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Promotion of Malignant Astrocytoma Cell Migration by Osteopontin Expressed in the Normal Brain: Differences in Integrin Signaling during Cell Adhesion to Osteopontin *Versus* Vitronectin[1]

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ABSTRACT

The extracellular matrix of the normal adult brain lacks expression of most of the adhesive glycoproteins that are known to promote cell attachment, and it has been thought that the malignant invasion of astrocytoma tumor is mediated primarily by remodeling of the matrix by the tumor cells. It has been reported, however, that normal brain neuropil does contain a protein(s) that promotes cell attachment. Therefore, we explored the possibility that the cell attachment protein, osteopontin, is expressed in the normal human brain. Here, we report that osteopontin is expressed in the cortical gray and white matter of normal adult brain, with the levels of osteopontin expression being equivalent to those in malignant astrocytic tumor biopsies as assessed by Western blot analysis. Immunoblotting identified osteopontin polypeptides with relative molecular weights of 60- and 65-kDa in normal brain white matter and in astrocytic tumors, with an additional 70-kDa polypeptide being identified in normal cortical gray matter and in some astrocytic tumors. Recombinant osteopontin was found to promote attachment of U-251MG human malignant astrocytoma cells in a process that was inhibited by anti-integrin monoclonal antibodies anti-$\alpha v\beta 3$ (75%), anti-$\alpha v\beta 5$ (80%), and anti-$\alpha 5$ (40%). On attachment, integrins $\alpha v\beta 5$ and $\alpha v\beta 3$ localized to focal adhesions, and there was an alteration in cell morphology with the formation of lamellae-like processes. The attachment was associated with activation of Rac in a slow and prolonged fashion and rapid activation of Rho. Similarly, integrins $\alpha v\beta 5$ and $\alpha v\beta 3$ localized to focal adhesions on attachment of the U-251MG cells to vitronectin, but on this substrate, the cells assumed a spread and flat morphology, and there was rapid activation of both Rac and Rho. Extracts of normal brain white matter were capable of promoting haptotactic migration, and this response was inhibitable by monoclonal antibodies anti-$\alpha v\beta 3$ and anti-$\alpha 5$. Depletion of the osteopontin in these extracts abrogated the haptotactic response significantly (50%). These data indicate that the cell attachment protein, osteopontin, is expressed in the normal adult brain and that it has the potential to promote malignant astrocytoma cell invasion.

INTRODUCTION

Malignant astrocytic tumors are capable of remodeling their extracellular matrix through the synthesis of vitronectin, and this process most likely promotes the cell attachment and migration of these tumors (1, 2). The extracellular matrix (neuropil) of the normal adult brain lacks expression of most of the adhesive glycoproteins that are known to promote cell attachment (reviewed in Ref. 2). It has been reported, however, that malignant astrocytoma cells can attach to normal brain extracts that contain crude preparations of myelin but lack laminin, fibronectin, collagen, and tenascin (3). This suggests that the normal brain neuropil does contain a protein(s) that promotes cell attachment and may contribute to malignant invasion. Therefore, we considered the possibility that expression of osteopontin in the neuropil of normal brain fulfills this function.

Osteopontin, also known as Eta-1 (early T-lymphocyte antigen), is a secreted, cell attachment protein, as well as a cytokine, that is highly conserved among mammals and is expressed in normal mineralized bone, the mammary gland, smooth muscle, kidney, placenta, and in some neoplastic tissues (4). Extensive studies of the expression of osteopontin protein in the normal human adult brain have not been undertaken to date; however, osteopontin protein has been reported to be expressed in astrocytic tumors from patient biopsies (5). In addition, osteopontin undergoes extensive post-translational modification that is tissue specific (4).

We have shown previously that human malignant astrocytoma cells express integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ *in vitro* and *in vivo* and that these two receptors are markers of the malignant astrocytic cell phenotype *in vivo* (1, 6). It is known that osteopontin contains an RGD peptide (amino acids 166–168) that has been shown to promote integrin-mediated cell attachment and migration of several nonglial cell types (4, 7). Several integrin receptors, including $\alpha v\beta 3$ and $\alpha v\beta 5$, as well as $\alpha v\beta 1$, $\alpha 8\beta 1$, $\alpha 9\beta 1$, $\alpha 5\beta 1$, and $\alpha 4\beta 1$, can recognize osteopontin as a ligand, but this interaction is influenced by the cell type and milieu (4, 7–11). In addition, integrin $\alpha v\beta 3$ recognizes thrombin-cleaved osteopontin (4). Most of these integrins recognize the RGD peptide in osteopontin, although $\alpha 4\beta 1$ and $\alpha 9\beta 1$ recognize the SVVYGLR peptide (9, 10). CD44, a cell surface proteoglycan, also has been reported to mediate the cell attachment to osteopontin in some cell types, and this occurs in an RGD-independent manner (4).

It has been shown in other cell types that integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ recognize vitronectin, as well as osteopontin, and integrin $\alpha v\beta 3$ also recognizes a number of other matrix proteins as ligands (1, 4, 6, 7, 11, 12). Although the ligand specificity of integrin receptors is generated, in part, on dimeric pairing, it also is influenced by other factors, including the cell type, the availability of certain divalent cations, the functional state of the receptor, the available cytokines, other cell surface receptors, and cross-talk from other integrins (1, 6–15). Thus, although the expression of the $\alpha v\beta 3$ and $\alpha v\beta 5$ receptors on the astrocytoma cells is suggestive of osteopontin binding based on data from other cell types, it cannot be assumed that this is the case. The functions of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ are regulated differently, and similarly, the cellular response to integrin receptor ligation is variable.

Integrin receptor-mediated attachment of a cell to an extracellular matrix protein typically results in the formation of focal adhesions with the formation of bundled actin fibers (actin cables; Ref. 7). Cytoskeletal organization in cells requires activation of the small GTPases, Cdc42, Rac, and Rho (16). In fibroblasts, RhoA activation is necessary for the formation of bundled actin fibers (stress fibers; Ref. 16). The cytoskeleton must undergo reorganization for cell migration and tumor invasion to occur, and Cdc42, Rac, and Rho modulate these processes. Although activation of these molecules are potential markers of cellular responses to integrin ligation, their activation has not been characterized in astrocytoma cells in response to specific integrin ligation.

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In this study, we examined the expression of osteopontin in the normal brain and the ability of rec and normal brain osteopontin to promote malignant astrocytoma cell attachment and migration. We found that osteopontin protein is expressed in the normal brain at levels equivalent to those expressed by malignant astrocytic tumors. Attachment and migration of malignant astrocytoma cells toward both rec-osteopontin and normal brain homogenates containing osteopontin were found to be mediated by the integrins that are markers of this phenotype, the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, as well as by integrin $\alpha 5\beta 1$. Furthermore, we found that the morphology of the malignant astrocytoma cells is altered on attachment to rec-osteopontin as compared with vitronectin; osteopontin cells exhibit lamellae-like processes, and cytoskeletal organization is characterized by a rapid activation of Rho and a slower but prolonged activation of Rac.

MATERIALS AND METHODS

Reagents and Proteins. Rec-rat osteopontin was produced and purified as described previously (17). Rat long bone osteopontin was purified as described (18) and was a kind gift from Dr. William T. Butler (University of Texas Health Sciences Center, Houston, TX).

Antibodies. The following neutralizing mAbs[3] were purchased: anti-integrin $\alpha v\beta 5$ (P1F6), anti-integrin $\alpha v\beta 3$ (LM609), anti-integrin $\alpha 5$ (PID6), anti-integrin $\beta 1$ (P4C10), and anti-integrin αv (mAb L230). The mAb antihuman osteopontin (mAb 53) has been described (19). The following antibodies were purchased: mAb anti-integrin $\beta 3$ subunit hybridoma (AP3), mAb anti-vinculin, and the following antibodies directed toward the cytoplasmic tails of integrins: rabbit anti-integrin $\beta 5$, rabbit anti-integrin $\beta 3$, and rabbit anti-integrin $\beta 1$. mAb 7E3 was produced at Centocor Corp. (20, 21).

Immunohistochemistry. Brain biopsy samples for immunohistochemical analyses were obtained from the University of Alabama at Birmingham Hospital and were formalin fixed and paraffin embedded. They represented surgical cases from 1991 to 1995 and were chosen based on the quantity of tissue in the paraffin blocks available for study. Tissue sections were subjected to antigen retrieval followed by immunohistochemical analysis using the indirect immunoperoxidase technique, as described previously (12).

Cell Culture, Attachment, and Haptotactic Migration Assays. U-251MG human malignant astrocytoma cells were obtained from the American Type Culture Collection. These cells are representative of grade IV malignant astrocytoma tumors, as they proliferate rapidly *in vitro* and *in vivo* in the intracerebral C.B.17 SCID mouse model and show some invasion in this animal model (1, 6). Mouse brain microvessel endothelial cells (IBE cells) isolated from SV40 large T-antigen transgenic mice were a kind gift from Dr. Lena Claesson-Welsh (Ludwig Institute for Cancer Research, Uppsala, Sweden; Ref. 22). Cell attachment assays were performed as described previously (1, 6, 12, 15). The optimal concentration of ligand to promote attachment was determined for each ligand. After harvest, cells were resuspended in adhesion assay buffer (serum free) with the divalent cations, 1 mM $MgCl_2$ and 100 μM $MnCl_2$. Migration assays were performed in two-well Boyden-type chambers as described previously (1). Western blot and immunofluorescent analysis were performed as described previously (12, 23). For immunofluorsecent analysis, cells were plated in adhesion assay buffer with the divalent cations, 1 mM $MgCl_2$ and 100 μM $MnCl_2$, for 5 h.

Homogenization of Normal Human Brain and Immuno-Depletion of Osteopontin. Cortical gray and white matter from three frozen normal human brains from autopsy were separated and homogenized into DMEM with amphotericin B, penicillin, and streptomycin, as well as protease inhibitors (60 μg/ml aprotinin, 30 μg/ml leupeptin, 100 μM Nα-p-tosyl-l-lysine-chloromethyl ketone, and 300 μM phenylmethylsulfonyl fluoride) over 3 min on wet ice using a Polytron, followed by the pooling of the gray matter samples, and of the white matter samples. Subsequently, the homogenates were centrifuged (3200 rpm, 30 min, 4°C), and the aqueous upper phase (normal brain homogenate) was harvested. The aqueous phase was then recentrifuged to remove insoluble material (35,000 rpm, 1 h, 4°C), and the supernatant (normal brain homogenate) was dialyzed extensively against PBS, followed by determination of the protein concentration. To deplete the normal brain homogenate of osteopontin, the homogenate was reacted with rabbit antiosteopontin IgG-Protein A-Sepharose conjugate or with rabbit IgG-Protein A-Sepharose conjugate (1 h, 4°C, rocking), followed by pelleting of the Sepharose beads, and reaction of the supernatant with fresh rabbit antiosteopontin Sepharose conjugate or rabbit IgG Sepharose conjugate X8. Cell surface biotinylation was accomplished by treatment with sulfosuccinimidyl-6-biotinamido hexanoate (sulfo-NHS-LC-biotin), followed by immunoprecipitation analysis, as described previously (11).

Rac and Rho Activation Assays. Cells were plated onto rec-osteopontin or vitronectin in adhesion assay buffer with 1 mM $MgCl_2$ and 100 μM $MnCl_2$ for the time indicated (37°C, 5% CO_2) and lysed. Equivalent micrograms of lysate reacted with p21-activated kinase-1 binding domain coupled to agarose or Rhotekin Rho-binding domain coupled to agarose, and immunoprecipitates subjected to 10% SDS-PAGE, transferred to Immobilon, and Western blotted with anti-Rac IgG or anti-Rho IgG, respectively, as per the instructions in the kits from Upstate Biotechnology (Lake Placid, NY).

RESULTS

Osteopontin Expression in Normal Adult Brain. The expression of osteopontin in normal adult brain was first evaluated by immunohistochemical analysis. Osteopontin protein was detected in the cortex and white matter of normal adult brain obtained from autopsy as well as brain biopsies from patients with seizures and mesial sclerosis. The pattern of expression of osteopontin in the cortical gray matter was perineuronal and resembled the branching of neuronal processes, whereas in the white matter, the pattern was characteristically diffuse (10 of 15 samples). As expression of osteopontin mRNA in two malignant astrocytoma biopsies has been reported (5), we also evaluated tumor biopsies of various grades of astrocytoma. In low-grade diffuse astrocytomas (grade II), osteopontin was detected in five of eight biopsies. In grade III and grade IV malignant astrocytoma biopsies (anaplastic astrocytoma and glioblastoma, respectively), osteopontin protein was detected in eight of nine and in eight of eight biopsies, respectively. The osteopontin protein was localized predominantly in the neuropil of the tumors, although a cytoplasmic distribution was observed in some tumor cells. The expression of vitronectin and von Willebrand factor proteins was used as a control. Consistent with our previous study (1, 2, 6), the expression of vitronectin protein correlated most closely with the malignant astrocytic phenotype, and von Willebrand factor protein was detected only in endothelial cells.

Western blot analyses with mAb antiosteopontin were then performed, followed by reprobing with mAb antiactin, to confirm the immunohistochemical identification of osteopontin protein in the normal brain and to estimate the heterogeneity of osteopontin in the normal brain and tumors. The expression of osteopontin protein in non-neoplastic brain samples was confirmed as it was detected in 14 of 14 non-neoplastic brain samples, which included 6 normal brains from autopsy and 8 brain biopsies from patients with seizure disorders and mesial sclerosis on histopathological examination (Fig. 1, *A, C,* and *E*). Similarly, the expression of osteopontin protein was confirmed in nine of nine malignant astrocytoma biopsies (all anaplastic astrocytomas, grade III; Fig. 1, *C* and *E*). The levels of expression in the malignant astrocytomas were equivalent to those in the non-neoplastic brain (Fig. 1, *C* and *E*), based on the ratio of osteopontin protein (summing all three forms) to actin protein for each sample from the same blot.

In the Western blot analyses of samples of non-neoplastic and normal brain, the mAb antiosteopontin reacted with three bands migrating with apparent relative molecular weights of 60, 65, and 70 kDa (Fig. 1, *A* and *C*). This migration pattern was also typical of some of the tumor samples (Fig. 1, *C* and *E*). Identification of these bands as osteopontin was confirmed by stripping and reprobing of the membranes with rabbit antiosteopontin IgG (data not shown). No

[3] The abbreviations used are: mAb, monoclonal antibody; FAK, focal adhesion kinase; PI3, phosphatidylinositol 3-hydroxyl.

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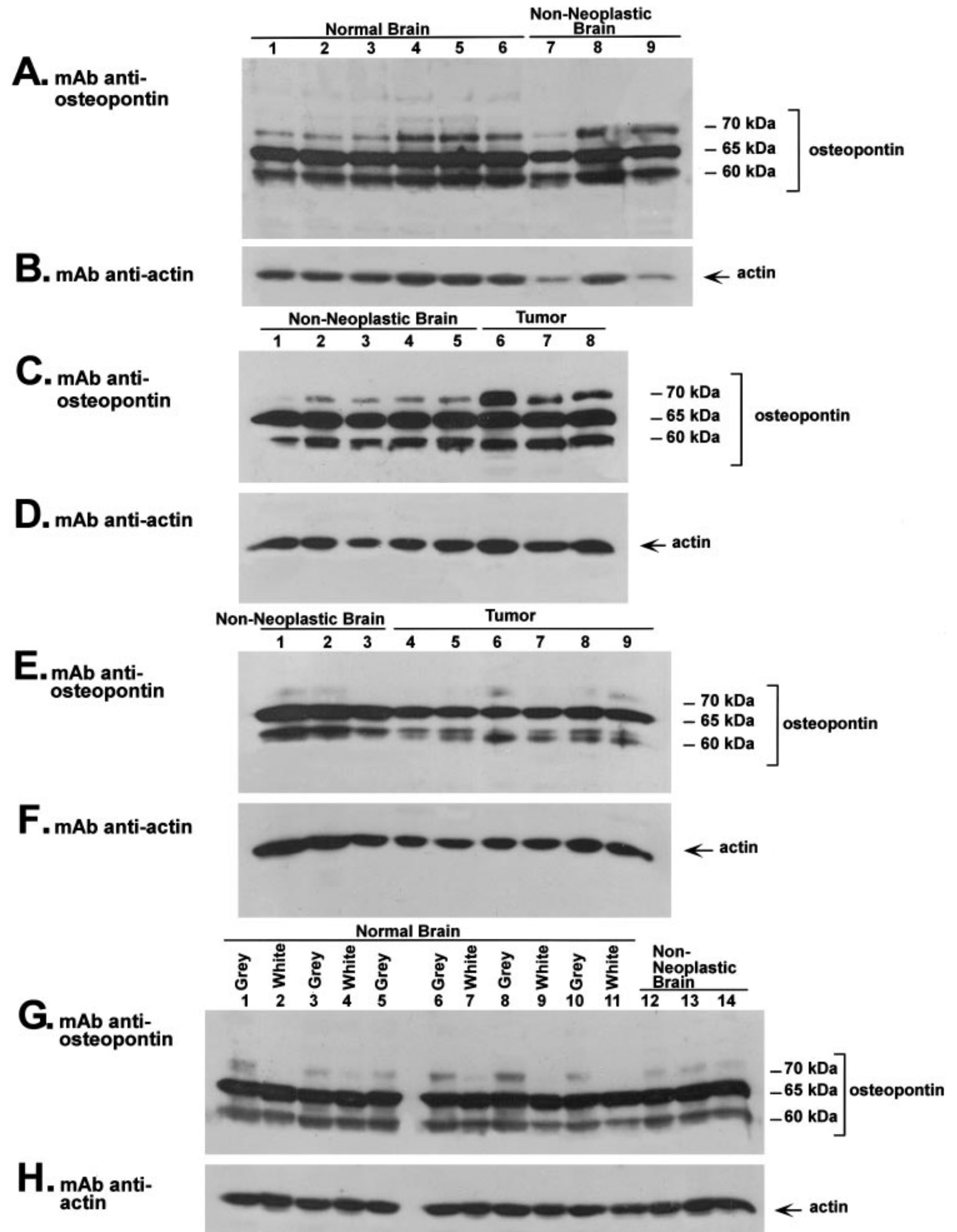

Fig. 1. Western blot analysis of osteopontin expression in the normal brain. Frozen samples of normal brain from autopsy (six samples) and non-neoplastic brain biopsies from patients with seizures and mesial sclerosis (eight samples) were homogenized into RIPA lysis buffer, and 15 μg were subjected to 7.5% SDS-PAGE, followed by Western blot analysis with mAb antiosteopontin (0.5 μg/ml; *A, C, E,* and *G*), stripped, and reprobed with mAb antiactin (0.5 μg/ml; *B, D, F,* and *H*). *A* and *B,* nine samples of normal or non-neoplastic brain (*Lanes 1–9*). *C* and *D,* five samples of non-neoplastic brain (*Lanes 1–5*) and three grade III anaplastic astrocytoma tumor biopsies (*Lanes 6–9*). *E* and *F,* three different samples of non-neoplastic brain (*Lanes 1–3*) and six different samples of grade III anaplastic astrocytoma tumor biopsies (*Lanes 4–9*). *G* and *H,* six samples of normal brain from autopsy separated as to gray and white matter (*Lanes 1–11*) and three samples of non-neoplastic brain (*Lanes 12–14*).

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cleavage product of osteopontin was detected in the normal brains, non-neoplastic brains, or the tumors on Western blot analysis with either antiosteopontin antibody. As the intensity of the band migrating with a relative molecular weight of 70 kDa varied somewhat among the Western blots of the normal brain and the non-neoplastic brain specimens, the frozen normal brain specimens from autopsy were reassessed after separation of the cortical gray and white matter. Although all three bands, including the 70-kDa band, were detected in all of the samples of gray matter, only the 60- and 65-kDa bands were detected in the samples of white matter (Fig. 1*G*). Thus, there may be differential expression of osteopontin in normal brain cortical gray matter and the underlying white matter.

Integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ Mediate Attachment of U-251MG Human Malignant Astrocytoma Cells to a Rec-Osteopontin Substrate. The ability of osteopontin to promote attachment of malignant astrocytoma cells was demonstrated by the attachment of the U-251MG cells to rec-osteopontin (Fig. 2*A*). We have established that integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$, as well as $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 6\beta 1$, are expressed on U-251MG human malignant astrocytoma cells (1, 6, 12, 15). Incubation of these cells with 25 μg/ml anti-integrin antibody before plating onto rec-osteopontin-coated wells indicated that mAbs anti-integrin $\alpha v\beta 3$ (LM609 and 7E3), anti-integrin $\alpha v\beta 5$ (P1F6), anti-integrin $\alpha 5$, and anti-integrin $\beta 1$ were all capable of inhibiting attachment (75, 80, 40, and 50% inhibition, respectively; Fig. 2*A*). In combination, mAbs anti-$\alpha v\beta 5$ (mAb P1F6) and anti-$\alpha v\beta 3$ (LM609) inhibited the attachment by 85%, as did anti-αv (mAb L230) (data not shown), and the combination of mAbs anti-αv (mAb L230) and anti-$\beta 1$ resulted in a 75% inhibition of attachment (Fig. 2*A*). In contrast, the mAbs anti-$\alpha 1$, anti-$\alpha 2$, anti-$\alpha 3$, and anti-$\alpha 6$ all failed to inhibit attachment. When 1 mM $CaCl_2$ was added to the adhesion assay buffer, mAb anti-$\alpha v\beta 3$ (LM609) failed to inhibit attachment, consistent with the report of Hu *et al.* (14) that integrin $\alpha v\beta 3$-mediated attachment to osteopontin is inhibited by Ca^{2+}. The presence of Ca^{2+} did not substantially effect the ability of

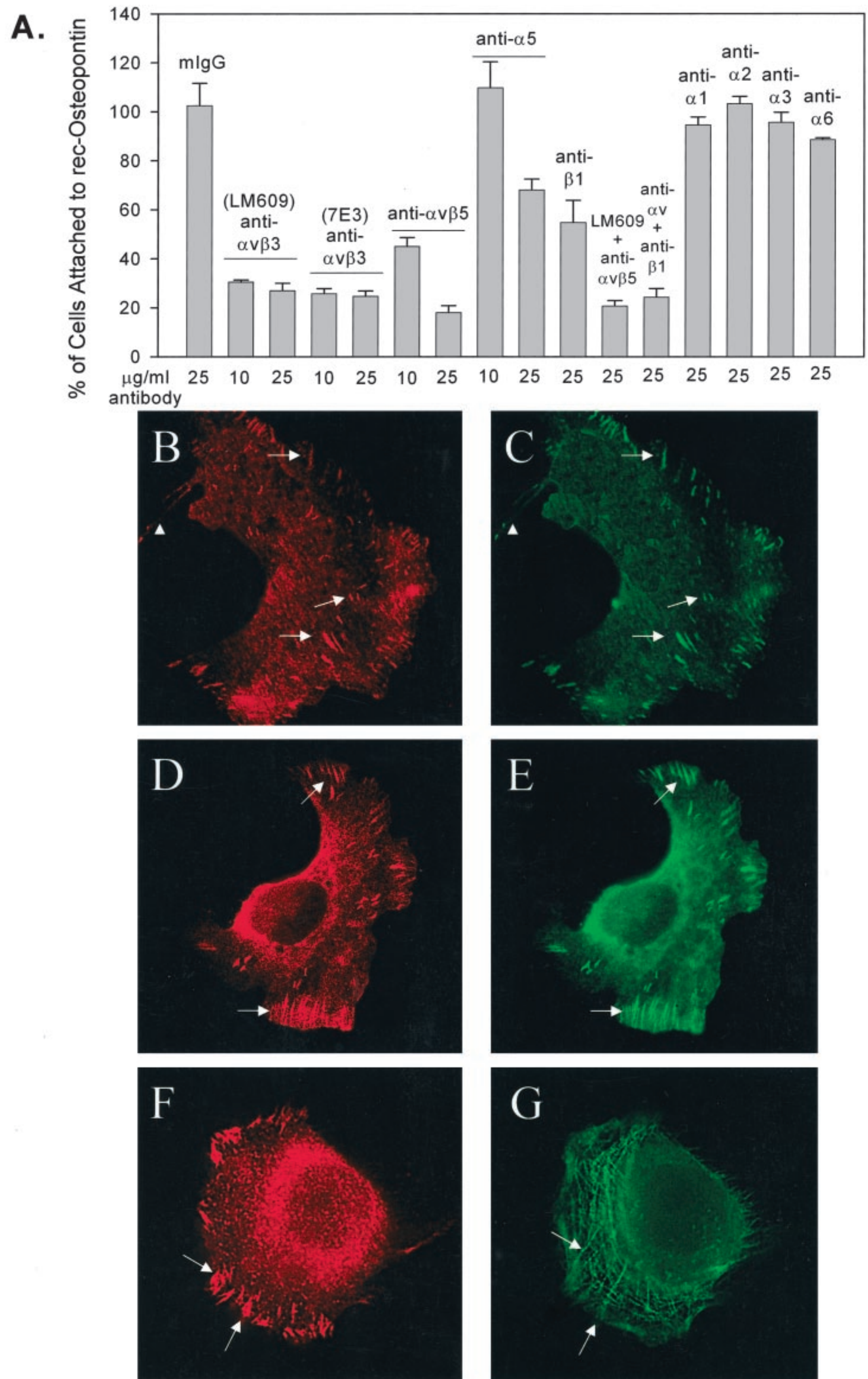

Fig. 2. U-251MG human malignant astrocytoma cell attachment to rec-osteopontin is mediated by integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$. Cell attachment results in an altered morphology. In *A,* U-251MG cells were incubated with the indicated anti-integrin antibody (20 min, 22°C) and then plated onto wells coated with 10 μg/ml rec-osteopontin or ovalbumin (30 min, 37°C, 5% CO_2). Background attachment to ovalbumin was subtracted. Samples were assayed in replicas of five, and the data are presented as the mean ± s.e.m. In *B–G,* U-251MG cells were plated onto coverslips coated with 10 μg/ml rec-osteopontin, followed by immunofluorescent analysis. *Arrows,* denotes focal adhesions. *B* and *C,* sequential staining with rabbit anti-$\beta 5$ IgG (*B*) and mAb antivinculin (*C*). *D* and *E,* sequential staining with rabbit anti-$\beta 3$ IgG (*D*) and mAb antivinculin (*E*). *F* and *G,* sequential staining with mAb antivinculin (*F*) and Oregon-Green-conjugated Phalloidin (*G*). Magnification: ×100.

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mAb anti-$\alpha v\beta 5$ or mAb anti-$\beta 1$ to inhibit attachment of the cells to rec-osteopontin, and the number of cells attaching was not significantly different. Taken together, these data indicate that integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ mediate U-251MG cell attachment to osteopontin. As the attachment of integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ to osteopontin is mediated by the RGD peptide on vascular cells (7), the effect of incubation with a GRGDSP hexapeptide during attachment was determined. This RGD-containing hexapeptide inhibited U-251MG cell attachment to osteopontin with an IC_{50} of 3 μM, whereas a scrambled peptide failed to inhibit attachment (data not shown), indicating that U-251MG cell attachment to osteopontin is, indeed, RGD-peptide dependent.

To ascertain whether integrins $\alpha v\beta 1$, $\alpha 8\beta 1$, or $\alpha 9\beta 1$ are expressed on the U-251MG cells and, thus, could potentially participate in the attachment to osteopontin, the cells were subjected to biotin cell-surface labeling, followed by cell lysis and immunoprecipitation using antibodies specific for the αv and $\alpha 8$ integrin subunits, as well as anti-$\alpha 9\beta 1$ IgG and control antibodies. Integrins $\alpha 9\beta 1$ and $\alpha 8\beta 1$ were not detected in the cell lysates (data not shown); anti-$\alpha 8$ IgG and anti-$\alpha 9\beta 1$ IgG successfully immunoprecipitated $\alpha 8\beta 1$ from neonatal

rat astrocytes (11) and α9β1 from neutrophils. As anticipated from our previous studies, immunoprecipitation with anti-αv yielded proteins with relative molecular weights of 150 and 95 kDa, which are consistent with the relative molecular weights of the αv and β3/β5 subunits, respectively (1, 6; data not shown). However, a protein with a relative molecular weight corresponding to that of the β1 subunit (120 kDa) was not detectable, indicating that these cells do not express integrin αvβ1. Immunoprecipitation with mAb anti-αvβ5 or mAb anti-αvβ3 (mAb 7E3) yielded proteins with relative molecular weights of 150 and 95 kDa, confirming precipitation of the αvβ5 and the αvβ3 integrins, respectively (1). Thus, there was no evidence of expression of the integrin receptors αvβ1, α8β1, or α9β1 on the U-251MG cells.

U-251MG Cells Adherent to a Rec-Osteopontin Substrate Demonstrate an Altered Morphology: Integrins αvβ5 and αvβ3 Localize to Focal Adhesions. Functional engagement of the integrin receptors typically results in their clustering in the cell membrane and their subsequent localization to focal adhesions with the formation of bundled actin cables or stress fibers. Immunofluorescent microscopy analysis of U-251MG cells plated onto rec-osteopontin showed an altered morphology with lamellae-like processes (Fig. 2, *B–G*). The β5 subunit and vinculin colocalized to focal adhesions (Fig. 2, *B* and *C*, respectively), as did the β3 subunit and vinculin (Fig. 2, *D* and *E*, respectively). The α5 subunit remained diffusely expressed in a punctate manner in the cytoplasm (data not shown), although the α5 subunit does localize to focal adhesions in the U-251MG cells adherent to fibronectin (24). Phalloidin staining demonstrated actin fibers that paralleled the lamellae-like processes and actin fibers that ended in vinculin-containing focal adhesions (Fig. 2, *G* and *F*, respectively). To rule out an effect on cytoskeletal organization that was specific for this rec-osteopontin, we plated IBE brain microvascular endothelial cells onto rec-osteopontin under identical conditions and found bundled actin fibers indicating cytoskeletal organization (Fig. 3*F*), consistent with the results of Liaw *et al.* (7) in studies of vascular cell attachment to osteopontin. As osteopontin is modified extensively by post-translational processing *in vivo,* we compared the morphology of the U-251MG cells adherent to rec-osteopontin and native rat long bone osteopontin, and a similar morphology was observed (data not shown). We have reported previously that integrins αvβ3 and αvβ5 on the U-251MG cells mediate attachment to vitronectin (1, 6); therefore, we compared the U-251MG cell morphology and cytoskeletal organization on vitronectin and rec-osteopontin. The change in the cell morphology and actin cytoskeleton observed on attachment to rec-osteopontin appeared to be substrate specific. When U-251MG cells were allowed to adhere to vitronectin under identical conditions, the cells appeared flat; integrins αvβ5 and αvβ3 colocalized with vinculin to focal adhesions (Fig. 3, *A–D*) and organized the cytoskeleton, resulting in the formation of bundled actin fibers that spanned the cell (Fig. 3*E*).

Attachment of Malignant Astrocytoma Cells to a Rec-Osteopontin Substrate Results in a Slow Prolonged Activation of the Small GTPase Rac and a Rapid Activation of Rho. To identify the signals that regulate cytoskeletal organization on malignant astrocytoma cell attachment to rec-osteopontin, we investigated the activation of Rac and Rho. U-251MG cell attachment to vitronectin and rec-osteopontin showed a strikingly different time course for activation of Rac (Fig. 3*G*); Rac was maximally activated by 1 h in the cells adherent to vitronectin, whereas it was not maximally activated until 5 h in the cells adherent to rec-osteopontin. In contrast, Rho was maximally activated at 1 h in U-251MG cells attaching to either vitronectin or rec-osteopontin (Fig. 3*H*). As a control, we plated the IBE brain microvascular endothelial cells onto rec-osteopontin or vitronectin and determined the kinetics of Rac and Rho activation. Rac and Rho were both maximally activated by 1 h in the IBE cells plated onto rec-osteopontin or vitronectin (data not shown), consistent with our detection of stress fibers when these cells adhere to rec-osteopontin (Fig. 3*F*). As other investigators have shown that integrin αvβ3 on prostate cancer cells differentially activates PI3-kinase on a vitronectin *versus* an osteopontin substrate (13), we also investigated the kinetics of focal adhesion kinase (FAK) and PI3-kinase activation. Phosphorylation of Akt was used as a measure of PI3-kinase activation. Both FAK (Fig. 3, *I–K*) and PI3-kinase (data not shown) were activated in U-251MG cells adhering to either vitronectin or rec-osteopontin and exhibited only a slightly different time course of activation. These data indicate that U-251MG cell attachment to rec-osteopontin promotes a slow or prolonged activation of Rac and a rapid activation of Rho.

Integrins αvβ3, αvβ5, and α5β1 Mediate Haptotactic Migration of U-251MG Cells toward Rec-Osteopontin. Haptotactic migration assays using Boyden-type two-well chambers indicated promotion of migration of U-251MG cells toward rec-osteopontin protein. Significant inhibition of this migration was observed on the addition of 25 μg/ml mAbs anti-integrin αvβ3 (mAb LM609 or mAb 7E3), anti-integrin αvβ5 (mAb P1F6), anti-integrin αv (mAb L230), anti-integrin β1 (mAb P4C10), and anti-integrin α5 (mAb P1D6) (85, 85, 65, 80, 50, and 60%, respectively; data not shown). Moreover, a combination of mAbs anti-αvβ3 and anti-αvβ5 inhibited migration by 80% (data not shown), similar to anti-αv.

Integrins αvβ3, αvβ5, and α5β1 Mediate Migration of U-251MG Cells toward Normal Brain Homogenate from White Matter and Cortical Gray Matter. As osteopontin can undergo extensive post-translational modification that is tissue specific, the effects of osteopontin may differ significantly among cells in different regions of the brain. To address the question of the potential effects of post-translational processing, the relative ability of rec-osteopontin to promote migration compared with that of native rat long bone osteopontin or vitronectin was determined using these proteins in the same haptotactic migration assay. No significant difference was observed in the number of cells migrating to these substrates at 3 or 6 h (data not shown). The ability of normal brain homogenates to promote the migration of U-251MG cells was then assessed. Homogenates of normal brain from the white matter (Fig. 4*A*) and the cortical gray matter (Fig. 4*C*) were capable of promoting the migration of these cells, and depletion of the osteopontin in the homogenate from white matter resulted in a significant reduction (≈50%) in this response (Fig. 4*A*). Depletion of the osteopontin in the homogenate from the gray matter did not significantly reduce U-251MG cell migration (Fig. 4*C*). Addition of specific anti-integrin antibodies indicated that the promotion of the migration toward normal brain homogenates from the white matter and the cortical gray matter is mediated by integrins αvβ3, α5β1, and αvβ5 (Fig. 4, *B* and *D*, respectively), the same integrins that mediate the migration toward rec-osteopontin. However, inhibition studies with the anti-integrin antibodies indicated that the above three integrins differentially mediate migration toward homogenates of normal brain white matter, cortical gray matter, and rec-osteopontin. Western blot analysis of these brain homogenates for osteopontin showed an estimated 75% reduction in osteopontin protein after immunodepletion of the white matter (Fig. 4*E, Lanes 1–2*) and of the gray matter (Fig. 4*E, Lanes 3–4*).

DISCUSSION

The detection of expression of osteopontin protein in the neuropil of the normal adult brain white matter by immunohistochemical analysis suggests that it is a part of the normal brain extracellular matrix (neuropil), and Western blot analysis confirmed this. The

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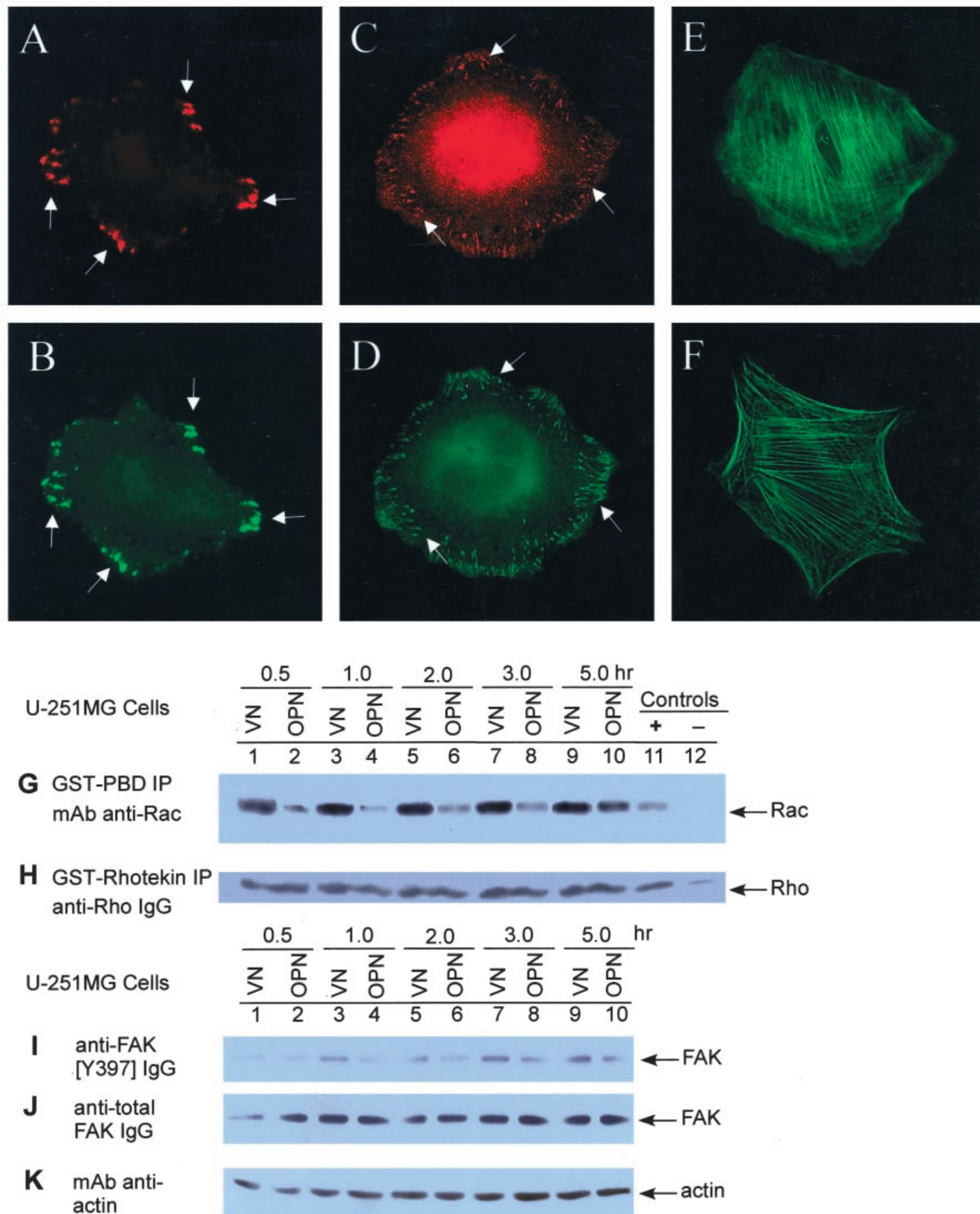

Fig. 3. U-251MG cell attachment to rec-osteopontin results in a slow prolonged activation of Rac and a rapid activation of Rho. U-251MG cells were plated onto coverslips coated with 5 μg/ml vitronectin (*A–E*), or IBE cells were plated onto coverslips coated with 10 μg/ml rec-osteopontin (*F*), followed by immunofluorescent analysis. *Arrows* denote focal adhesions. In *A* and *B,* cells were stained sequentially with rabbit anti-β5 IgG (*A*) and mAb antivinculin (*B*). In *C* and *D,* cells were stained sequentially with rabbit anti-β3 (*C*) and mAb antivinculin (*D*). In *E* and *F,* cells were stained with Oregon-Green-conjugated phalloidin (*E,* U251MG cells; *F,* IBE cells). *G–K,* U-251MG cells were plated onto 10 μg/ml rec-osteopontin or 5 μg/ml vitronectin for the indicated time and then detergent lysed. In *G* and *H,* lysate was incubated with p21-activated kinase-1 binding domain (PBD) coupled to agarose or Rhotekin Rho-binding domain coupled to agarose, washed, subjected to SDS-PAGE, and Western blotted with anti-Rac IgG or anti-Rho IgG, respectively. In *I–K,* lysate from each sample (20 μg) was Western blotted with rabbit anti-FAK [Y397] IgG (*I*), stripped and reprobed with anti-FAK IgG (*J*), and then stripped and reprobed with mAb antiactin (*K*).

distribution of osteopontin in the cortical gray matter suggests a specific interaction of osteopontin with a receptor or other protein on neuronal processes that could be organizing osteopontin on the cell surface or in the extracellular matrix. Integrin $\alpha5\beta1$ may be a receptor for osteopontin in the cortical gray matter, as it has been reported to be expressed on neuronal cell bodies and processes in the telencephalon (25). CD44, which is an osteopontin receptor on some cell types *in vitro,* is unlikely to be a receptor for osteopontin in the cortical gray matter, as it is expressed in the normal adult human brain predominantly on fibrous astrocytes in the white matter (26). Other investigators, using Northern blot analysis, have reported that osteopontin mRNA was up-regulated in two grade IV malignant astrocytoma

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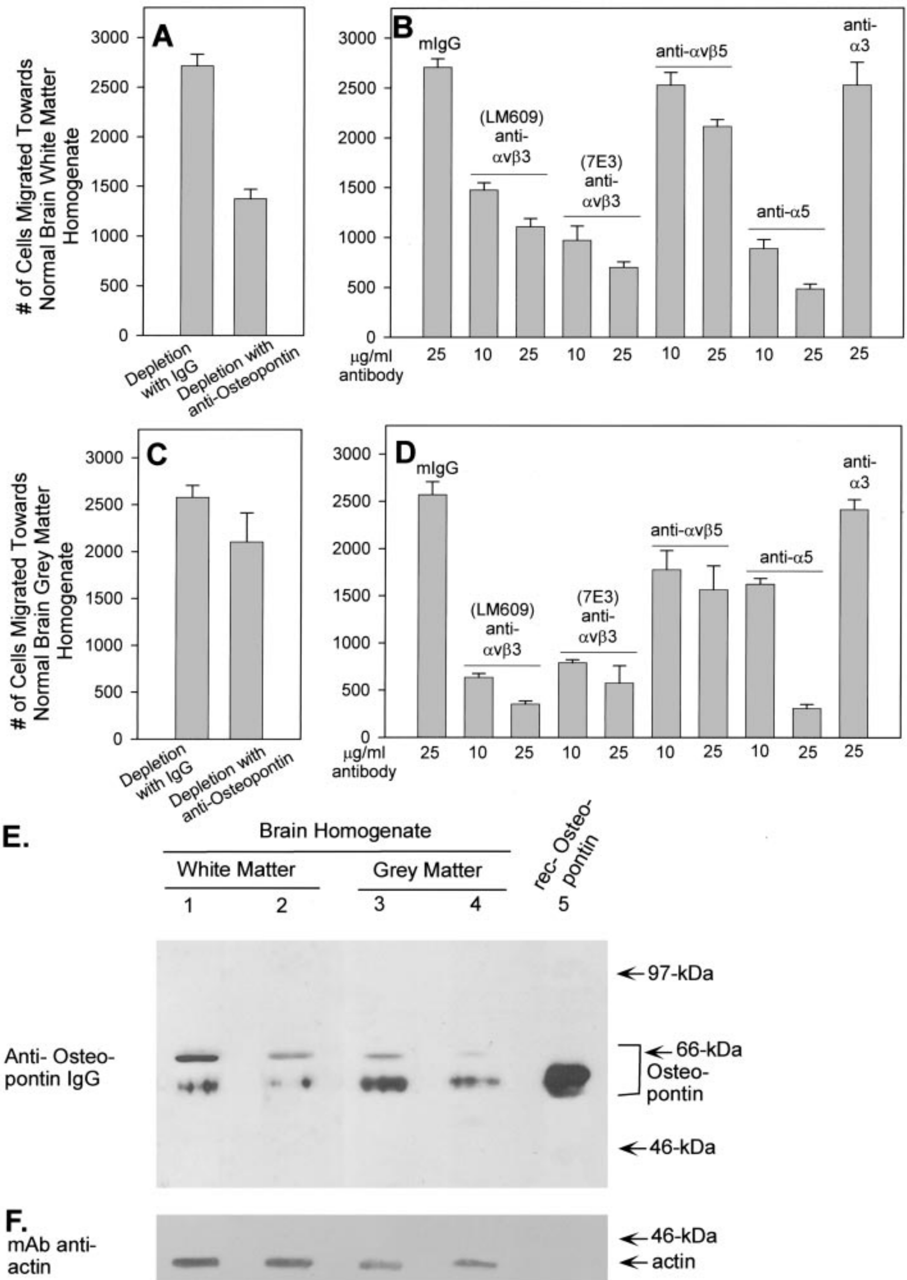

Fig. 4. U-251MG malignant astrocytoma cell migration toward homogenates of normal human brain white matter is inhibited by immunodepletion of osteopontin. U-251MG cells in serum-free DMEM were incubated with the indicated anti-integrin-specific antibody (20 min, 22°C) and then plated onto filters coated on the bottom surface with 60 μg/ml detergent-free normal brain homogenate from white matter (*A* and *B*) or cortical gray matter (*C* and *D*), before or after immunodepletion of osteopontin (*A* and *C*). Cells were allowed to migrate for 48 h (37°C, 5% CO_2). Samples were assayed in replicas of four, and the data were analyzed and presented as the mean ± s.e.m. In *E* and *F,* brain homogenates (15 μg), or rec-osteopontin (300 ng), were subjected to 7.5% disulfide-reduced SDS-PAGE and blotted with 0.3 μg/ml antiosteopontin IgG (*E*), stripped, and reprobed with mAb antiactin (*F*). *Lane 1,* white matter; *Lane 2,* white matter immunodepleted of osteopontin; *Lane 3,* cortical gray matter; *Lane 4,* cortical gray matter immunodepleted of osteopontin; *Lane 5,* rec-osteopontin.

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tumors as compared with one normal brain (5). Although our results do not address the level of mRNA in normal brain *versus* malignant astrocytoma, they do indicate that osteopontin protein is expressed in both normal brain and grade III malignant astrocytic tumors at similar levels. Differences in mRNA half-life could account for the relatively low level of osteopontin mRNA in the one normal brain, as compared with the two grade IV malignant astrocytomas (glioblastomas) in the previous study (5). The up-regulation of the expression of osteopontin is regulated largely at the level of transcription, which is susceptible to modulation by several different cytokines, hormones, and oncogenes (4). Recently, we identified two elements in the 5′ proximal promoter region of osteopontin that are required for its basal transcription in both primary rat astrocytes and human malignant astrocytoma cells (27).

Our Western blot analyses further revealed a differential distribution of osteopontin between normal brain cortical gray matter and white matter. Three bands of relative migration 60, 65, and 70 kDa were observed in the normal brain cortical gray matter. In contrast, in the normal brain white matter, only the bands of relative molecular weight of 60 and 65 kDa were observed, and the 70-kDa band could not be detected. Similar results were observed using two different antibodies directed toward osteopontin in the Western blot analysis. Osteopontin isolated from various tissues and cells exhibits electrophoretic mobilities consistent with a protein of apparent molecular mass between 44 and 75 kDa and typically migrates as more than one band on SDS-PAGE (4). Furthermore, the banding pattern of osteopontin on SDS-PAGE is thought to reflect extensive post-translational modification, including glycosylation, phosphorylation, and sulfation (4). Thus, the different bands of osteopontin detected by Western blot in the normal brain cortical gray and white matter may reflect different post-translational processing.

The morphology of the U-251MG cells adherent to a rec-osteopontin substrate was altered as compared with the morphology on plating onto a vitronectin substrate. On the rec-osteopontin substrate, the cells exhib-

ited lamellae-like processes, and phalloidin staining demonstrated some actin fibers that paralleled the lamellae-like processes. However, on the vitronectin substrate, the cells were smaller and flatter, and phalloidin staining demonstrated bundled actin fibers or actin cables (stress fibers) that spanned the cell. As the small GTPases Rac and Rho regulate cytoskeletal organization, we investigated their activation in U-251MG cells adherent to rec-osteopontin as compared with vitronectin. Attachment to rec-osteopontin resulted in a slow prolonged activation of Rac (maximal activation at 5 h), as compared with a rapid activation in cells adhering to vitronectin (maximal activation at 1 h). In contrast to that found with Rac, Rho was maximally activated at 1 h in U-251MG cells adherent to either vitronectin or rec-osteopontin. Thus, our data indicate differential activation of Rac and an altered cell morphology and cytoskeletal organization in U-251MG cells adherent to rec-osteopontin, as compared with vitronectin. As integrins $\alpha v\beta 5$ and $\alpha v\beta 3$ organize the cytoskeleton (localize to focal adhesions) and mediate haptotactic migration in the U-251MG cells adhering to rec-osteopontin or vitronectin, the different signals generated on U-251MG cell attachment to these two substrates are likely caused by differential regulation of these two integrins by other molecules or the involvement of another receptor recognizing osteopontin. There is precedent for substrate specificity in integrin signaling; other investigators have reported that integrin $\alpha v\beta 3$ on $\beta 3$-transfected LNCaP prostate cancer cells can exist in multiple functional states differentially activating PI3-kinase and mediating cell migration, depending on whether the cells are adherent to osteopontin or vitronectin (13). To our knowledge, independent of growth factors, no molecule has been identified that specifically regulates the function of integrin $\alpha v\beta 5$. However, depending on the cell type, integrin $\alpha v\beta 3$ can be regulated by integrin-associated protein or cross-talk from other integrins (15, 28), and these possibilities will be examined in future studies.

Migration toward detergent-free homogenates from normal brain white matter was confirmed, and it was shown that this was reduced significantly by depletion of osteopontin and was inhibited by antibodies directed toward integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ but not toward integrins $\alpha v\beta 5$ and $\alpha 3\beta 1$, indicating that the osteopontin expressed in the brain white matter is capable of promoting the migration of malignant astrocytoma cells. The significance of this finding is underscored by the long-standing observation of microscopists that the predominant histological pattern of malignant astrocytoma cell invasion *in vivo* is migration/invasion into adjacent brain white matter (2). In contrast to migration toward rec-osteopontin, migration toward homogenates from normal brain white matter showed the greatest inhibition ($\approx$85%) with mAb anti-integrin $\alpha 5$. Integrin $\alpha 5\beta 1$ has been reported to be expressed on malignant astrocytoma cells *in vivo* and *in vitro* (reviewed in Ref. 2); thus, this finding suggests a role for integrin $\alpha 5\beta 1$ in malignant astrocytoma cell invasion of normal brain. Migration toward homogenates from normal brain gray matter was also found; however, depletion of the osteopontin failed to significantly alter the migration, suggesting another ligand(s) is promoting U-251MG cell migration.

In summary, our data suggest a role for normal brain osteopontin in promoting malignant astrocytoma cell migration and, thus, invasion *in vivo*. This tumor cell migration is mediated by integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$, expressed on these tumor cells. We demonstrate for the first time that the signals generated on malignant astrocytoma cell attachment to rec-osteopontin *versus* vitronectin result in the differential activation of Rac. The slow prolonged activation of Rac on attachment to rec-osteopontin likely contributes to the difference in cytoskeletal organization that we have observed and could modulate nonhaptotactic cell migration and/or cell proliferation, a possibility that will be pursued in future studies.

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