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Developmental Regulation of Annexin II (Lipocortin 2) in Human Brain and Expression in High Grade Glioma

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ABSTRACT

In experiments to identify molecules that might be important in the pathogenesis of glioblastoma multiforme, the most common malignant brain tumor, we found that annexin II (Lipocortin 2, p36), a likely second messenger in several different mitogenic pathways, was highly expressed in tumor tissue of glioblastoma multiforme (9 of 9) and highly anaplastic astrocytoma (2 of 6), but not in astrocytomas of lower patho-

physical grade (0 of 6). We also detected high levels of annexin II expression in fetal brain during the period when radial glia proliferate, although annexin II expression was not detected in normal adult brain.

These data demonstrate that annexin II expression is developmentally regulated in the human central nervous system and suggest that the early progenitor radial glia share important characteristics with highly malignant glial tumors.

INTRODUCTION

Annexin II is a substrate for both cellular and viral encoded protein tyrosine kinases including the EGFR3 and pp60c-src (1–3), and for protein kinase C (4). These findings and the ability of annexin II to inhibit PLA2 (5, 6) and PLC, (7) activity and bind phospholipid membranes in a CaE-dependent manner (8, 9) suggest that it is an important second messenger for the transduction of extracellular signaling pathways. During the course of experiments utilizing subtractive hybridization cloning and differential hybridization screening to identify genes preferentially expressed in high grade glioma, we isolated a cDNA clone, psub14, from the GBM-derived cell line HTB17.

DNA sequencing and a search of the Genbank data base showed that this cDNA contained sequences identical to those of annexin II (6, 10). Expression of annexin II has not previously been detected in the mammalian brain. We therefore have examined the expression of annexin II during normal human fetal development and in glioma of different histological grades.

MATERIALS AND METHODS

Isolation of Annexin II Using Subtractive Hybridization and Differential Hybridization Screening. Total and poly(A)+ RNA were isolated as described (11, 12). 32P-labeled cDNA was synthesized using mRNA from the glioma cell line HTB17 as described (13). Biotinylated cRNA from the nonglial tumor cell line, TE671, was prepared by converting 5 #g of plasmid DNA were digested with EcoRI and XbaI (BRL), co-

linkers. Double-stranded cDNA and single-stranded molecules were removed by adding 10 #g of streptavidin-UTP (BRL) according to the manufacturer’s recommendation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed.
2 The abbreviations used are: EGFR, epidermal growth factor receptor; PLA2, phospholipase A2; PLC, phospholipase C; cDNA, complementary DNA; GBM, glioblastoma multiforme; poly(A)+ RNA, polyadenylated RNA; HAA, highly anaplastic astrocytoma; MAA, moderately anaplastic astrocytoma; GFAP, glial fibrillary acidic protein; CNS, central nervous system.

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Northern Blot Analysis. Total RNA was isolated from tumor cell lines, primary tumor specimens, and normal brain tissues at various developmental stages as described above. Total RNA samples (20 #g) were electrophoretically fractionated on 1% agarose/2.2 M formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell). Hybridization and washing conditions were as described previously (14). The psub14 and pHGP-1 (a cDNA encoding human GFAP) cDNA probes were 32P-radiolabeled in vitro to a specific activity of ~2.5 x 108 cpm/ug by nick-translation using [o-32P]dCTP. The final hybridization wash was in 15 mM NaC1/1.5 mM sodium citrate, pH 7, containing 1% sodium dodecyl sulfate at 65°C for 1 h. Ethidium bromide staining of gels was used to confirm that similar amounts of RNA were loaded per lane.

Preparation of Annexin II-specific Peptide Antisera. The annexin II-specific NH2-terminal peptide, KLSLEGDHSSIPSAV, was synthesized and conjugated to keyhole limpet hemacycin with glutaraldehyde as described (17). The conjugated peptide was then used to immunize two rabbits of an initial dose of 0.250 mg/rabbit. Then 0.125 mg/rabbit was used for three additional boosts at 3-week intervals (Babc, Richmond, CA). Anti-annexin II peptide antisera was performed on lysates from glial cell lines in which annexin II mRNA was detectable by Northern blot analysis (U343MGA CL 2.6 and SNB19). In these cell lines, the anti-annexin II peptide antisera recognized a specific protein with a molecular weight of approximately 36,000 (data not shown) consistent with that previously described for annexin II (1–4).

Immunohistochemistry. Neuropathological biopsies were fixed in Carnoy’s solution at 0–4°C, embedded in paraffin, and cut in 5-um sections using standard laboratory techniques. Annexin II and EGFR were evidenced by the streptavidin-biotin technique. Briefly, tissue sections were deparaffinized in xylene twice for 3 min and then rehydrated for 1
min each in 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol, 
H$_2$O twice, and then phosphate-buffered saline for 10 min. Endoge-

nous peroxidases were blocked with methanol containing 0.5% hydro-
gen peroxide. Immunohistological staining was performed using the 
Zymed (South San Francisco, CA) reagents and protocol. Both annexin 
II and EGFR (ICN, clone 29.1.1) antisera were used at dilutions of 
1:1000 and incubated with tissue sections at room temperature over-

night. After this incubation, the substrate-chromogen mixture consist-
ing of 6 mg of 3,3-diaminobenzidinetetro-HCl in 10 ml 50 mM Tris-
HCl, pH 7.6, and 0.1 ml 3% hydrogen peroxide was incubated with the 
tissue sections for 3–5 min to stain for annexin II and 7–10 min for 
EGFR staining. Sections were then washed twice in H$_2$O, stained with 
Harris hematoxylin, and mounted.

RESULTS

We isolated a partial cDNA encoding annexin II from the 
glioma cell line HTB17 using a subtractive hybridization and 
differential hybridization screening procedure. Annexin II can 
be a substrate for tyrosine phosphorylation by the EGFR which 
is frequently amplified and overexpressed in glial tumor speci-
mens (18, 19) and may be a component of the pathway that 
mediates EGF stimulated growth. We therefore surveyed glial 
tumor cell lines and cell lines derived from nonglial tumors to 
determine their level of annexin II mRNA expression. In these 
experiments we used a cDNA probe corresponding to the 3'-
untranslated region of annexin II, psub14, and thus specific for 
annexin II and not the other members of the annexin family. 
The experiment shown in Fig. 1a indicates that high levels of 
annexin II mRNA (approximate size of 1.4–1.6 kilobases) ex-
pression were detected in all nine glial cell lines examined (Fig. 
1a, Lanes 1–9), which were established from highly malignant 
glial tumors. Additionally, we detected high levels of annexin II 
mRNA in a human fetal glial cell line, SVG, established by 
transfection with SV40 DNA (20) (Fig. 1a, Lane 10). In a 
survey of cell lines from tumors that arise in other nervous 
system tissues (Fig. 1b), we were unable to detect annexin II 
expression in seven different neuroblastoma cell lines (Fig. 1b, 
Lanes 3–6), although very low level expression could be 
detected in 4 of 4 neuroepithelioma cell lines (Fig. 1b, Lane 9) 
and in 4 of 8 cell lines derived from Ewing’s sarcoma, a very 
undifferentiated tumor closely related to neuroepithelioma that 
expresses neuronal features (Fig. 1b, Lanes 7–8). Higher mo-

lecular weight bands are detectable on these blots but we at-
tribute them to unprocessed nuclear forms of annexin II and not 
due to hybridization with other members of the annexin family.

We then examined 21 primary glial tumors for annexin II 
expression: 9 GBM, 6 HAA, and 6 MAA, which are of high, 
intermediate, and low pathological grade, respectively. Fig. 2a 
shows data representative of our results and Table 1 is a com-
parison of this survey comparing the levels of annexin II ex-
pression in these 21 glial tumors. Annexin II mRNA was de-
tectable in 9 of 9 GBM, 2 of 6 HAA, and 0 of 6 MAA, although 
the level of expression was variable within the tumors in which 
it was detectable. GFAP is expressed in both the normal adult 
CNS and in malignant astrocytes. In the more anaplastic astro-
cytic tumors, variable expression is typically observed (21). As 
a control for the integrity of RNA analyzed per lane, we reex-
amined the Northern blot shown in Fig. 2a with a probe specific 
for GFAP. Fig. 2b shows that GFAP mRNAs are detectable in 
all of the glial tumors demonstrating that the RNAs are indeed 
intact for each sample analyzed. On the basis of these findings,

we determined that enhanced annexin II expression is closely 
associated with increased pathological grade.

We observed variable amounts of annexin II mRNA in the 
HAA and GBM tumors (Table 1). In this regard, it is of interest 
that cellular pleomorphism and phenotypic heterogeneity are 
well-described features of high grade astrocytic tumors (22–24). 
To better understand this variability at the cellular level, we 
developed an anti-peptide antibody (LCII-2.2) that was specific 
for annexin II and that would not recognize other members of 
the lipocortin family. We used this antisera to evaluate annexin 
expression immunohistochemically in a series of high grade 
gliomas. Fig. 3 (a, b, and c) shows the immunostaining patterns 
that were typically found in GBM tumors evaluated with LCII-
2.2. In the GBM shown in Fig. 3a, LCII-2.2 is diffusely reactive, 
although a subset of the tumor cells are more intensely 
stained (Fig. 3, large arrows). In the GBM shown in Fig. 3c, 
LCII-2.2 is apparently equally reactive against the majority 
of the tumor cells (Fig. 3, small arrows), albeit faintly. In both 

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Footnote: 3 Our unpublished data.
GBMs staining localizes to the membrane and cytoplasm portions of the tumor cells. Note the multinucleated, bizarre giant cell that is a distinctive feature of GBM also stains with LCII-2.2 (see open arrow in Fig. 3A). The variable annexin II immunoreactivity observed among the gliomas we examined is consistent with the variable levels of mRNA we detected in our Northern blot analysis (Fig. 2A; Table 1). Moreover, this variable expression is consistent with the extreme cellular and biochemical heterogeneity that has previously been observed in high grade gliomas (22–24).

Since amplification of the gene encoding EGFR occurs in more than 40% of GBM analyzed (18, 19) and annexin II is a major substrate for this tyrosine kinase (1, 2) we attempted to characterize the expression of EGFR in brain tumor specimens. Rehybridization of the blots shown in Fig. 2A with a probe that recognizes EGFR RNA revealed RNA of numerous different kilobases.

In pools of RNA isolated from human fetal brain between 67 days of gestation (Fig. 4B, Lane 3) and annexin II mRNA cannot be detected (Fig. 4A, Lane 3). This increase in GFAP mRNA coincides with the proliferation and maturation of radial glia (25–28) and with the expression of annexin II mRNA (Fig. 3A, Lanes 3–4). At 118 days (Fig. 4B, Lane 2) when the majority of the radial glia are thought to be maturing into the adult forms of astrocytes (25–27) and annexin II mRNA cannot be detected (Fig. 4A, Lane 3), GFAP mRNA levels have decreased to approximately 5-fold that observed between 46 and 67 days, consistent with the previously reported biphasic pattern of GFAP expression (26, 29).

Table 1 Summary of annexin II mRNA expression in 21 gliomas

<table>
<thead>
<tr>
<th>Name</th>
<th>Tumor</th>
<th>Annexin II expression</th>
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<tbody>
<tr>
<td>SF951</td>
<td>GBM</td>
<td>1.00</td>
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<tr>
<td>SF905</td>
<td>GBM</td>
<td>1.00</td>
</tr>
<tr>
<td>SF941</td>
<td>GBM</td>
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<tr>
<td>SF466</td>
<td>HAA</td>
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<td>SF918</td>
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<td>SF949</td>
<td>GBM</td>
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<tr>
<td>SF889</td>
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</tr>
<tr>
<td>SF921</td>
<td>GBM</td>
<td>0.21</td>
</tr>
<tr>
<td>SF831</td>
<td>GBM</td>
<td>0.20</td>
</tr>
<tr>
<td>SF629</td>
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<tr>
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</tr>
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<td>MAA</td>
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</table>

clonal antibody 29.1.1 (Fig. 3, B and D). In the tumor GM671 (Fig. 3B, the majority of the tumor cells stain diffusely (Fig. 3, small arrows) with this antibody, although a small population of tumor cells stain more intensely (Fig. 3, large arrows). The pattern of staining observed with 29.1.1 is very similar to that observed for the annexin II antibody in an adjacent section of the same tumor (Fig. 3A). As with EGFR staining, annexin II immunoreactivity is confined to the membrane and cytoplasm of the tumor cells. A second GBM, GM457, also has a 29.1.1 staining pattern similar to that seen with LCII-2.2 where the majority of the tumor cells are stained equally well (Fig. 3D).

Tumors may arise from precursors of fully differentiated cells. Since we detected high levels of annexin II mRNA expression in a human radial glia cell line, SVG, established from fetal brain (20) (Fig. 1A, Lane 10), we examined the expression of annexin II during human CNS development. As shown in Fig. 4A, although expression of annexin II was not detectable in RNA isolated from either adult brain (Fig. 4, Lane 1) or fetal brain at 118 days of gestation (Fig. 4, Lane 2), it was detectable in pools of RNA isolated from human fetal brain between 46 and 96 days of gestation (Fig. 4, Lanes 3 and 4). In the human CNS, primitive radial glia proliferate extensively in the cerebrum early in gestation and then with the onset of astrocytic differentiation begin to express high levels of GFAP (25–28). To correlate annexin II expression with the proliferation and maturation stages of radial glia, we reexamined the Northern blot shown in Fig. 4A for evidence of GFAP expression. GFAP mRNA was detected at low levels between 46 and 67 days of gestation (Fig. 4B, Lane 4) but increased approximately 20-fold between 52 and 96 days of gestation (Fig. 4B, Lane 2). This increase in GFAP mRNA coincides with the proliferation and maturation of radial glia (25–28) and with the expression of annexin II mRNA (Fig. 3A, Lanes 3–4). At 118 days (Fig. 4B, Lane 2) when the majority of the radial glia are thought to be maturing into the adult forms of astrocytes (25–27) and annexin II mRNA cannot be detected (Fig. 4A, Lane 3), GFAP mRNA levels have decreased to approximately 5-fold that observed between 46 and 67 days, consistent with the previously reported biphasic pattern of GFAP expression (26, 29).
In the adult brain, which normally contains large numbers of mature GFAP-positive astrocytes (26), the mRNA level is about 200 times greater than the levels observed at earlier stages of development (Fig. 4b, Lane 1), although annexin II expression is not detectable (Fig. 4a, Lane 1).

DISCUSSION

While the enhanced expression of annexin II may be of pathological importance in high grade glial tumors, the expression of annexin II during early stages of CNS development raises the possibility that annexin II expression in highly malignant glial tumors reflects its expression in astrocytic progenitor cells (i.e., highly proliferative radial glia). Such a model suggests that these tumors may arise in rare descendants of glial precursors that do not mature but remain in the postnatal CNS possibly as glial stem cells important for reactive gliosis. In this regard it is of particular interest that we were able to detect annexin II expression in glial scar tissue (data not shown), since it is composed primarily of astrocytic cells that share many of the same characteristics of radial glia, such as hypertrophy, hyperplasia, and increased migratory behavior (30, 31). Since human fetal tissues are difficult to obtain, we were unable to define the exact period(s) during which annexin II is expressed during human CNS development. However, future experiments are planned to better define the pattern of expression and the role annexin II plays in the developing CNS by using a rodent model system.

Annexin II has been shown to regulate the activity of PLA2 and PLC. Both of these enzymes are clearly involved in intracellular signal transduction, since they are responsible for the generation of several second messenger molecules.
PLA₂ deacylates phosphatidylinositol within the phospholipid membranes releasing arachidonic acid, a precursor of prostaglandin and leukotriene biosynthesis. Using reconstitution assays, other investigators (32) have shown that phosphorylation of PLC, by the EGFR tyrosine kinase activates PLC, which is then able to cleave phosphatidylinositol 4,5-bisphosphate to form inositol trisphosphate and diacylglycerol. It is of interest that inhibition of PLA₂ and PLC, by annexin II appears to be at the level of binding the phosphatidylinositol substrates of these enzymes (4, 8).

Annexin II expression was easily detectable in all the GBM tumors we examined, although it has not been shown to be expressed in normal CNS tissue of adults. It has also been shown that the EGFR gene is amplified and or overexpressed in a significant proportion of GBM; however, as with annexin II, EGFR expression is not detectable in normal glia (24) and is thereby also associated with gliomagenesis. These data and the very similar patterns of annexin II and EGFR immuno-

staining in the GBMs we examined suggest a likely relationship between the expression of these genes, although experiments to demonstrate the pathological importance of their coexpression are beyond the scope of this study. Since annexin II is expressed in all GBM and EGFR is expressed in less than 50%, it is possible that other growth factor tyrosine kinase receptors, such as the platelet-derived growth factor receptor, which is expressed in glioma cell lines (33) and has been shown to phosphorylate annexin II (34), will also be found to be of importance for the pathogenesis of GBM.

REFERENCES

ANNEXIN II IN HUMAN AND HIGH GRADE GliOMA


Developmental Regulation of Annexin II (Lipocortin 2) in Human Brain and Expression in High Grade Glioma

Steven A. Reeves, Cindy Chavez-Kappel, Richard Davis, et al.