Renin-angiotensin System-regulating Aminopeptidases in Tumor Growth of Rat C6 Gliomas Implanted at the Subcutaneous Region

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Abstract. *Background: Angiotensin peptides play roles in brain tumor infiltration and associated angiogenesis. Materials and Methods: We explored the roles of soluble and membrane-bound forms of renin–angiotensin system-regulating aminopeptidase N (APN)-, aminopeptidase B (APB)-, glutamate aminopeptidase- and aspartate aminopeptidase (AspAP)-specific activities on tumor growth in the rat C6 glioma model with implantation into the subcutaneous abdomen of Wistar rats, evaluating these activities as biological markers. The tumor volume was assessed for three weeks and a sample of tumor was obtained every seven days to obtain the soluble and membrane-bound fraction, in order to assay enzyme activities fluorometrically using their corresponding aminoacyl-β-naphthylamide as substrates. Results: We found a time-dependent decrease in soluble and membrane-bound APN and APB. Soluble AspAP increases with tumor growth in a time-dependent manner. Conclusion: Although gliomas are heterogeneous tissues, angiotensin peptides are involved in this model of tumor growth and their role could be analyzed through their corresponding regulatory proteolytic enzymes.*

Malignant gliomas, the most frequent type of brain tumor, are currently non-curable central nervous system neoplasias and unfortunately there has been little improvement in the efficacy of adjuvant therapies for such tumors (1, 2). A critical step leading to clinical trials of treatments for

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gliomas after *in vitro* studies, is to examine the efficacy and toxicity of therapeutic agents in *in vivo* animal models. Rat C6 glioma models are widely used to evaluate the effects of novel therapies. The implantation of C6 gliomas into a rat brain is a popular and proper animal model mimicking human central nervous system tumors, thus many investigations using this model have been reported (3-4). To overcome the disadvantages of the intracerebral C6 glioma model (5), in one study C6 glioma cells were implanted into the rat abdominal subcutaneous region. The growth pattern and pathological findings in the subcutaneous region were compared with those of cells implanted in the cerebral region, and it was demonstrated that the subcutaneous model was a useful tool (6).

Gliomas show extensive proliferation of vascular endothelium and angiogenesis, essential for tumoral growth and invasiveness (7). Tumor endothelial cells play an active role in angiogenesis through secretion of growth factors (8, 9). A therapeutic possibility in the treatment of cancer is through inhibition of angiogenesis (10), the principal route by which tumor cells exit the primary tumor site and enter the circulation (11). Angiotensin peptides of the renin-angiotensin system (RAS) are involved in the control of cell growth and vascular permeability (12). In fact, angiotensin II stimulates angiogenesis and tumor growth (13, 14). Clasically, angiotensin II has been considered as the effector peptide of RAS, but it is not the only active peptide. Several of its degradation products, including angiotensins III and IV, also possess biological functions. These peptides are formed *via* the action of several aminopeptidases. Thus, angiotensin III derives from angiotensin II by deletion of the N-terminal aspartic residue by glutamyl-aminopeptidase (G1uAP; E.C: 3.4.11.7) and aspartyl-aminopeptidase (AspAP; E.C: 3.4.11.21). AspAP and GluAP have classically been named together as angiotensinase or aminopeptidase A (APA), but these activities reflect two different enzymes. However, angiotensin III is further converted to angiotensin

IV by arginyl-aminopeptidase (aminopeptidase B, APB) (EC 3.4.11.6) or alanyl-aminopeptidase (aminopeptidase N, APN) (EC 3.3.11. 14) (15-18).

Because it has been suggested that several angiotensin peptides may be essential steps in both brain tumor infiltration and in brain tumor-associated angiogenesis, the present study explores the role of the soluble and membrane-bound forms of the RAS-regulating aminopeptidases APN, APB, GluAP and AspAP on tumor growth in the rat C6 glioma model, with implantation into the subcutaneous abdomen and evaluates their activities as biological markers.

Materials and Methods

Animals. Six male Wistar rats (470±52 g body weight) were used in this study. The animals were provided from the animal house-care of the University of Jaen, and maintained in a controlled environment under constant temperature (25°C) with a 12-h light/12-h dark cycle. Rats were housed in cages and given free access to standard laboratory rat food and water. The experimental procedures for animal use and care were in accordance with the European Community Council directive (86/609/EEC). Protocols were approved by the Bioethical Committee of the University of Jaen.

Implantation of C6 glioma cells. A rat glioma cell line (C6 cells) was obtained from the American Type Culture Collection (no. CCL-107) (ATTC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium/HAM F-12 mixture, supplemented with 5% fetal bovine serum (FBS), without antibiotics. Five million cells were injected subcutaneously into both dorsal flanks of Wistar rats using a Hamilton syringe with a 26-gauge needle.

Measurement of tumor volume and collection of the tissue and serum. The size of the abdominal tumor was measured with slide calipers every seven days for three weeks. The tumor volume was defined as ½ $(ab)^2$, where *a*: long diameter and *b*: short diameter (19). Rats were anesthetized with equitensin (2 ml/kg body weight) by intraperitoneal injection and then shaved and sterilized with 10% povidone-iodine. Samples of tumors were obtained every seven days, quickly removed and frozen at –80°C until use. Blood samples were obtained from the left cardiac ventricle and centrifuged for 10 min at 3000 ×*g* to obtain the serum, which was frozen and stored at –80°C until use.

Measurement of the specific activity of aminopeptidases. Samples of tumors were used to obtain the soluble and membrane-bound fractions, to assay for enzyme activities fluorometrically in triplicate, by using their corresponding aminoacyl-β-naphthylamide as substrates. To obtain the soluble fraction, tissue samples were homogenized in 10 volumes of 10 mM HCl–Tris buffer (pH 7.4) and ultracentrifuged at 100,000 ×*g* for 30 min (4°C). The resulting supernatants were used to measure the soluble enzymatic activity and the protein content in triplicate. To solubilize membrane proteins, the pellets were homogenized again in HCl–Tris buffer (pH 7.4) plus 1% Triton X-100. After centrifugation (100,000 ×*g* for 30 min at 4°C), supernatants were used to determinate solubilized membrane-bound activities and proteins in triplicate (20), similarly to serum samples (21). AspAP and GluAP were determined fluorimetrically using aspartyl-β-naphthylamide

(AspNNap) and glutamyl-β-naphthylamide (GluNNap) as substrates, according to the method previously described by Carrera *et al*. (20, 21). Briefly, 10 μl of each sample was incubated in triplicate for 30 min at 37°C with 100 μl of the substrate solution for AspAP [100 μM AspNNap, 1.3 μM ethylenediaminetetraacetic acid (EDTA) and 2 mM $MnCl_2$ in 50 mM of phosphate buffer, pH 7.4] and for GluAP [100 μM GluNNap, 0.65 mM dithiothreitol (DTT) and 50 mM $CaCl_2$ in 50 mM of phosphate buffer, pH 7.4]. APN and APB were also measured fluorometrically using alanyl-β-naphthylamide (AlaNNap) or arginyl-β-naphthylamide (ArgNNap) as the substrate, as previously described by Garcia *et al*. (22). Ten microlitres of each sample were incubated for 30 min at 37°C with 100 μM of the substrate solution: 100 μM AlaNNap or 100 μM ArgNNap and 0.65 mM dithiothreitol (DTT) in 50 mM of phosphate buffer, pH 7.4. All the reactions were stopped by adding 100 μl of 0.1 M acetate buffer, pH 4.2. The amount of β-naphthylamine, released as the result of the enzymatic activities was measured fluorimetrically at a 412 nm emission wavelength with an excitation wavelength of 345 nm. Proteins were also quantified in triplicate by the method of Bradford, using bovine serum albumin (BSA) as a standard. Specific soluble and membrane-bound APN, APB, AspAP and GluAP activities were expressed as nanomoles of Ala-, Arg-, Asp- and Glu-β- naphthylamide, hydrolysed per minute per milligram of protein, by using a standard curve prepared with the latter compound under corresponding assay conditions.

Histopathological examination. The resected tumors were perfused with formalin and the specimens were dehydrated and embedded in paraffin. The sections were stained by hematoxylin and eosin (H&E) using a Nikon imaging system. Images were performed using x10 and ×100 digital magnification.

Statistical analysis. To analyze the differences on the specific activity of aminopeptidases during the three weeks of the study, we used one-way analysis of variance (ANOVA). *Post-hoc* comparisons were made using the Newman–Keul's test; comparisons with $p<0.05$ were considered significant.

Results

Tumor formation and growth rate. C6 glioma cells were implanted into the subcutaneous region of each of six rats on day 0. Taking account of the volume capacity for the implantation, 5×10^6 cells were implanted into the subcutaneous region. On day 7, tumor was visible and the volume was measurable in all six rats. This indicated that the success rate for implantation of C6 glioma cells was 100% in Wistar rats. The volumes of subcutaneous tumors are shown in Figure 1.

Histopathological examination of the implanted tumors. We sliced each resected specimen into sections and examined the histopathological features of the subcutaneous-implanted tumors. All the histological findings by H&E staining were similar, and Figure 2 shows representative sections of a tumor on day 21 after the implantation. Microscopic images of H&E staining demonstrated tissue necrosis (Figure 2a). Examination of the central necrosis revealed the presence of tumor cells invading the subcutaneous tissue as well as infiltration of skin

cells in the tumors. In addition, there were polygonal to spindle-shaped cells and large areas with atypical cells in loosely-cohesive clusters with nuclear pleiomorphisms and intranuclear inclusion (Figure 2b). Fibrillary processes extending from these atypical cells were apparent (Figure 2c). Tumor area infiltration with cellular atypia exhibited abundant cellularity and necrosis (Figure 2d).

Aminopeptidase activity assays. Specific soluble and membrane-bound tumoral aminopeptidase activities in rats with C6 gliomas implanted at the subcutaneous region are shown in Figure 3. Specific soluble APN and APB activities decreased significantly by 43.92% ($p<0.001$) and 47.88% ($p<0.01$), respectively, at the second week of tumor growth. At the third week, these activities significantly decreased ($p<0.001$) by 43.03% and 53.97% respectively. In the same way, specific membrane-bound APN and APB activities significantly decreased ($p<0.01$) by 37.73% and 58.92%, respectively, at the second week of tumor growth. At the third week, these activities significantly decreased ($p<0.001$) by 77.66% and 77.44%, respectively. On the other hand, specific soluble and membrane-bound APA activities did not exhibit any significant changes in relation tumor growth. On the contrary, only the specific activity of soluble (but not membrane-bound) AspAP increased with tumor growth in a time-dependent manner. At the second week, this activity increased significantly by 100% ($p<0.001$) and at the third week by 177.78% ($p<0.01$). Circulating serum levels of these specific aminopeptidase activities were studied, but there were no significant differences in activity before and after tumor induction (data not shown).

Discussion

This study proved that the rat C6 glioma model by implantation at the subcutaneous region is feasible and applicable to tumorigenecity assays. Injected cells formed tumors at the subcutaneous site and were visible externally on the seventh day. Moreover, it was easy to measure the volume of the tumor without sacrificing the rat during the study. Thus, the rat C6 glioma model by implantation at the subcutaneous region constitutes a useful tool for studying the initiation, promotion and progression of tumorigenesis. In addition, to our knowledge, time the present work describes for the first time changes in RAS-regulating aminopeptidase activities in relation to tumor growth in the rat model by C6 gliomas implanted at the subcutaneous region, but not in the circulating RAS. Thus, we found a time-dependent significant decrease in specific activity of soluble and membrane-bound APN and APB, whereas no changes were found in those of soluble nor membrane-bound GluAP. On the contrary, only the specific activity of soluble (but not membrane-bound) AspAP increased
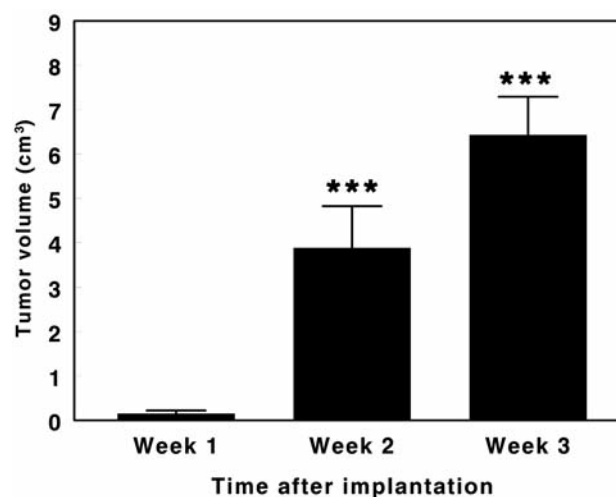

Figure 1. *Growth curve of C6 glioma implanted in the subcutaneous region. The volumes of implanted tumors were measured on days 7, 14 and 21. The values represent the mean volume (cm^3) ±SEM ($n=6$) (***$p<0.001$ compared to week 1).*

concomitantly with tumor growth in a time-dependent way. AspAP and GluAP classically have been named together as aminopeptidase A or angiotensinase, but G1uAP and AspAP are in fact two enzymes with different properties and roles (16, 17). These aminopeptidases degrade angiotensin II to form angiotensin III. Angiotensin III is also produced from angiotensin I through the production of des-Asp1-Angiotensin I, which is further converted to angiotensin III by the action of angiotensin-converting enzyme (ACE) (23). APB and APN are the enzymes responsible for the degradation of angiotensin III to form angiotensin IV (18). These two main bioactive peptides of the RAS, Ang II and its direct metabolite Ang III, exert their actions through their receptors AT1 and AT2, two membrane-bound receptors which are functionally distinct polypeptides with 30% sequence homology, belonging to the seven-transmembrane G protein-coupled receptor (GPCR) family (24, 25).

Proteins of the RAS are expressed, independently of the circulating RAS proteins, in normal non-vascular tissues, including the nervous system (26, 27), where they play a role in regulating cardiovascular functions (28) and other physiological processes such as cell growth, cell differentiation and apoptosis (29). It has been shown that some neurons or glial cells and glioma cells express the components of the RAS and their receptors, although the participative mechanisms were unclear (12, 26, 30-32). It has been described that APA was up-regulated and enzymatically active in blood vessels of human tumors, but was not detected in normal blood vessels (33). In addition, APA expression was found on dysplastic cells and was found to be increased in precancerous lesions and invasive cervical
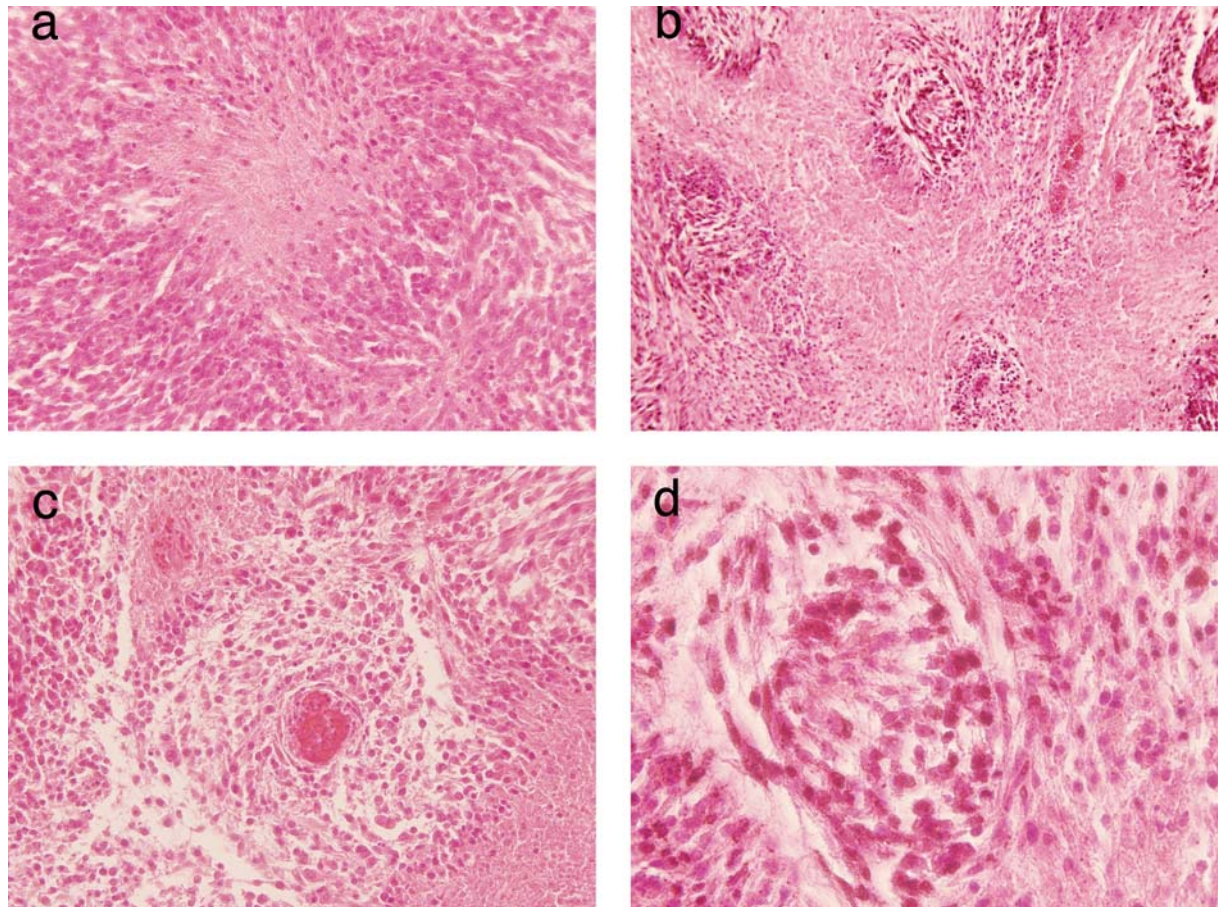
Figure 2. *Histopathological studies showing representative specimens of the implanted tumors of C6 glioma. Microscopic view with H&E staining and using ×10 (a, b and c) and ×100 (d) digital magnifications.*

cancer (34). These data and others regarding the expression of APA on prostate cancer cells (35), suggest that APA may play a regulatory role in neoplastic transformation and disease progression in various types of cancer. On the other hand, others have reported that several kinds of carcinomas including those of colon, kidney, breast and lung, exhibited little expression of APN (36, 37). Although APN has been considered as a proteolytic enzyme with the ability to facilitate tumor cell invasion through the extracellular matrix (38), lower expression of APN implies that APN may enzymatically function in ways other than extracellular matrix degradation. In accordance with the angiotensin converting cascade, our findings of no change in APA, increase in AspAP and a decrease in APN/APB expression suggest that the metabolism of angiotensin II to angiotensin III is constant, whereas the metabolism of angiotensin III to angiotensin IV is slow, resulting in the predominant actions of angiotensin II and angiotensin III in C6 gliomas. We, as other authors (35), hypothesize that these angiotensins may synergistically stimulate cell growth and angiogenesis.

Circulating serum levels of these specific aminopeptidase activities were studied (data not shown) but there were no significant differences between them, before and after tumor induction. Because of this, we can asses that changes in RAS proteins, through their regulating aminopeptidases, could be responsible, at least in part, for the progression of C6 gliomas and the development of the angiogenic processes *via* the local RAS in cancer tissue.

It is known that gliomas are accompanied by extensive angiogenesis, essential for tumoral growth and invasiveness (7); they also produce vast amounts of growth factors, as well as their receptors. The presence of these cytokines in normal brain is minimal, but in gliomas, their concentration is positively-related to vascular density, radioresistance, cell proliferation, degree of malignancy and the patient's survival (8, 9). Some reports indicate that angiotensin II, due to the up-regulation of these growth factors, induces neovascularization (13, 14, 39) and the expression of proto-oncogenes (40). Interestingly, many of these effects can be blocked by the AT1 antagonists but not by AT2 antagonists
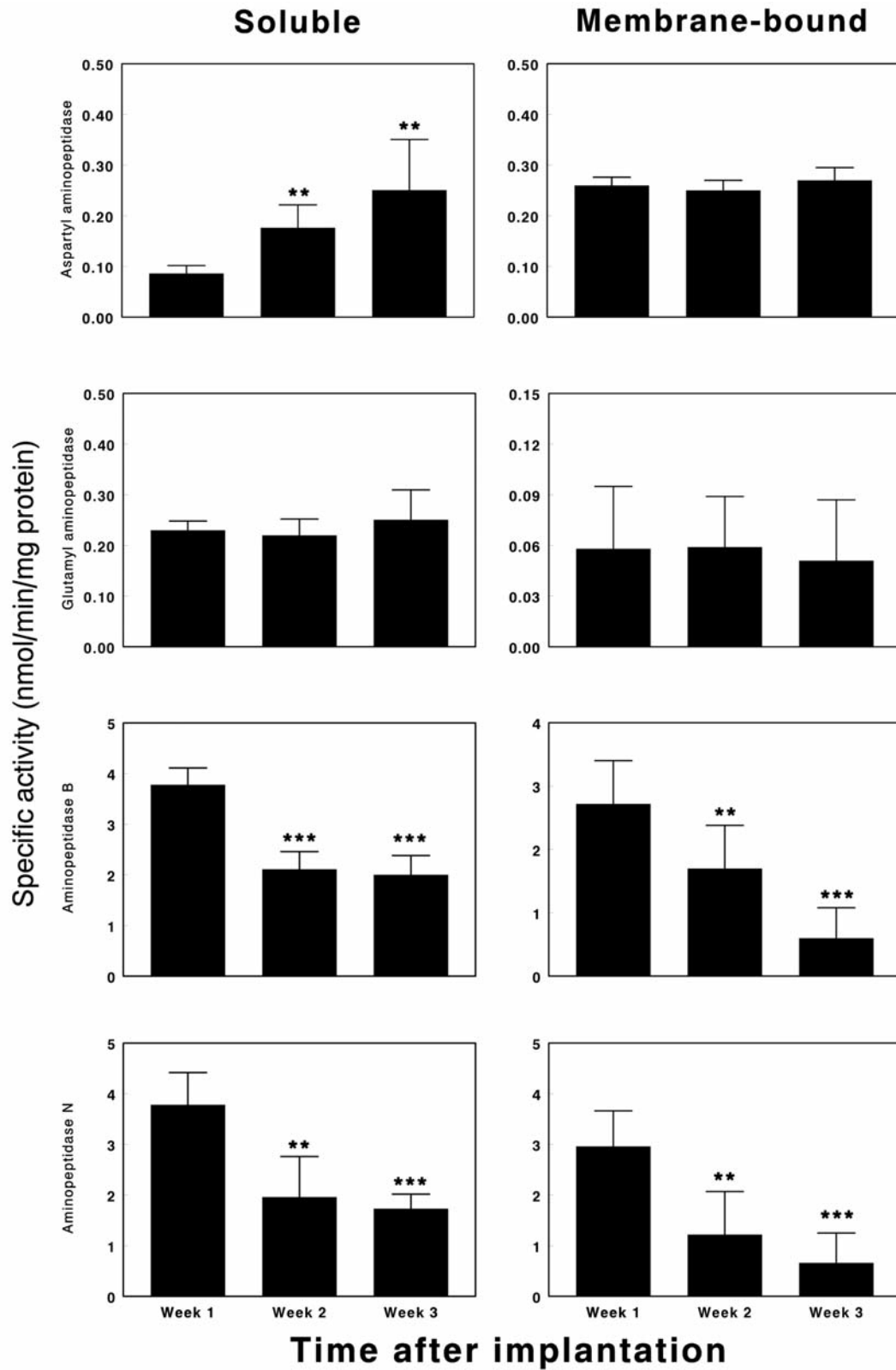
Figure 3. *Specific soluble and membrane-bound tumoral aspartyl aminopeptidase, glutamyl aminopeptidase, aminopeptidase N and aminopeptidase B activities in rats with C6 gliomas implanted at a subcutaneous region. Results are expressed in nanomoles of their corresponding aminoacyl-β-naphthylamide, hydrolyzed per min and per mg of protein (mean±SEM; n=6; **p<0.01 and ***p<0.001 compared to week 1).*

(39, 41). The presence of AT1 and AT2 in C6 glioma cells or rats with C6 glioma has been previously described, suggesting a possible relation between both receptors, and that selective blockage of one receptor could increase the effect of the other (32, 42). Although angiotensin II is considered the only effector of the RAS, other peptides, such as angiotensin III (or des[Asp^1] angiotensin II) and angiotensin IV, also demonstrate biological activities (43). Some actions originally attributed to angiotensin II, such as vasopressin release, are attributable to angiotensin III (44). Interestingly, angiotensin III was shown to be several times more effective in the brain than angiotensin II, where it plays an important role (43, 45). On one hand, angiotensin II participates in vasoconstriction, cell proliferation and angiogenesis (14) through AT1 functioning as anti-apoptotic (42, 46, 47); by reducing the synthesis of growth factors, the stimulation of AT1 increases the transcription of these growth-related factors (7). AT1 antagonists block this signal (46, 48), while AT2 has a counter-regulatory effect, functioning as proapoptotic (47). On the other hand, angiotensin III binds mainly to AT2 receptors and exhibits lower affinity for AT1 receptors compared with angiotensin II (47). AT2 receptors are involved in cell growth inhibition and are highly expressed in pathological conditions, whereas AT1 receptors are present in all tissues (47, 49). AT1 antagonists are commonly used for the treatment of hypertension, heart failure and renal diseases (47). It seems that the selective blockage of AT1 may lead to disequilibrium of the AT1/AT2 relation because it may also increase the bioavailability of angiotensin II, producing up-regulation and an overstimulation of the AT2 receptor, which in turn, could produce some beneficial effects, including vasodilatation and antiproliferative/apoptotic responses (30, 42, 47). The clinical precedent to this finding is an apparent low cancer incidence in hypertensive patients receiving ACE inhibitors (50) and a decrease of cell proliferation in C6 malignant glioma after the inhibition of the biochemical pathway of growth factors mediated by angiotensin II with the angiotensin II receptor antagonist Losartan (30). Other studies have demonstrated that similarly to angiotensin II, angiotensin III activated cell growth of prostate cancer, and interestingly, it was inhibited by Olmesartan, another angiotensin II receptor antagonist (35).

Although the participative mechanisms are unclear because gliomas are heterogeneous tissues, we conclude that angiotensin peptides are involved in tumor growth in the rat model of C6 gliomas implanted at a subcutaneous region, and that their role in tumor growth can be analyzed through their corresponding proteolytic regulatory enzymes. The fact that the RAS influences tumor growth and angiogenesis through their regulating aminopeptidases provides interesting pathways to the study of gliomas, proposing the RAS as an attractive therapeutic target.

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