In Situ Visualization of Intratumor Growth Factor Signaling

Immunohistochemical Localization of Activated ERK/MAP Kinase in Glial Neoplasms

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Abnormal growth factor signaling is implicated in the pathogenesis of gliomas. The extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway is a likely target, linking receptor tyrosine kinase activation to downstream serine/threonine phosphorylation events regulating proliferation and differentiation. Signaling within heterogeneous cell populations of gliomas cannot be adequately assessed by traditional biochemical enzyme assays. Immunohistochemical detection of doubly phosphorylated (activated) ERK/MAPK permitted visualization of spatially discrete cellular patterns of ERK/MAPK activation, compared with the relatively uniform expression of total ERK/MAPK protein. The astrocytic tumors, regardless of grade, had the highest overall degree of enzyme activation, whereas oligodendrogliomas had the least. Anaplastic progression in oligodendrogliomas resulted in a larger number of cells with active ERK/MAPK. Within glioblastomas, microvascular hyperplasia and necrosis were associated with ERK/MAPK activation in adjacent tumor cells. In addition to spatial patterns of intratumor paracrine signaling, a possible cell-cycle-associated regulation was detected: mitotic and actively cycling tumor cells showed diminished activation relative to cells in G0. Although ERK/MAPK activation was not restricted to neoplastic glia, consistent patterns of selective activation in tumor cells suggests that sustained activation may contribute to the neoplastic glial phenotype. (*Am J Pathol* 1998, 153:1411–1423)

The diffuse-type astrocytomas are the most common group of primary brain tumors and have a strong propensity for anaplastic progression to more malignant forms.¹

A growing body of evidence implicates up-regulated and abnormal growth factor signaling, due to either genetic or epigenetic changes, in both early and late stages of pathogenesis.^{2–9} Genes for growth factor receptors of the protein-tyrosine kinase family, including those for the epidermal growth factor (EGF) receptor and the platelet-derived growth factor (PDGF) receptor are amplified and/or overexpressed in many malignant astrocytomas. Of particular interest is the finding that approximately one-half of glioblastomas with amplification of the EGF receptor gene harbor mutations conferring constitutive receptor activation.⁴ Other receptors, such as those for insulin-like growth factors (IGFs), may contribute to the malignant astrocytic phenotype via autocrine/paracrine mechanisms.⁹ However, thus far, there is little direct evidence for altered signaling downstream of growth factor receptors in human glial tumors.

The extracellular signal-regulated kinases (ERKs) are the most thoroughly studied group of the larger mitogen-activated protein kinase family, which includes ERKs, stress-activated protein kinases/JNKs, and p38 mitogen-activated protein kinase (MAPK). The ERKs constitute a family of highly conserved and ubiquitously expressed serine/threonine kinases that are crucial components of signaling pathways activated either by growth factors binding to tyrosine-kinase-linked receptors or by ligands binding to G-protein-linked receptors.^{10–14} ERK/MAPK activation has been shown to lead either to differentiation or proliferation, depending on the cellular context and temporal features of the activating signal.¹⁵ In cultured astrocytes, ERK/MAPK can be activated by multiple factors, including EGF, fibroblast growth factor, PDGF, IGF-1, brain-derived neurotrophic factor, thrombin, endothelin, and cholinergic agonists.^{16,17} Moreover, experimental models suggest that persistent activation of ERK/MAPK can lead to cellular transformation in some, but not all cell types.^{18–21} Constitutive activation of ERK/MAPK,

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as assessed by electrophoretic mobility shift and kinase assays, has been described in renal cell carcinomas.²² A recent report suggests that ERK/MAPK is overexpressed and activated in breast carcinomas.²³ The present study was undertaken to assess ERK/MAPK expression and activation in human astrocytomas. We examined both low-grade and malignant gliomas to assess potential roles for the signaling pathway in both neoplastic transformation and malignant progression. We used a newly developed antibody specific for activated ERK/MAPK (dually phosphorylated on both threonine and tyrosine within the activation domain). The use of immunohistochemistry to specifically detect activated ERK/MAPK in tumor tissue sections enabled visualization of spatial patterns of pathway activation within heterogeneous tumor tissues, previously not possible with biochemical enzymatic assays. Our results indicate that ERK/MAPK is highly activated in both low-grade and malignant gliomas. Within glioblastomas, there appears to be a more complex spatial activation of the signaling pathway within distinct tumor zones as well as within specific cell cycle subpopulations.

Materials and Methods

Antibodies

The phospho-specific antibody to dually phosphorylated ERK/MAPK (dp-ERK/MAPK) was prepared commercially (Quality Control Biochemicals, Hopkinton, MA) by immunizing rabbits with the phosphopeptide CTGFL(pT)E(pY)VATRW conjugated to keyhole limpet hemocyanin. Affinity purification was performed by first adsorbing serum with the unphosphorylated peptide and then positively selecting for phospho-specific antibodies with the doubly phosphorylated peptide. Specific antiserum was eluted in glycine buffer, pH 2.0, and used at 1 μ g/ml for immunohistochemistry and 0.2 μ g/ml for immunoblotting. Other primary antibodies were obtained from the following sources and used at the indicated concentrations for immunohistochemistry: polyclonal affinity-purified rabbit anti-MAPK (ERK1 plus ERK2; ZS61-7400, Zymed Laboratories, South San Francisco, CA) at 2.5 μ g/ml for immunohistochemistry and 0.5 μ g/ml for immunoblotting, monoclonal anti-Ki-67 (Mib-1; Immunotech, Marseilles, France) at 1:50 for immunohistochemistry, and monoclonal antibody MPM-2²⁴ (provided by Dr. Gary Gorbsky, University of Virginia) at 1:40,000 from ascites fluid for immunohistochemistry.

Immunoblotting

Immunoblotting was performed on snap-frozen tumor or non-neoplastic cortex specimens using both the phospho-specific and total ERK/MAPK antibodies. Specimens were homogenized in lysis buffer (50 mmol/L Hepes, pH 7.5, 100 mmol/L NaCl, 2 mmol/L EDTA, 1 μ mol/L pepstatin, 1 μ g/ml leupeptin, 0.2 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 2 μ g/ml aprotinin, 40 mmol/L P-nitrophenyl phosphate, and 3.5

mg/ml dithiothreitol and then cleared by centrifugation at $15,000 \times g$ for 10 minutes. Protein (100 μ g/lane) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% gel. After electrophoretic transfer to nitrocellulose, antibody binding was detected with horseradish-peroxidase-coupled goat anti-rabbit antibodies using the enhanced chemiluminescence technique according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

Immunohistochemistry

Tumor and non-neoplastic neurosurgical specimens were obtained from the Neuropathology archives or consultation files of the University of Virginia. All tissues were fixed either in buffered neutral formalin or buffered zinc formalin and processed routinely into paraffin. After deparaffinization in xylenes and alcohols, quenching of endogenous peroxidase activity was performed for 45 minutes in 0.5% hydrogen peroxide/methanol. Microwave antigen enhancement (in 10 mmol/L citrate buffer, pH 6.0, for 10 minutes at 1.15 kW), although not required for detection of either ERK/MAPK or dp-ERK/MAPK immunoreactivity, improved nuclear staining and was used for all experiments. Alkaline phosphatase pretreatment was performed as previously described.²⁵ Peptide competition controls were performed by preincubating diluted anti-dp-ERK/MAPK (1 μ g/ml) for 2 hours at room temperature with 15 μ mol/L of either the dp-ERK/MAPK peptide (competitor), the corresponding unphosphorylated peptide (control), or a doubly phosphorylated MAPK/ERK-Kinase (MEK) peptide (control). Immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method according to the manufacturer's instructions (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) using diaminobenzidine as the chromogen. Sections were lightly counterstained with hematoxylin. Double-label immunohistochemistry was performed sequentially using the peroxidase substrates aminoethyl-carbazole and Vector SG, according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA).

Digital images were acquired with a Dage DC-330 three-color CCD camera controlled by Image Pro-Plus software. Adjustments in image brightness and/or contrast were made using Adobe Photoshop 3.0 and were performed identically and in parallel on both phospho-MAPK and corresponding total ERK/MAPK images. Composite images were made using Aldus Freehand and printed on a Codonics NP-1600 dye sublimation printer.

Characterization of the ERK/MAPK Phosphopeptide in Histopathological Specimens

Comparison of neurosurgical specimens (rapidly fixed) and postmortem (long delay until fixation) revealed evidence for lability of the ERK/MAPK phosphopeptide. For example, surgical specimens containing metastatic tumors or iatrogenic injuries invariably showed strong cy-

toplasmic and nuclear dp-ERK/MAPK immunoreactivity within surrounding reactive astrocytes (J.W. Mandell and S.R. VandenBerg, unpublished observations). In contrast, postmortem specimens containing morphologically similar reactive astrocytes revealed no detectable dp-ERK/MAPK, suggesting time-dependent dephosphorylation and loss of epitope. The phosphorylation-independent ERK/MAPK epitope was uniformly preserved in both autopsy and surgical material (data not shown). Moreover, large (>1 cm diameter) surgical astrocytic tumor specimens sometimes showed a central zone of diminished or absent dp-ERK/MAPK immunoreactivity, in contrast to spatially uniform total ERK/MAPK immunoreactivity. This pattern suggests that loss of phosphoepitope may be partly fixation dependent. The ERK/MAPK phosphoepitope thus appears to be somewhat labile, most likely with a half-life in the range of one to several hours in tissue specimens. This time course is probably due to the continued activity of cellular phosphatases in the face of ATP depletion and consequent diminishing kinase activity. A similar phenomenon has been described in detail for the microtubule-associated protein tau in human brain tissues.^{26,27} Because of the apparent lability of the ERK/MAPK phosphoepitope, we compared our routine fixative (10% zinc-buffered formalin) with one containing acrolein, a rapidly acting cross-linking fixative,²⁸ with or without a permeabilizing detergent and a tyrosine phosphatase inhibitor (10% zinc-buffered formalin, 0.5% acrolein, 0.1% Triton X-100, 1 mmol/L orthovanadate). We found no significant differences in retention of dp-ERK/MAPK immunoreactivity between the two fixatives when used on small fragments of fresh astrocytic tumor material (not shown). Acrolein fixation of a glioblastoma cell line (U-251 MG) did result in an increase in basal dp-ERK/MAPK immunoreactivity, with a higher percentage of positive cells, but caused no obvious qualitative differences in the subcellular pattern of immunolabeling (not shown).

Results

Immunoblotting

Specificity of both ERK/MAPK antibodies for ERK1 and ERK2 was confirmed by immunoblotting. In addition, these experiments revealed evidence for elevated phosphorylation, but not overexpression of ERK/MAPK in astrocytic tumors relative to non-neoplastic cortex (Figure 1). In both non-neoplastic and astrocytic tumor specimens, the phospho-insensitive ERK/MAPK antibody revealed a doublet at 42 to 44 kd, consistent with the specificity for both ERK1 and ERK2 as reported by the manufacturer (Zymed). There appeared to be no significant differences in ERK/MAPK protein levels between non-neoplastic cortex and tumors or among astrocytomas, both circumscribed (pilocytic, WHO grade I) and diffuse types (WHO grades II to IV). The phospho-specific antibody also labeled a doublet at the appropriate position, with a tendency for greater intensity of the p42 band. In non-neoplastic cortex samples, dp-ERK/MAPK intensity varied from undetectable to low levels. In con-

Figure 1. Immunoblot analysis of ERK/MAPK protein expression and phosphorylation in non-neoplastic cortex and astrocytomas. ERK/MAPK protein expression levels (**bottom panel**; total ERK/MAPK) are similar in non-neoplastic cortex (**lanes 1 to 3**) and astrocytic tumors of all grades (**lanes 4 to 7**). In contrast, dually phosphorylated ERK/MAPK (**top panel**; phospho-MAP kinase) is generally elevated in astrocytic tumors compared with the barely detectable levels in three different normal cortex samples. Although there was great variation in dp-ERK/MAPK levels among tumors, intense dp-ERK/MAPK bands like those in the tumor samples illustrated were never observed in non-neoplastic cortex samples, obtained from lobectomy specimens for intractable epilepsy. Equal quantities of protein were loaded for each specimen.

trast, many astrocytic neoplasms, including all histological grades, tended to have elevated dp-ERK/MAPK levels, although there was significant variation from sample to sample. This variation could be due to the inadvertent sampling of necrotic tissue in some of the glioblastoma specimens or of predominantly normal cortex in some of the lower-grade tumor samples. Alternatively, the finding of occasional tumor specimens with very low dp-ERK/MAPK could represent biologically significant variations. However, it is not possible to determine the source of variation from the immunoblot analysis. In any case, the high level of dp-ERK/MAPK detected in many astrocytomas was never observed in non-neoplastic cortex specimens.

Figure 2. Demonstration of dp-ERK/MAPK antibody specificity in immunohistochemistry. dp-ERK/MAPK (phospho MAPK) immunoreactivity is abolished by preadsorption with the doubly phosphorylated immunizing peptide (B) but not the unphosphorylated peptide (A). Alkaline phosphatase pretreatment almost completely eliminates phospho-MAPK immunoreactivity (D) compared with buffer-only control (C). Staining with the phospho-insensitive general ERK/MAPK antibody is unaffected by phosphatase treatment (E and F). Immunoperoxidase staining with hematoxylin counterstain; original magnification, $\times 50$.

Immunohistochemical Specificity of Phospho-Specific ERK/MAPK Antibody

Two different types of control experiments were performed to demonstrate specificity of the phospho-specific ERK/MAPK antibody in immunohistochemistry. First, immunostaining was almost completely eliminated by preincubation of antibody with the doubly phosphorylated but not the unphosphorylated peptide (Figure 2, A and B). Preincubation of the dp-ERK/MAPK with an irrelevant doubly phosphorylated peptide (corresponding to the MEK phosphorylation site; gift of Dr. Andreas Nelsbach, New England Biolabs, Beverly, MA) did not affect immunolabeling (data not shown). This control demonstrates that the specific amino acid sequence surrounding the phospho-amino acids, and not merely the presence of phosphate groups, was recognized by the antibody in tissue sections. Second, immunostaining with the phospho-specific but not the phospho-insensitive antibody was greatly diminished by pretreatment of tissue sections with alkaline phosphatase, which is expected to cleave nonspecifically all phosphate groups (Figure 2, C and D).

Immunohistochemical Localization of ERK/MAPK Phosphorylation in Non-Neoplastic Cortex

The distribution of total and phospho-MAPK immunoreactivity was examined in nine non-neoplastic temporal and frontal lobe specimens resected for intractable epilepsy. Areas depicted in Figure 3 were from portions of lobes that were histologically normal. Immunostaining using the phospho-insensitive ERK/MAPK antibody was similar to that previously reported in human brain,²⁹ with highest expression in cytoplasm of pyramidal neurons (Figure 3, B and D). In addition, we found significant immunoreactivity in neuropil, subpial and parenchymal astrocytes, and white matter. Phosphorylated ERK/MAPK (dp-ERK/MAPK), on the other hand, was undetectable throughout most non-neoplastic temporal lobe specimens. Pyramidal neurons, although strongly immunoreactive for ERK/MAPK protein, never showed detectable dp-ERK/MAPK immunoreactivity. Occasional capillary endothelial cells in gray and white matter (arrow, Figure 3C) and some subpial astrocytes in regions showing mild subpial gliosis (corner inset, Figure 3A) demonstrated detectable dp-ERK/MAPK immunoreactivity. The presence of subpial gliosis in these specimens was

Figure 3. Localization of activated ERK/MAPK in non-neoplastic human cortex. dp-ERK/MAPK (pMAPK) immunoreactivity is undetectable in non-neoplastic gray (A) and white (C) matter, with the exception of subpial astrocytes in regions of mild gliosis (corner inset, A) and occasional capillary endothelial cells (arrows, A and C). In contrast, total ERK/MAPK (MAPK) immunoreactivity is uniformly present in gray (B) and white (D) matter, especially in pyramidal cell somata (B), and in the processes of subpial astrocytes (inset, B). Immunoperoxidase staining with hematoxylin counterstain; original magnification, $\times 100$.

Table 1. Summary of Immunohistochemical Analysis of ERK/MAPK Phosphorylation in Human Gliomas

	1 (<10%)	2 (10–50%)	3 (>50%)
Pilocytic astrocytoma (I)	2	8	4
Pleomorphic xanthoastrocytoma (II/III)	1	2	2
Diffuse-type astrocytoma (II/III)	0	5	6
Glioblastoma multiforme (IV)	0	11	9
Oligodendroglioma (II)	5	3	1
Anaplastic oligodendroglioma (III)	1	3	4
Ependymoma (II)	0	3	2
Anaplastic ependymoma (III)	0	1	0

Tumor sections immunolabeled with an antibody recognizing doubly phosphorylated (activated) ERK/MAPK were visually scored as 1 (<10% of tumor cells positive), 2 (10 to 50% of tumor cells positive), or 3 (>50% of tumor cells positive). Either cytoplasmic and/or nuclear immunoreactivity was considered as positive. The WHO tumor grade is indicated in parentheses.

confirmed by glial fibrillary acidic protein immunohistochemistry (not shown).

Immunohistochemical Localization of ERK/MAPK Phosphorylation in Glial Neoplasms

A total of 89 neurosurgical tumor specimens was examined by immunohistochemistry. The tumors included 52 astrocytomas (16 pilocytic astrocytomas, 5 pleomorphic xanthoastrocytomas, 11 WHO grade II or III, and 20 WHO grade IV (glioblastoma multiforme), 9 oligodendrogliomas, 8 anaplastic oligodendrogliomas, 5 ependymomas, 1 anaplastic ependymoma, 11 primary and metastatic adenocarcinomas, and 3 CNS lymphomas. For each specimen, adjacent paraffin sections were processed simultaneously for immunohistochemistry using both the phospho-specific (dp-ERK/MAPK) and phospho-insensitive (total ERK/MAPK) antibodies.

Astrocytomas

Relative to non-neoplastic and quiescent astrocytes, astrocytic tumor cells from all grades of tumors showed elevated ERK/MAPK phosphorylation (Table 1; Figures 4 to 6). Both nuclear and cytoplasmic dp-ERK/MAPK immunoreactivity was detected in subpopulations of tumor cells in all specimens examined, with significant cell-to-cell variation in the degree of nuclear localization. As detailed in Materials and Methods, some of the large-scale spatial heterogeneity could be attributed to fixation-related artifact, such as the absence of immunoreactivity in the core of large (>1 cm) tumor specimens. However, interesting patterns of heterogeneous labeling were observed in large areas where immunoreactivity was preserved, as described below. In contrast to dp-ERK/MAPK immunoreactivity, total ERK/MAPK immunoreactivity was

more uniformly expressed throughout tumor specimens and in all cells.

Diffuse astrocytomas, both grade II and III (anaplastic), showed selective labeling of a subset of infiltrating tumor cells by the phospho-specific ERK/MAPK antibody (Figure 4, A and C). Nuclear labeling was much more prominent and cytoplasmic process labeling less prominent than in pilocytic astrocytomas. Anaplastic astrocytomas with gemistocytic features showed especially high somatic cytoplasmic dp-ERK/MAPK immunoreactivity (Figure 4C). Reactive brain near the infiltrating tumor edge often showed marked elevation of dp-ERK/MAPK in reactive astrocytes (not shown). Quiescent non-neoplastic astrocytes, oligodendrocytes, neurons, and neuropil distant from infiltrating tumor were devoid of dp-ERK/MAPK immunoreactivity. In contrast to dp-ERK/MAPK, total ERK/MAPK immunoreactivity was more evenly expressed in all tumor cells and in surrounding brain (Figure 4, B and D).

Grade IV astrocytomas (glioblastoma multiforme), in keeping with their wide range of morphological variation, showed a spectrum of patterns of ERK/MAPK phosphorylation (Figures 4, G and I, and 5, A and C). In general, these tumors exhibited the highest percentage of dp-ERK/MAPK-immunoreactive cells. Glioblastomas with biphasic cell subpopulations, like the tumor illustrated with multinucleated giant cell transformation, showed intriguing differences in dp-ERK/MAPK immunoreactivity. Whereas the large, bizarre, multinucleated tumor cells had strong dp-ERK/MAPK immunoreactivity, the small cell population was completely negative (Figure 4I). Both small and large cell populations showed uniform ERK/MAPK protein immunoreactivity (Figure 4J), suggesting differentially activated ERK/MAPK signaling and not merely elevated protein expression.

Selective Dephosphorylation of ERK/MAPK within Mitotic and Cycling Glioblastoma Cells

Immunoreactivity for dp-ERK/MAPK was consistently noted to be diminished or undetectable within individual mitotic tumor cells in all glioblastoma specimens examined. Examples from three different glioblastomas are illustrated (Figure 4, I, K, and L). Many, but not all, adjacent interphase cells contained abundant cytoplasmic and nuclear dp-ERK/MAPK immunoreactivity. Mitotic phosphoepitopes in general do not appear to be lost in routinely processed surgical specimens. Immunolabeling of adjacent sections with monoclonal antibody MPM-2, which recognizes a family of mitosis-specific phosphoepitopes,²⁴ revealed strong cytoplasmic and perichromosomal MPM-2 staining in all morphologically recognizable mitotic cells (Figure 4M). Thus, at least some mitotic phosphoepitopes are preserved in routinely processed paraffin-embedded tumor specimens. To assess the state of ERK/MAPK phosphorylation in other cycling tumor cells, we performed double-label immunohistochemistry for dp-ERK/MAPK and Ki-67, an antigen expressed in all phases of the cell cycle except G0. Examination of two glioblastoma specimens revealed non-colocalization; ie, cycling tumor cells with Ki-67-positive nuclei

Figure 4. Immunohistochemical demonstration of ERK/MAPK phosphorylation in astrocytic neoplasms. For each tumor, adjacent sections were immunolabeled with the phospho-specific ERK/MAPK antibody (pMAPK; **left panels**) and a phospho-insensitive ERK/MAPK antibody (MAPK; **right panels**). Elevated nuclear and cytoplasmic dp-ERK/MAPK immunoreactivity, relative to normal quiescent astrocytes, was detected in a subset of tumor cells within a variety of astrocytic neoplasms. In contrast, ERK/MAPK immunoreactivity was more uniformly expressed. **A and B:** Astrocytoma, WHO grade II. Some diffusely infiltrating astrocytoma cells show increased nuclear and cytoplasmic dp-ERK/MAPK immunoreactivity. Note the absence of labeling in adjacent normal neurons. ERK/MAPK protein is more evenly expressed in tumor cell cytoplasm and in neurons. **C and D:** Anaplastic astrocytoma, WHO grade III. A subset of gemistocytic tumor cells shows high cytoplasmic and nuclear dp-ERK/MAPK immunoreactivity. **E and F:** Ependymoma. A subpopulation of highly fibrillar ependymoma cells, especially those forming perivascular pseudorosettes, shows elevated dp-ERK/MAPK immunoreactivity; compare with the relatively uniform expression of ERK/MAPK protein. **G and H:** Glioblastoma multiforme (astrocytoma, WHO grade IV). Tumor cells near the hyperplastic microvasculature show the most intense nuclear and cytoplasmic pMAPK immunoreactivity. Some fine immunoreactive tumor cell processes appear to be oriented toward the microvasculature (**corner inset**). Total MAPK immunoreactivity is much more uniform, and not restricted to the perivascular tumor cells. **I and J:** Glioblastoma with giant cell transformation (astrocytoma, WHO grade IV). Only the large, bizarre, multinucleated tumor cells have detectable dp-ERK/MAPK immunoreactivity; the small cell population is completely negative. Note the absence of dp-ERK/MAPK immunoreactivity in a mitotic tumor cell (**left side**). In contrast, both small and large tumor cell populations show uniform ERK/MAPK protein immunoreactivity. Also, a mitotic cell is visible that has detectable MAPK immunoreactivity (**right side**). **K and L:** Immunoreactivity for dp-ERK/MAPK is absent within individual mitotic tumor cells in two additional glioblastoma specimens; adjacent interphase cells show abundant cytoplasmic and nuclear immunoreactivity. **M:** Evidence that mitotic phospho-epitopes in general are not lost in routinely processed surgical specimens comes from immunolabeling with monoclonal antibody MPM-2, which recognizes a number of mitosis-specific phosphorylated proteins. Strong perichromosomal staining is present in mitotic cells. **N:** Double-label immunohistochemistry for dp-ERK/MAPK (red-brown) and Ki-67 (dark blue) reveals essentially complete non-co-localization. This suggests that cycling cells (those with nuclear Ki-67 immunolabeling) and those with activated ERK/MAPK (dp-ERK/MAPK immunoreactive) are generally mutually exclusive populations. **A to M:** Immunoperoxidase staining (DAB) with hematoxylin counterstain. **N:** Double-label immunoperoxidase with aminoethylcarbazole and Vector SG substrates; no counterstain. Scale bar in **A** represents 50 μ m and applies to **A** to **J** and **M**; bar in **N** represents 20 μ m and applies to **K**, **L**, and **N**.

Figure 5. ERK/MAPK in glioblastomas, showing increased phosphorylation in tumor cells near hyperplastic microvessels and surrounding zones of tumor necrosis. Glioblastoma cells in contact with or near hyperplastic microvessels show increased dp-ERK/MAPK immunoreactivity relative to those at a distance (A). Total ERK/MAPK immunoreactivity in the same microscopic field is uniform in all tumor cells (B). Increased dp-ERK/MAPK is present in a zone of viable tumor cells surrounding a focus of tumor necrosis (C), whereas total ERK/MAPK immunoreactivity is uniform in the same microscopic field (D). Immunoperoxidase with hematoxylin counterstain. Scale bar in D represents 175 μ m.

generally lacked nuclear or cytoplasmic dp-ERK/MAPK staining (Figure 4N). Conversely, cells with strong cytoplasmic and/or nuclear dp-ERK/MAPK staining had no detectable Ki-67 immunoreactivity. These findings suggest that elevated ERK/MAPK phosphorylation occurs in a subset of G0 cells, and that a relative dephosphorylation of ERK/MAPK occurs in M-phase and cycling cells.

ERK/MAPK Activation Surrounding Hyperplastic Microvasculature and Tumor Necrosis

Many glioblastoma specimens demonstrated patterns of tumor cell activation spatially related to the vascular stroma or areas of tumor necrosis. Tumor cells intimately associated with hyperplastic tumor microvasculature often showed markedly elevated nuclear and cytoplasmic dp-ERK/MAPK immunoreactivity relative to other cells (Figures 4G and 5A). In some cells, dp-ERK/MAPK immunoreactivity could be seen in fine processes oriented toward the vessel wall (inset, Figure 4G). Similarly, some tumors exhibited elevated dp-ERK/MAPK in a rim of palisading tumor cells around zones of tumor necrosis (Figure 5C). The expression of total ERK/MAPK protein, on

the other hand, was relatively uniform among all glioblastoma cells (Figures 4H and 5, B and D).

Focal ERK/MAPK Activation in Non-Infiltrating (Circumscribed) Astrocytomas

Non-diffuse-type astrocytomas, namely, pilocytic astrocytomas and pleomorphic xanthoastrocytomas, generally displayed more focal ERK/MAPK activation than high-grade diffuse tumors (Figure 6, A and C). In pilocytic astrocytomas, cells of both stellate and piloid morphologies showed evidence of cytoplasmic ERK/MAPK activation. In contrast to the diffuse-type astrocytomas, nuclear labeling was significantly less prominent whereas labeling of processes was more conspicuous. Another significant difference, with respect to diffuse-type astrocytomas, was the absence of conspicuous ERK/MAPK activation in tumor cells surrounding the hyperplastic microvessels of pilocytic astrocytomas. The microvascular glomeruloid proliferations in these tumors were, like their counterparts in glioblastomas, devoid of the phosphorylated ERK/MAPK. The small number of pleomorphic xanthoastrocytomas examined ($n = 5$) showed quite

Figure 6. Focal ERK/MAPK activation in non-infiltrating (circumscribed) astrocytomas. Immunoreactivity for activated ERK/MAPK is focally prominent in cytoplasm and coarse processes of pilocytic astrocytoma cells (A). A glomeruloid vessel (v) is devoid of the phosphorylated ERK/MAPK epitope. Total ERK/MAPK immunoreactivity is present in all cells in this tumor (B). A pleomorphic xanthoastrocytoma shows focally elevated cytoplasmic dp-ERK/MAPK immunoreactivity and occasional nuclear labeling in tumor cells (C); adjacent non-neoplastic brain (br) is negative. Total ERK/MAPK immunoreactivity is present in all tumor cells as well as non-neoplastic brain (br; D). Original magnification, $\times 100$.

variable staining. Two tumors had only rare cells with cytoplasmic dp-ERK/MAPK immunoreactivity whereas the other three showed more widespread cytoplasmic and occasional nuclear labeling, indistinguishable from higher-grade astrocytomas.

Immunohistochemical Localization of ERK/MAPK Phosphorylation in Non-Astrocytic Brain Tumors

Oligodendrogliomas generally showed low dp-ERK/MAPK immunolabeling, with occasional immunoreactive tumor cell nuclei and only rare detectable cytoplasmic staining (Figure 7A). The few cells that did show cytoplasmic immunoreactivity often had gliofibrillary or minigemistocytic features. In contrast, anaplastic oligodendrogliomas (WHO grade III) often exhibited intense cytoplasmic and nuclear dp-ERK/MAPK immunoreactivity in a large fraction of tumor cells (Figure 7C). In contrast to glioblastomas, the activated tumor cells did not show

any obvious association with the tumor vasculature. Ependymomas, both grade II and III, generally showed a significant proportion of cells with markedly elevated dp-ERK/MAPK immunoreactivity (Figure 4E). The most prominent immunostaining was in the cytoplasm of fibrillary cells surrounding blood vessels and forming perivascular pseudorosettes. Ependymomas thus shared with glioblastomas a strong angiocentric pattern of elevated ERK/MAPK activation.

Compared with astrocytomas, ependymomas, and anaplastic oligodendrogliomas, ERK/MAPK activation in non-glial brain neoplasms was much less conspicuous. Primary CNS lymphomas, for example, showed almost undetectable dp-ERK/MAPK immunoreactivity (Figure 7E). The presence of intensely immunoreactive entrapped reactive astrocytes provided evidence that the specimens were fixed adequately to preserve phosphoepitopes. Similarly, metastatic poorly differentiated adenocarcinomas (Figure 7G) and melanomas (not shown) had detectable but low dp-ERK/MAPK immunoreactivity

pMAPK

MAPK

compared with the intense labeling of surrounding reactive astrocytes.

Discussion

Molecular genetic and biochemical studies of gliomas, especially astrocytomas, have revealed abundant evidence for aberrant growth factor signaling, thus implicating downstream intracellular signaling pathways in glioma pathogenesis. Immunohistochemical application of a phosphorylation-state-specific antibody to ERK/MAPK enabled the morphological imaging of dynamic intratumor signaling events. Unlike traditional immunohistochemical studies, which assess only levels of protein expression, this molecular morphological technique allows determination at the cellular level of signaling enzyme activation state. Using this approach, we demonstrated significant activation of a growth-factor-stimulated signaling pathway in a broad range of glial tumors at both early (low-grade) and late (high-grade) stages of malignant progression. Tumor cells appear to exhibit elevated ERK/MAPK phosphorylation, but not notable protein overexpression, relative to normal non-neoplastic glia. Significant ERK/MAPK phosphorylation was detected in a variety of glial tumors, with the exception of oligodendrogliomas, and in reactive non-neoplastic astrocytes. It is interesting to note that of the glial tumor types examined, only oligodendrogliomas showed a reproducible increase in ERK/MAPK activation with malignant progression. Additional studies are needed to determine the functional significance and prognostic utility of this preliminary finding.

This immunohistochemical approach revealed selective activation of subsets of tumor cells, not previously possible using standard biochemical assays. The finding of elevated ERK/MAPK phosphorylation in glioblastoma cells surrounding tumor neovasculature and around focal tumor necrosis, for example, suggests the existence of spatially complex intratumor signaling. The finding of diminished or absent ERK/MAPK phosphorylation in mitotic tumor cells suggests the existence of cell-cycle-associated regulation of ERK/MAPK activity. It is possible that some of the large spatial heterogeneity in ERK/MAPK activation could be due to the existence of spatially separated populations of tumor cells for which cell cycles are locally synchronized. The presence of ERK/MAPK activation in reactive non-neoplastic astrocytes suggests that pathway activation is not sufficient for neoplastic transformation and may have a role in the maintenance of the reactive astroglial phenotype. We have confirmed that activation is present in reactive astrocytes within a variety of non-neoplastic neurosurgical specimens, including examples of direct injury, infarct, and infection (J.W. Mandell and S.R. VandenBerg, unpublished data). Finally, the

presence of highly activated ERK/MAPK in low-grade gliomas also suggests that pathway activation is not necessarily tied to malignant progression. The more focal patterns of activation observed in these nonmalignant states may reflect local autocrine/paracrine signaling, such as that mediated by PDGF^{5,30,31} or IGF-1³²⁻³⁵ whereas the more widespread activation observed in high-grade astrocytomas could reflect constitutive activation mediated by EGF receptor overexpression and mutation.^{3,4}

It is conceivable that the patterns of ERK/MAPK phosphorylation detected in routinely fixed surgical specimens do not accurately reflect those *in vivo*. As detailed in Materials and Methods, some of the heterogeneity could be attributed to fixation-related artifact, such as the absence of immunoreactivity in the core of large tumor specimens. It could be argued that uniformly high levels of ERK/MAPK phosphorylation are present in all tumor cells *in vivo*, but cell-to-cell differences in dephosphorylation kinetics after surgical resection give rise to the heterogeneities observed in fixed tissue sections. Evidence arguing against such an artifact comes from experiments in which we maintained astrocytic tumor specimens in culture for periods up to 24 hours, to revive cell metabolism, and then used either routine or rapid acrolein fixation. Patterns of dp-ERK/MAPK immunoreactivity were similar when comparing the fixed cultured tumor tissue with additional portions of the the same tumors routinely fixed and processed. This result suggests that the patterns of dp-ERK/MAPK immunoreactivity revealed in routine tissue sections provide an accurate snapshot of cell signaling that takes place on a time scale of minutes to hours. A probable exception is rapid intracellular signaling taking place on a time scale of seconds or less, for example, that which occurs between neurons. Changes in ERK/MAPK phosphorylation on this rapid time scale probably would not be captured by the relatively slow fixation process, possibly explaining our failure to detect ERK/MAPK phosphorylation by immunohistochemistry in neurons. ERK/MAPK phosphorylation is known to occur on glutamate stimulation of neurons, for example, and can be detected by biochemical techniques.³⁶ In fact, we have detected strong phospho-MAPK immunoreactivity in cortical neurons in rapidly fixed adult rat brain (J.W. Mandell, unpublished results). Even if some of the dp-ERK/MAPK heterogeneity we observed in tumors was due in part to postsurgical loss of phosphoepitope, the fact that the differential dephosphorylation takes place in biologically meaningful patterns indicates at least the existence of interesting cell-to-cell differences in phosphorylation/dephosphorylation kinetics. A similar problem has been faced in the investigation of neuronal tau protein hyperphosphorylation in Alzheimer's Disease.^{26,27}

The vast majority of published studies in cell culture models emphasize the nuclear localization of activated

Figure 7. ERK/MAPK phosphorylation in non-astrocytic brain tumors. **A** and **B:** A grade II oligodendroglioma shows generally low dp-ERK/MAPK immunolabeling, with a few immunoreactive nuclei. **C** and **D:** In contrast, an anaplastic oligodendroglioma (grade III) shows greatly elevated cytoplasmic and nuclear dp-ERK/MAPK immunoreactivity in a large subpopulation of tumor cells. **E** and **F:** A primary CNS lymphoma shows no detectable dp-ERK/MAPK immunoreactivity; note the intense immunolabeling within an entrapped reactive astrocyte. **G** and **H:** A metastatic poorly differentiated adenocarcinoma shows detectable but low dp-ERK/MAPK immunoreactivity compared with the intense labeling of surrounding reactive astrocytes. Immunoperoxidase with hematoxylin counterstain. Scale bar in **H** represents 50 μ m.

ERK/MAPK.^{11,12} Our observation of nuclear activated ERK/MAPK in a variety of gliomas, especially malignant forms, is consistent with the idea of constitutively activated signaling pathways maintaining a malignant phenotype via transcriptional regulation. However, it is well known that these enzymes have cytoplasmic substrates in addition to the better characterized nuclear transcription factor substrates.^{10–12} Potential cytoplasmic substrates include cytoskeletal elements and regulatory enzymes, including microtubule-associated proteins and myosin light chain kinase.^{37–39} Thus, our observation of significant cytoplasmic activated ERK/MAPK, especially in astrocytomas and ependymomas, suggests possible nontranscriptional roles, such as the regulation of cytoarchitecture and cell motility.

Might overactivation of ERK/MAPK be related to upstream signaling defects, such as the presence of an overexpressed or mutated EGF receptor? Evidence that this could be the case for glioblastomas comes from the finding of increased ERK/MAPK activity in fibroblasts transfected with a mutant EGF receptor obtained from a human glioblastoma.⁴⁰ Our finding of highly activated ERK/MAPK in many low-grade astrocytomas and ependymomas, in which EGF receptor overexpression or mutation is rare, suggests the existence of alternative mechanisms for pathway activation. These might include increased activation or expression of other growth factor receptors or any of the subsequent components of the ERK/MAPK pathway. One such component is the adaptor protein Shc, one of the molecular links between receptor tyrosine kinase and ras activation. Shc is constitutively phosphorylated in many tumor cells, including a glioblastoma cell line, in which it is complexed with the activated PDGF receptor.⁴¹ Alternatively, the elevated ERK/MAPK phosphorylation in glial tumors could result from mutational inactivation or decreased expression of a relevant phosphatase. The recent discovery of a cytoplasmic phosphatase commonly mutated in glioblastomas raises an interesting candidate.^{42,43} Another possibility is that the elevated ERK/MAPK activation is not due to an intracellular defect but is secondary to a self-perpetuating autocrine/paracrine feedback loop involving tumor-derived or exogenous growth factors.

Our finding of a possible association of ERK/MAPK phosphorylation with cell cycle regulation is intriguing but will require an appropriate cell culture model for verification. In cultured fibroblasts, persistent ERK/MAPK activation induces cyclin D1 expression and is necessary for the G0/G1 to S transition.⁴⁴ A recent study implicates ERK/MAPK in the spindle assembly checkpoint but, interestingly, found no effect of ERK/MAPK dephosphorylation on progression through mitosis. The relationship of ERK/MAPK activation to astrocytic cell cycle regulation could be properly tested by pharmacological manipulation, antisense knockout, or expression of constitutively active or dominant negative enzymes in astrocytic tumor cell lines.

The studies on glioblastomas allowed the first direct confirmation of the predicted paracrine signaling interaction(s) between the hyperplastic tumor microvasculature and nearby tumor cells. The nature of the signaling inter-

actions between tumor cells and the hyperplastic microvasculature is only beginning to be elucidated. The putative signals could be serum-derived factors such as thrombin, a potent stimulator of the astrocytic ERK/MAPK pathway, or growth factors produced and secreted by the proliferating microvascular cells. An alternative view is that tumor cells produce and secrete factors (such as vascular endothelial growth factor) that both stimulate vascular hyperplasia and, in an autocrine manner, tumor cell ERK/MAPK activation. Similarly, the local activation of ERK/MAPK signaling surrounding zones of tumor necrosis could be due either to factors released by dying tumor cells or, alternatively, by cytokines produced by infiltrating macrophages. These hypotheses await testing in appropriate cell culture and *in vivo* models.

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