2+
J11	A.A. (III)	1+	2+	2+	−	1+	1+	−	−	−	2+	2+	2+
J47	A.A. (III)	2+	2+	2+	1+	1+	1+	1+	1+	1+	−	−	±
J60	A.A. (III)	2+	2+	2+	−	±	±	±	±	1+	−	1+	1+
J62	A.A. (III)	1+	2+	2+	1+	1+	2+	±	1+	2+	1+	2+	2+
J165	A.A. (III)	1+	2+	2+	1+	2+	2+	1+	2+	1+	1+	2+	1+
J16	A.O.D. (III)	1+	2+	2+	1+	2+	1+	−	±	−	1+	2+	1+
J17	A.O.D. (III)	2+	3+	3+	1+	2+	1+	±	1+	1+	1+	2+	2+
J95	A.O.D. (III)	1+	2+	2+	±	1+	3+	±	1+	1+	2+	2+	2+
J96	A.O.D. (III)	1+	2+	2+	±	1+	2+	−	1+	±	±	3+	3+
J18	A.O.A. (III)	2+	2+	2+	−	±	±	±	1+	−	2+	2+	3+
J97	A.O.A. (III)	1+	3+	3+	−	1+	1+	±	1+	1+	1+	2+	2+
J1	GBM (IV)	−	2+	2+	−	3+	2+	−	3+	2+	−	3+	2+
J2	GBM (IV)	1+	2+	2+	2+	2+	2+	1+	2+	2+	2+	2+	3+
J3	GBM (IV)	2+	3+	3+	−	1+	2+	±	2+	2+	1+	2+	2+
J5	GBM (IV)	1+	2+	2+	1+	1+	1+	−	±	±	2+	2+	2+
J6	GBM (IV)	2+	3+	2+	2+	2+	2+	±	1+	2+	2+	1+	2+
J7	GBM (IV)	±	2+	2+	1+	2+	2+	1+	1+	2+	1+	2+	2+
J58	GBM (IV)	1+	2+	2+	±	2+	2+	±	2+	1+	−	2+	2+
J64	GBM (IV)	±	2+	±	−	1+	1+	−	1+	−	±	2+	1+
J66	GBM (IV)	1+	2+	2+	2+	2+	2+	−	1+	1+	1+	1+	2+
J68	GBM (IV)	1+	2+	2+	2+	2+	2+	±	1+	1+	1+	2+	2+
J71	GBM (IV)	2+	3+	3+	±	2+	2+	−	1+	1+	±	±	1+
J72	GBM (IV)	±	2+	2+	1+	2+	1+	−	1+	1+	1+	1+	1+
J78	GBM (IV)	2+	2+	3+	1+	2+	1+	−	1+	1+	−	1+	1+
J79	GBM (IV)	2+	3+	3+	−	−	−	−	1+	1+	1+	2+	2+
J80	GBM (IV)	1+	2+	2+	±	2+	1+	−	±	±	1+	2+	2+
J81	GBM (IV)	1+	3+	3+	1+	2+	2+	−	1+	1+	1+	2+	2+
J82	GBM (IV)	2+	3+	3+	±	2+	1+	−	1+	1+	−	1+	1+
J83	GBM (IV)	±	2+	2+	±	1+	2+	−	1+	1+	2+	2+	2+
J85	GBM (IV)	2+	3+	2+	−	1+	1+	1+	1+	2+	1+	2+	1+
J86	GBM (IV)	2+	3+	3+	1+	2+	2+	±	1+	1+	1+	2+	1+
J87	GBM (IV)	1+	1+	2+	±	2+	1+	1+	2+	2+	2+	2+	±
J89	GBM (IV)	1+	2+	1+	1+	2+	2+	1+	2+	2+	±	1+	1+
J94	GBM (IV)	1+	2+	2+	1+	2+	2+	−	1+	1+	±	2+	2+
J98	GBM (IV)	1+	2+	2+	1+	1+	2+	1+	1+	2+	1+	1+	1+
J151	GBM (IV)	2+	2+	2+	−	2+	2+	−	1+	1+	1+	2+	2+
J152	GBM (IV)	1+	2+	2+	1+	2+	1+	1+	2+	2+	1+	2+	2+
J153	GBM (IV)	1+	3+	2+	±	2+	1+	−	1+	1+	2+	3+	3+
J154	GBM (IV)	1+	2+	2+	1+	3+	2+	1+	2+	2+	1+	2+	2+
J155	GBM (IV)	1+	2+	2+	1+	2+	1+	±	2+	2+	±	2+	1+
J156	GBM (IV)	2+	3+	3+	2+	2+	3+	1+	2+	2+	1+	2+	2+
J164	GBM (IV)	1+	2+	2+	1+	3+	2+	±	1+	1+	±	2+	1+

*Contains neurofibroma 1.
#Scores of normal brain were indicated in the columns of "Center" for convenience.
P.A., pilocytic astrocytoma; D.A., diffuse astrocytoma; O.A., oligoastrocytoma; O.D., oligodendroglioma; A.A., anaplastic astrocytoma; A.O.D., anaplastic oligodendroglioma; A.O.A., anaplastic oligoastocytoma; GBM, glioblastoma multiforme.

Microdissection of Paraffin-Embedded Tissue Sections of Human Glioma Specimens

Microdissection of paraffin-embedded primary human glioma tissues was performed as previously described.[20] Briefly, paraffin-embedded glioma specimens were sectioned separately at 5 μm and 50 μm thickness and mounted onto plain glass slides. To identify the center, border, and invasion regions within the glioma specimen, 5-μm-thick sections were stained by H&E. Three 50-μm-thick sister sections of each sample were then deparaffinized in xylenes, rehydrated in graded ethanol, immersed in distilled water, and air-dried. To exclusively collect center-tumor or border-tumor tissues, the targeted areas were cut microscopically under an Olympus SZ-STS stereomicroscope (Melville, NY) with a fine needle referring to the microscopic observation of the morphology of serial H&E-stained sections.

Extraction and Western Blot Analyses of Proteins from Microdissected Human Primary Glioma Tissues

Protein extraction from microdissected formalin-fixed, paraffin-embedded tissues was performed following protocols that were previously described.[21] Briefly, the collected tumor tissues were suspended in lysis buffer containing 25 mmol/L sodium phosphate buffer (pH 7.6), 150 mmol/L sodium chloride, 1% Triton X-100, 2% sodium dodecyl sulfate, 12 mmol/L sodium deoxycholate, 0.2% sodium azide, 0.95 mmol/L fluoride, 2 mmol/L phenylmethyl sulfonyl fluoride, 50 mg/ml aprotinin, and 50 mmol/L leupeptin and incubated at 100°C for 20 minutes followed by incubation at 60°C for 2 hours. After incubation, the tissue lysates were centrifuged at 15,000 × *g* for 20 minutes at 4°C, and the supernatants containing extracted proteins were collected. The protein extracts were then subjected to Western blot analyses using a sensitive protocol.[22] Briefly, 10 μg of total proteins were separated by NuPAGE 4 to 12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to Immobilon-P transfer membranes (pore size 0.45 μm; Millipore, Bedford, MA) according to the manufacturer's standard procedure. The membrane was blocked and separately probed with anti-MT1-MMP (AB8012, Chemicon), anti-LN 5 γ 2 (C-20, Santa Cruz Biotechnology), and anti-β-actin (Sigma) antibodies. Antibody detection was accomplished with SuperSignal West Femto maximum sensitivity substrate (Pierce).

Generation of U87MG Cell Lines That Stably Express Ang2 or LacZ

Transfected U87MG cell clones that stably express Ang2 were generated by transfecting U87MG cells with a cDNA for Ang2 in the pSecTagB/Myc-His(+) expression vector (Invitrogen, San Diego, CA). The clones that expressed exogenous biologically active Ang2 or LacZ were expanded and characterized as previously described.[17,18]

Tumorigenicity, Glioma Invasion, Mouse Brain Tissue Processing, and IHC

U87MG LacZ- or U87MG Ang2-expressing cell clones (5×10^5) were stereotactically implanted into individual nude mouse brains with five mice per group. When mice developed neurological symptoms because of disturbance of their central nervous system, mice were sacrificed and their brains were removed, processed, and analyzed by IHC as previously described.[17,18]

Quantification Analyses of IHC Data

To quantify microvascular density or the degree of staining by antibodies (for mouse xenograft tissues), five to seven serial-cut sections were stained with the anti-CD31, anti-vWF, anti-Ang2, anti-MMP-2, anti-MT1-MMP, or anti-LN 5 γ 2 antibodies. The stained images (10 or more random areas per section) were captured using an Olympus BX51 microscope equipped with a digital camera, imported into the Image Pro Plus program (Version 4.1; Media Cybernetics, Silver Spring, MD) and analyzed. The mean values of microvessel density or relative intensity of antibody staining from serial brain sections (five or more individual mice per group) in each group were used. Microvessel density was expressed as the ratio of positively stained areas to the total area of the image (object areas/mm^2).[19] The degree of antibody staining in U87MG/Ang2 gliomas was shown as fold of increase to that in U87MG/LacZ tumors.[17]

To quantify the intensities of IHC staining on primary human glioma specimens using anti-Ang2, anti-MMP-2, anti-MT1-MMP, and anti-LN 5 γ 2 antibodies, two experienced researchers (P.G. and Y.I.) independently examined all of the aforementioned sections stained with the four antibodies under ×200 and ×400 magnification. In each stained section, 5 to 10 identified areas (center area, border area, and invasive area) in each type were examined. The intensities of IHC staining with each antibody were defined as no stain (−), weakest (±), low (1+), medium (2+), and strong (3+) stains. The immunoactivities of each antibody on the paraffin-embedded sections were different. The anti-Ang2 and anti-MMP-2 antibodies produced excellent staining without nonspecific background staining. The anti-MT1-MMP had the weakest activity whereas the anti-LN 5 γ 2 antibody had nonspecific staining in some tissue areas. These variations were considered during the scoring. Greater than 95% of the scoring by the two examiners determined in a double-blinded manner was comparable. Discrepancies between the scores were resolved by re-examining the stained sections side-by-side followed by consultation between the examiners.

Western Blot and Zymography Analyses

Western blot and zymography analyses were performed as previously reported.[17] Briefly, six-well plates were coated with or without 10 μg/ml of recombinant Ang2 or bovine serum albumin in phosphate-buffered saline

(PBS) at 4°C overnight, washed three times with PBS, and then dried. U87MG cells or U87MG Ang2-expressing cells were seeded onto the plates and incubated in Dulbecco's modified Eagle's medium with 10% heat-inactivated cosmic calf serum for 4 hours at 37°C. The medium was then replaced by serum-free Dulbecco's modified Eagle's medium, and the cells were maintained in the medium for 24 hours at 37°C. Whole cell lysates containing 30 μg of total protein from the various cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred onto a nitrocellulose membrane. The membrane was blocked and probed with various antibodies. The reacted proteins were visualized by enhanced chemiluminescence reaction. Quantification of the expression of MT1-MMP, TIMP-2, and LN 5 γ 2 was performed by importing and analyzing the scanned images using the Image Pro Plus program.

The zymography analysis for proteolytic activities of MMP-2 toward gelatin was performed as previously described.[17] Briefly, serum-free conditioned medium from a 48-hour culture of various U87MG cells plated onto six-well plates coated with Ang2, bovine serum albumin, or noncoating was collected. Conditioned medium containing 20 μg of total proteins was separated at 4°C in a 7.5% sodium dodecyl sulfate polyacrylamide gel containing 0.2% gelatin. The sodium dodecyl sulfate in the gel was removed by washing the gel three times with a Tris-HCl buffer containing 2% Triton X-100. The gel was rinsed and developed in a gel-developing buffer containing 1% Triton X-100 and 5 mmol/L of $CaCl_2$ at 37°C for 16 hours. The gel was then stained by a Coomassie Blue solution, destained, and photographed. The proteolytic activities of MMP-2 toward gelatin appear as unstained areas.

Results

Preferential Expression of Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 in Invading Glioma Cells and Neovessels of Human Primary Glioma Specimens

We recently reported that Ang2 and MMP-2 are co-overexpressed in the invasive areas including tumor borders, but not in the central regions of primary human glioma biopsies.[17] To further demonstrate whether there is a significant association between up-regulated expression of Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 and tumor invasiveness displayed by various human gliomas in all four WHO grade stages, we conducted a comprehensive survey by IHC analyses. The IHC analyses were performed on a total of 96 human glioma specimens and 4 normal human brain biopsies in our collection. We tested several well-characterized antibodies against each of these molecules that have been reported in similar clinical investigations on human tumor specimens and chose goat anti-Ang2 (C-19),[11,12,15] rabbit anti-MMP-2 (AB809),[9,23] rabbit anti-MT1-MMP (AB8012),[9,23] and goat anti-LN 5 γ 2 (C-20) antibodies as described in the Materials and Methods section. For the negative controls, we applied isotype-matched IgG at the same concentration onto the sister sections of each specimen during the IHC staining, which all showed negative stains (see the insets in Figure 2). The specificity of each of these antibodies on detecting the corresponding protein products were verified by Western blot analyses using these antibodies on U87MG cells that express endogenous MMP-2, MT1-MMP, and LN 5 γ 2 proteins (Figure 4, and data not shown) and U87MG Ang2-expressing cells (for Ang2).[17] Table 1 summarizes our findings of the relative immunoactivities of Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 in 57 paraffin-embedded biopsies of human gliomas that display invasiveness or normal human brain specimens. The relative intensities of the immunostaining by each antibody were quantified as described in the Materials and Methods section.

Normal Human Brain Tissues

We performed IHC analyses on four normal brain samples that were from autopsied patients who did not have brain lesions (specimens J140 to J143, Table 1). All four specimens were mostly from the cerebral hemisphere or cortex of the brain where gliomas occur most frequently.[3] These specimens were homogenous normal brain tissue with similar morphology judged by H&E staining (Figure 1a). As shown in Figure 1 (specimen J141) and Table 1, no or very weak immunoactivities of the antibodies to Ang2 (Figure 1b), MMP-2 (Figure 1d), MT1-MMP (Figure 1e), or LN 5 γ 2 (Figure 1g) were detected. These data indicate that under normal physiological conditions, the expression of these molecules is below or near the detectable levels by IHC analyses.

Pilocytic Astrocytomas (WHO Grade I)

Eight PA biopsies were included in this study (specimens J39, J40, J42, J43, J49, J51, J182, and J183). By H&E staining, we found distinct invasive areas in seven of eight PA biopsies (87.5%; Table 1, data not shown). In addition, intratumoral hemorrhage is common in most of these grade I tumors (Figure 2A, b, data not shown). Figure 2A shows the IHC staining of specimen J42 using H&E and various antibodies. In the center regions of this PA, low expression of Ang2 (Figure 2A, d), MMP-2 (Figure 2A, g), MT1-MMP (Figure 2A, j), and LN 5 γ 2 (Figure 2A, m) was detected (Table 1). In the tumor border (Figure 2A, b), increased expression levels of Ang2 (Figure 2A, e), MMP-2 (Figure 2A, h), and LN 5 γ 2 (Figure 2A, n) are found while a low level of MT1-MMP expression was seen (Figure 2A, k). Interestingly, high levels of Ang2 and MMP-2, but not MT1-MMP and LN 5 γ 2, were seen in neovessels (arrowheads in Figure 2A, e and h), suggesting the role of Ang2 and MMP-2 in vascular growth. In invasive areas (0.25 mm away from the border, Figure 2A, c) where glioma cells are actively invading into the brain structure, only tumor cells express Ang2 (Figure 2A, f, arrows), MMP-2 (Figure 2A, i, arrows), MT1-MMP (Figure 2A, l, arrows), and LN 5 γ 2 (Figure 2A, o, arrows).

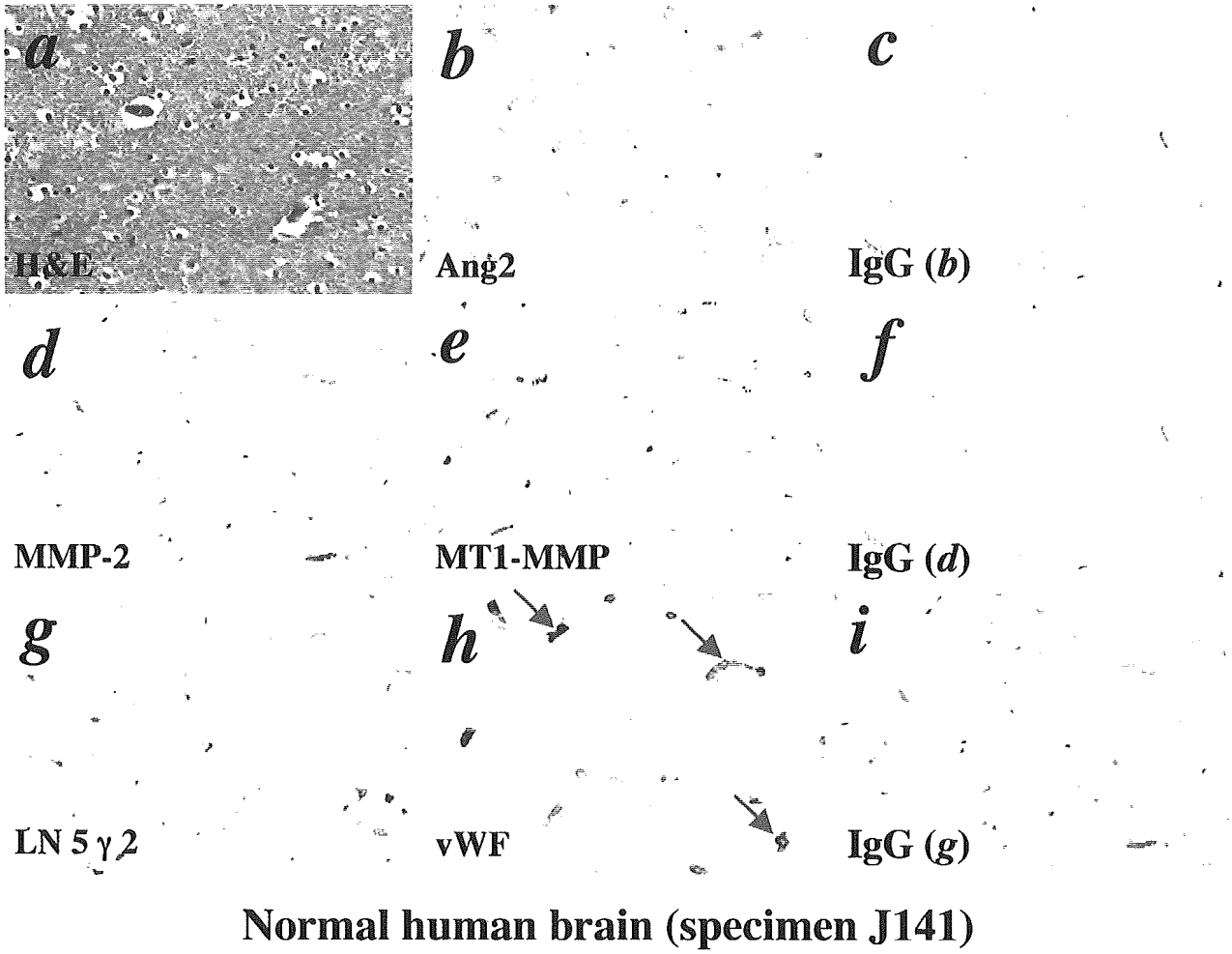

Figure 1. Expression of Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 in normal human brain tissue. IHC on serial sections of a normal human brain biopsy (specimen J141) using H&E (**a**), goat polyclonal anti-Ang2 (**b**), rabbit polyclonal anti-MMP-2 (**d**), rabbit polyclonal anti-MT1-MMP (**c**), goat anti-LN 5 γ 2 (**g**), and rabbit polyclonal anti-vWF (**h**) antibodies. The isotype-matched IgG controls (**c**, **f**, and **i**) are the identical areas shown in **b**, **d**, and **g**. **Arrows** in **h** indicate blood vessels. A total of four individual normal human brain tissues were analyzed. The experiments were repeated two additional times with similar results. Original magnifications, ×400.

Thus, co-expression of Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 are detected in the invading glioma cells and growing neovessels in tumor borders, but not in the central regions of PA tumors. These results suggest that at the early stage of glioma progression, up-regulation of these molecules correlates with the initial invasion displayed by PA.

Diffuse Astrocytomas (DA, Grade II), Oligoastrogliomas (OA, Grade II), and Oligodendrogliomas (OD, Grade II)

There were 8 DA, 3 OA, and 7 OD biopsies that are WHO grade II tumors in our collection. In the DA specimens, diffuse morphology of tumor cells is evident. In some tumors, diffusive tumor cells formed a cellular gradient toward the normal brain structure without identifiable borders (data not shown). Among the 18 grade II tumors, 7 tumor specimens (38.8%) contain identifiable tumor borders and clear invasive regions (at least 0.25 mm away from the tumor border, Table 1). Figure 2B shows the IHC staining of specimen J13 (a DA) using H&E and various antibodies. Consistent with the expression profiles in the WHO grade I gliomas (Figure 2A), strong expression of Ang2 (Figure 2B, e), MMP-2 (Figure 2B, h), MT1-MMP (Figure 2B, k), and LN 5 γ 2 (Figure 2B, n) were evident in tumor cells (arrows) and in neovessels (arrowheads) along the tumor border, but not in the center region of the tumors (Figure 2B; d, g, j, and m). Increased expression of Ang2 (Figure 2B, f), MMP-2 (Figure 2B, i), and LN 5 γ 2 (Figure 2B, o), and strong expression of MT1-MMP (Figure 2B, l) were detected in cells (arrows in Figure 2B; f, i, l, and o) and in vessels (arrowheads) in the invasive areas that are 0.25 mm away from the tumor border.

Anaplastic Astrocytomas (AA, Grade III), Anaplastic Oligodendrogliomas (AOD, Grade III), and Anaplastic Oligoastrocytomas (AOA, Grade III)

There were 10 AA, 6 AOD, and 3 AOA gliomas in our collection. All of the grade III gliomas displayed in-

creased cellularity and malignancy, especially in the central region (data not shown). Among the 19 grade III glioma biopsies, 12 tumors contain tumor borders and invasive areas (63.2%, Table 1). In contrast to the grade I and II glioma specimens, the tumor borders were distinct and with sharp edges (data not shown). Similar to that in the grade II gliomas (Figure 2B), up-regulated Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 were found in the tumor borders, but not in the central regions of these tumors (data not shown). In addition, increased expression of all four molecules were detected in cells in the invasive region 0.25 mm away from the tumor borders in the grade III gliomas (data not shown).

GBM (WHO Grade IV)

There were 51 GBM specimens in our collection. These grade IV glioma biopsies display the highest cellularity and contain evident necrotic areas and intratumoral hemorrhage (data not shown). Among the 51 GBM, 31 specimens contain tumor borders and invasive areas (60.8%, Table 1). The tumor borders are also sharp and easily distinguishable (Figure 2C, a). Figure 2C shows the IHC staining of specimen J1 (a GBM) using H&E and various antibodies. We found that protein expression profiles of Ang2 (Figure 2C, e), MMP-2 (Figure 2C, h), MT1-MMP (Figure 2C, k), and LN 5 γ 2 (Figure 2C, n) were located in the tumor borders similar to IHC staining of the grade II and III gliomas (Figure 2B and data not shown). In addition, increased levels of expression of Ang2 (Figure 2C, f), MMP-2 (Figure 2C, i), MT1-MMP (Figure 2C, l), and LN 5 γ 2 (Figure 2C, o) were also found in the invasive areas of the GBM tumors. The IHC results of WHO grade III and IV gliomas demonstrate that increased expression of Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 are further elevated in the tumor borders and invasive regions of high-grade human glioma specimens.

Glioma Angiogenesis

As shown in Figures 1 and 2, we stained all of the normal brain and glioma tissue specimens using an anti-vWF antibody that stains tumor endothelium. We evaluated glioma angiogenesis by quantifying the microvessel density in all of the stained tissue sections. In normal human brain tissues, vessels were apparent (Figure 1, h). In WHO grade I (Figure 2A, p and q) and II (Figure 2B, p and q) tumors, when compared with the MCD in normal brain tissues, a 1.8-fold increase of MCD was found. In WHO grade III gliomas, a 3.2-fold increase of MCD was seen. In WHO grade IV gliomas (Figure 2C, p and q), a further increase of MCD (7.1-fold) was found (data not shown). These data corroborate with previous studies showing that glioma angiogenesis is not evident in low-grade (I and II) gliomas, but significantly increased in high-grade (III and IV) tumors.[24]

Up-Regulated Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 Are Preferentially Associated with Tumor Invasiveness among the Clinical Glioma Specimens

As shown in Table 1, we have found that 57 glioma specimens contain identifiable tumor borders and nearby invasive regions. There are 39 additional glioma specimens that only contain the central region of the tumors. We hypothesize that there is a significant association between up-regulated expressions of Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 and tumor invasiveness displayed by all four WHO grades of human gliomas. To test our hypothesis, we examined the IHC analyses data on the cases that contain all three areas within the same sample: tumor center, border, and invasive areas, and determined the number of the cases analyzed that meet these criteria. As shown in Table 1, in seven grade I gliomas, there are five to six cases of seven samples (71 to 85.7%) that have expression scores in the areas both in the border and invasive areas higher than that of the center area. Similarly, the scores of protein expression level in the majority of cases in grade II to IV specimens meet the same criteria: grade II, 5 to 6 cases of 7 (71 to 85.7%); grade III, 7 to 9 cases of 12 (58 to 75%), and grade IV, 23 to 28 cases of 31 (74.1 to 90%). Together, in a total of 50 glioma grade II to IV specimens that contain all three areas (center, border, and invasion), 35 to 43 cases (70 to 86%) are available for testing our hypothesis (Table 2).

Next, we determined whether there is a distinct link between the up-regulation of the four molecules that are shown to be vital in the invasiveness of human cancers. A χ^2 test for trend was performed to examine the association between positive staining for Ang2, MMP-2, MT1-MMP, LN 5 γ 2, and each area of grade II to IV glioma specimens (center area, border area, and invasion area). A two-sided P value was calculated on the basis of the χ^2 test. As shown in Table 2, significant links are found between the positive immunoactivities of Ang2 ($P < 0.0001$ for both comparisons), MMP-2 ($P < 0.0001$ for border versus center and $P = 0.0001$ for invasion versus center, respectively), MT1-MMP ($P < 0.0001$ for both comparisons), and LN 5 γ 2 ($P < 0.0001$ for border versus center and $P = 0.0002$ for invasion versus center, respectively), and the invasiveness displayed by these gliomas. In contrast, there are no differences in the expression profiles of these four proteins between the tumor borders and invasive regions (between $P = 0.5120$ to $P = 0.7880$). Because the pathogenesis of PAs is different from that of grade II to IV gliomas,[3] we separately performed the χ^2 test for trend on the IHC data for PA gliomas. No significant association of the expression levels of these four molecules and tumor invasiveness could be found, possibly because of the small sample size analyzed (data not shown). In addition, because the majority of the glioma specimens analyzed in Table 2 are grade IV GBM (31 of 50, 62%), we separately performed the χ^2 test for trend analyses on the IHC data of these 31 grade IV GBM samples. A statistically significant link

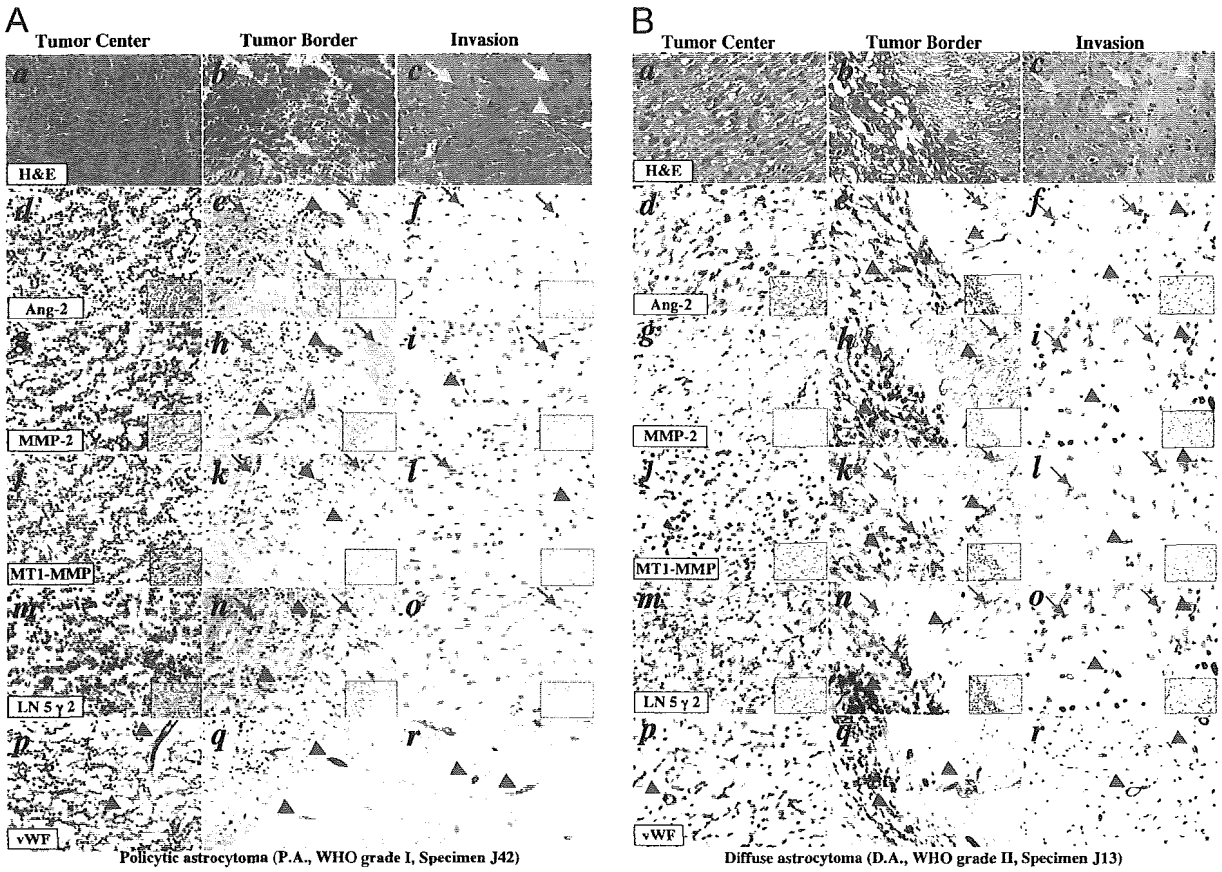

Figure 2. Co-overexpression of Ang2, MMP-2, MT1-MMP, and LN 5 γ 2 in the invasive areas of primary human glioma tissues. IHC on serial sections of PA (**A:** specimen J42, WHO grade I), DA (**B:** specimen J13, grade II), and GBM (**C:** specimen J1, grade IV) tissues using H&E (**a** to **c**), goat polyclonal anti-Ang2 (**d** to **f**), rabbit polyclonal anti-MMP-2 (**g** to **i**), rabbit polyclonal anti-MT1-MMP (**j** to **l**), goat anti-LN 5 γ 2 (**m** to **o**), and rabbit polyclonal anti-vWF (**p** to **r**) antibodies. **Insets** in **d** to **o** are the isotype-matched IgG controls that are the identical areas shown in each panel. **a**, **d**, **g**, **j**, **m**, and **p:** Central regions of the tumor mass; **b**, **e**, **h**, **k**, **n**, and **q:** tumor borders; and **c**, **f**, **i**, **l**, **o**, and **r:** areas that are 0.25 mm away from the tumor edge shown in **b**, **e**, **h**, **k**, **n**, and **q**. **Blue arrowheads** in **b** indicate the border between the tumor mass (**left**, high cellular density) and normal brain tissue (low cellular density). **Red** or **yellow arrowheads** in **b** to **r** indicate tumor vessels. **Arrows** in **b** to **o** are glioma cells or activated neural cells that were stained by anti-Ang2 (**e**, **f**), anti-MMP-2 (**h**, **i**), anti-MT1-MMP (**k**, **l**), and anti-LN 5 γ 2 (**n**, **o**) antibodies, respectively. A total of 96 individual primary tumor specimens (WHO grades I to IV) were analyzed. The experiments were repeated two additional times for each section with similar results. Original magnifications, ×400.