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Altered expression of antithrombotic molecules in human glioma vessels

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Abstract A total of 14 surgical specimens, including 7 glioblastomas, 3 anaplastic astrocytomas, 2 brains adjacent to glioblastoma and 2 grossly normal brains, were investigated immunohistochemically for the expression of antithrombin III (AT-III), heparan sulfate proteoglycan (HSPG) and thrombomodulin (TM) in the endothelium of microvessels. The immunoreaction to AT-III was of moderate intensity in grossly normal brains, brains adjacent to glioblastoma, and anaplastic astrocytomas, but was only weak in glioblastomas, especially in the capillaries. The immunoreaction to HSPG was constantly intense in the microvessels in all the specimens. Although the immunoreaction to TM was negative or only faint in the microvessels in grossly normal brains, it was moderately to strongly intense in anaplastic astrocytomas and brains adjacent to glioblastoma. The intensity of immunoreaction to TM was variable, from faint to strong in the capillaries, and moderate to strong in larger microvessels in glioblastomas. The present study suggested that the alterations in the expression of those antithrombotic molecules could explain, at least in part, the tendencies for intratumoral hemorrhage as well as intravascular thrombosis in the different areas of malignant gliomas.

Key words Antithrombin-III · Heparan sulfate proteoglycan · Thrombomodulin
Vascular endothelium · Malignant glioma

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The normal blood vessels maintain their antithrombotic property on the endothelial surface by several mechanisms [1, 13]. These include the antithrombin-III (AT-III)-heparan sulfate proteoglycan (HSPG) system, thrombomodulin (TM)-protein C system, and production of prostaglandin I (PGI), tissue-type plasminogen activator (t-PA) and protease nexin 1 (PN-1), etc. [6, 13, 18]. They also have thrombotic mechanisms like production of platelet-activating factor (PAF), tissue factor (TF), coagulation factor and plasminogen activator inhibitor-1 (PAI-1) [13]. Under normal conditions the activities of these mechanisms are balanced to prevent spontaneous intravascular thrombosis and bleeding [13]. In malignant gliomas, however, intratumoral microvascular thrombosis or hemorrhage are not infrequently encountered [4, 14, 17]. The structural abnormalities of the tumor blood vessels, like the presence of retiform capillaries, are reportedly associated with the intratumoral hemorrhage [17]. However, the other important factors of the blood vessels, like the antithrombotic and thrombotic activities of the endothelium, have not been studied. To evaluate the alterations in the antithrombotic activity of blood vessels, we studied the expression of three major antithrombotic molecules, AT-III, HSPG and TM, in the vascular endothelium of malignant gliomas. The immunoreaction to factor VIII-related antigen (FVIIIRAg) was used as a marker for vascular endothelium [13].

Materials and methods

A total of 14 surgical specimens, including 7 glioblastomas, 3 anaplastic astrocytomas, 2 brains adjacent to glioblastoma and 2 grossly normal brains, were investigated. Grossly normal brains were obtained at lobectomy in patients with epilepsy. Tissues were frozen in isopropylalcohol, cooled by dried ice, immediately after the removal and stored at –80 °C. Serial cryostat sections of 6-μm thickness were mounted on 3-aminopropryltriethoxysilane-coated glass slides and kept at –80 °C until used for immunohistochem-

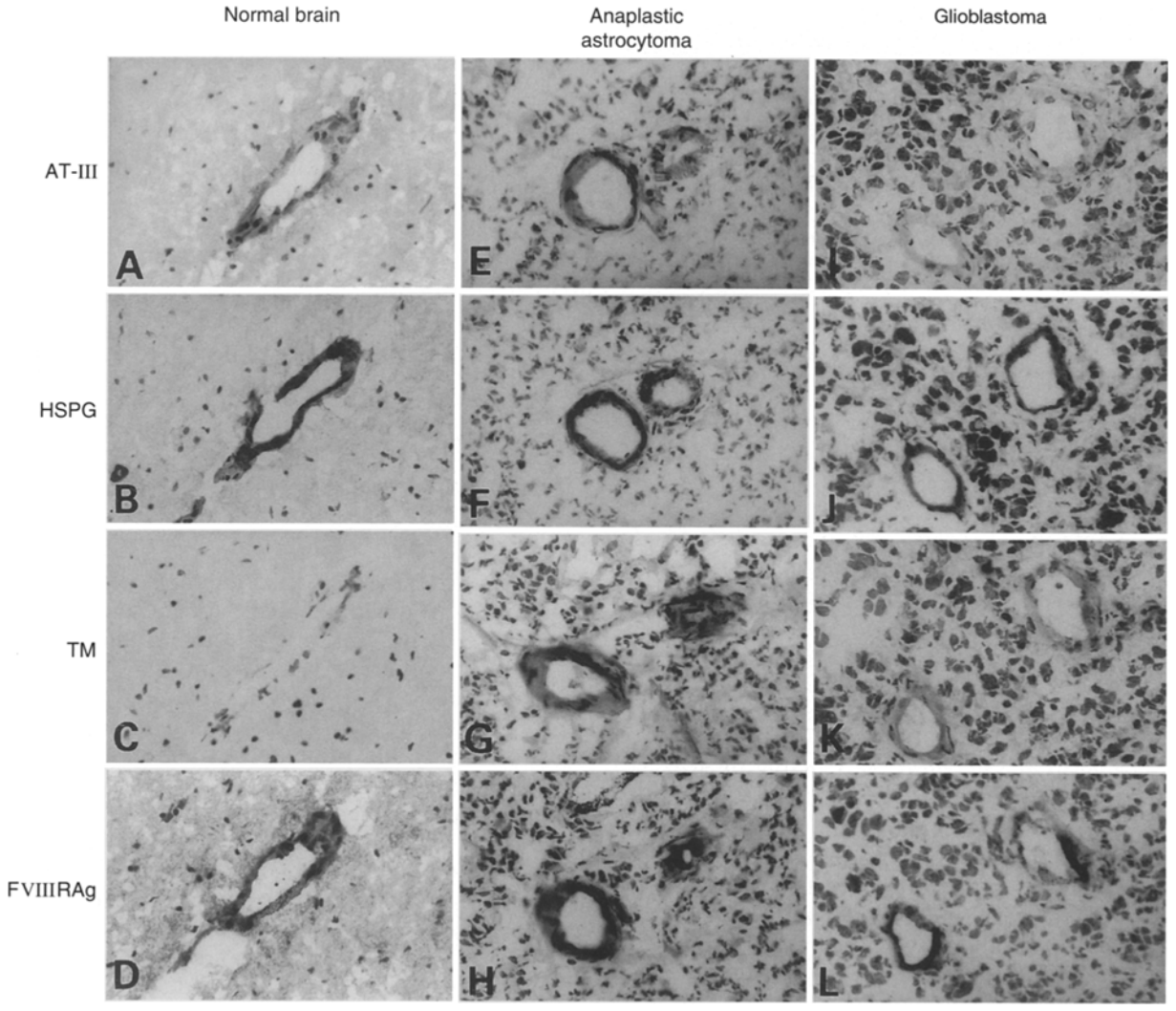

Fig. 1 The immunoreaction to antithrombin (AT-III; **A, E** and **I**), heparan sulfate proteoglycan (HSPG; **B, F** and **J**), thrombomodulin (TM; **C, G** and **K**) and factor VIII-related antigen (FVIIIRAg; **(D, H** and **L)** in successive section of case 1 (normal brain, **A** to **D**), case 3 (anaplastic astrocytoma, **E** to **H**) and case 6 (glioblastoma, **I** to **L**). The immunoreaction to AT-III was of moderate intensity in case 1 **(A)** and case 3 **(E)**, but was decreased in case 6 **(I)**. The immunoreaction to HSPG was strong in all the specimens (**B, F** and **J**). The immunoreaction to TM was faint in case 1 **(C)**, but was increased in case 3 **(G)** and case 6 **(K)**. The immunoreaction to FVIIIRAg was strong in all the specimens **(D, H** and **L)**. Avidin-biotin-peroxidase complex. (ABC) methods × 200

istry. For immunohistochemical study, sections were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 5 min, washed twice in PBS for 5 min each, and incubated for 15 min in TRIS-HCI buffer, pH 7.6, containing 0.3% hydrogen peroxide. After two washes in TRIS-HCI buffer, pH 7.6, for 5 min each, an avidin-biotin-peroxidase complex (ABC) method was employed with Vectastain ABC kit (Vector, Calif., USA). Nonspecific binding of the antibody was blocked by incubation in 1.5% normal horse, goat or rabbit serum in 1% bovine serum albumin containing 0.3% Triton X-100 for 20 min. Sections were then incubated overnight at 4°C with mouse anti-human FVIIIRAg monoclonal antibody (501840-13, Nichirei Corp., Tokyo, Japan), mouse anti-bovine HSPG monoclonal antibody (MAB 458, raised against a core protein of basement membrane of kidney glomeruli, Chemicon Corp., Calif. USA), rabbit anti-human TM polyclonal antibody [12] or goat anti-human AT-III polyclonal antibody (G 15, Biomeda Corp., Calif., USA). The commercially supplied prediluted anti-FVIIIRAg monoclonal antibody was used without further dilution. Anti-HSPG, anti-TM and anti-AT-III antibodies were diluted to make a protein concentration of 1 μg/ml. In control sections, mouse or rabbit IgG, or normal goat serum, was used instead of the primary antibody at the same protein concentration. After incubation with biotinylated secondary antibody for 30 min, the sections were reacted for 30 min with ABC, and were finally reacted with 3,3-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. In each section, blood vessels smaller than about 100 μm in diameter were examined. These blood vessels were tentatively divided into two groups; the capillaries (diameter less than approximately 15 μm) and other larger microvessels.

Results

On control sections, no nonspecific reactions were observed for any antigens. The vascular endothelium was readily identified by the positive immunoreaction to FVIIIRAg. Since the immunoreaction to FVIIIRAg was constantly intense in the microvessels in nearly every specimen (Fig. 1D, H and L), we arbitrarily

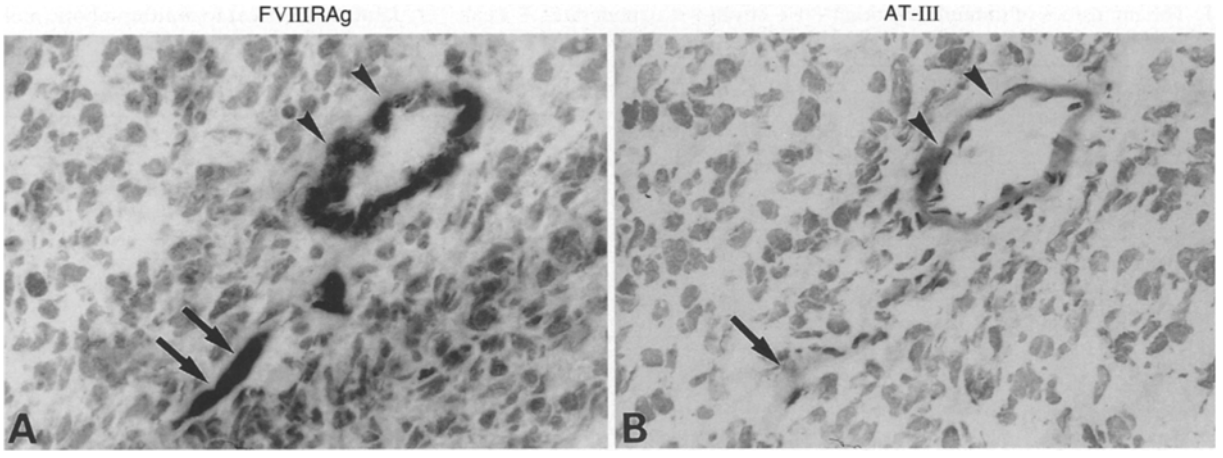

Fig. 2 The immunoreaction to FVIIIRAg **(A)** and to AT-III **(B)** in successive sections of glioblastoma (case 8). The immunoreaction to FVIIIRAg was of strong intensity in larger microvessels (*arrowheads,* **A**) and capillaries (*arrows,* **A**). The immunoreaction to AT-III was faint or negative in the capillaries (*arrow,* **B**), but of only moderate intensity in the larger microvessels (*arrowheads,* **B**). ABC methods × 200

graded this intensity as strong (+++). Other weaker intensities were graded as moderate (++), weak (+), faint (+/–) and negative (–) (Table 1). In grossly normal brains, the immunoreaction of vascular endothelium to AT-III was of moderate intensity (++) (Fig. 1A). This immunoreaction was of similar intensity (++) in anaplastic astrocytomas (Fig. 1E). The immunoreaction was weaker in glioblastomas (Fig. 1I), especially in their capillaries (– to +) (Fig. 2). The immunoreaction of the microvessels in the brains adjacent to glioblastoma was of similar intensity (++) to that of normal brains. The immunoreaction to HSPG was constantly intense (+++) in the microvessels in all the specimens including normal brain, malignant glioma and adjacent brain (Fig. 1B, F and J). Although the immunoreaction to TM was negative (–) or only faint (+/–) in the endothelium of microvessels in normal brains (Fig. 1C), it was increased to moderate (++) to strong (+++) intensity in anaplastic astrocytomas (Fig. 1G). The intensity of immunoreaction to TM was variable, from faint (+/–) to strong (+++) in the endothelium of capillaries, and moderate (++) to strong (+++) in larger microvessels in glioblastomas (Fig. 1K). The microvessels in the adjacent brain demonstrated moderate (++) to strong (+++) immunoreaction.

Although the immunoreactions to FVIIIRAg, AT-III and TM were limited to vascular endothelium, the immunoreaction to HSPG was also noted in the basement membrane beneath the vascular endothelium. The localization of HSPG was especially obvious in the thick basement membrane surrounding the larger microvessels of arteriole size. On smaller vessels of capillary size, the surrounding basement membrane was so thin that the localization of HSPG was not clear. No tumor cells were positive for these antithrombotic molecules in the specimens examined.

Discussion

AT-III, one of the important antithrombotic molecules, is synthesized in the liver and secreted into the circulating plasma [8]. This glycoprotein readily binds to heparan sulfate (HS) of the vascular endothelium (Fig. 3) [2, 18] and dramatically inhibits the activities of thrombin and clotting factor X (FXa) [18]. HSPG is synthesized in the vascular endothelium [18]. HSPG is composed with a core protein and glycosaminoglycan (HS) (Fig. 3) [7], and HS provides a binding site for AT-III [16, 18]. Since AT-III and HSPG have important functions in maintaining the antithrombotic property of the vascular endothelium, we investigated the expression of these two molecules in the endothelial cells of normal and pathological tissues. The immunoreaction to AT-III was not limited on the luminal surface, but diffusely located in the cytoplasm of endothelial cells. This finding is in good accordance with the observation by previous investigators [15]. The plasma AT-III supposedly penetrates into the endothelial cytoplasm relatively freely [2] and binds to the cytoplasmic HSPG. The localization of HSPG was carefully compared with that of FVIIIRAg, which clearly demonstrated that the HSPG is localized not only on the vascular endothelium but also on the basement membrane surrounding the microvessels, especially of arteriole size. Although the immunoreaction to AT-III was of moderate intensity in the vascular endothelium of normal brains, the immunoreaction was decreased in the capillaries in glioblastomas. The immunoreaction to HSPG was, on the other hand, equally intense in the normal brains and in glioblastomas. Since anti-HSPG antibody recognizes the core protein of HSPG and the direct binding site of AT-III is glycosaminoglycan (HS), the decreased AT-III in the presence of constantly intense HSPG immunoreaction may suggest that the amount of HS linked to the core protein is decreased [20], or its affinity for AT-III is decreased in the capillaries of glioblastomas [20]. The conversion of HSPG-bound AT-III to thrombin-AT-III complex is facilitated by the presence of TF, which subsequently leads to the release in AT-III [13]. Thus, altered activity

Table 1 The intensities of immunoreaction (+++ strong; ++ moderate; + weak; +/– faint; – negative) to antithrombotic molecules in vascular endothelium (*C.* capillary, *L.M.V.* larger microvessels)

Case no.	Histology	AT-III		HSPG		TM		FVIIIRg	
		C.	L.M.V.	C.	L.M.V.	C.	L.M.V.	C.	L.M.V.
1.	normal brain	++	++	+++	+++	–~ +/–	+/–	+++	+++
2.	normal brain	++	++	+++	+++	–~ +/–	+/–	+++	+++
3	anaplastic astrocytoma	++	++	+++	+++	++	++	+++	+++
4	anaplastic astrocytoma	++	++	+++	+++	++	++	+++	+++
5	anaplastic astrocytoma	++	++	+++	+++	+++	+++	+++	+++
6	glioblastoma	–~ +/–	+	+++	+++	+/–	++	+++	+++
7	glioblastoma	–~ +/–	+	+++	+++	+/–	++	+++	+++
8	glioblastoma	–~ +/–	++	+++	+++	++	+++	+++	+++
9	glioblastoma	+	++	+++	+++	+/–	++	+++	+++
10	glioblastoma	+	++	+++	+++	++	+++	+++	+++
11	glioblastoma	+	++	+++	+++	+++	+++	+++	+++
12	glioblastoma	+	++	+++	+++	+++	+++	+++	+++
13	adjacent brain	++	++	+++	+++	++	++	+++	+++
14	adjacent brain	++	++	+++	+++	+++	+++	+++	+++

of TF, if present, may also affect the localization of AT-III. TM is a glycoprotein localized on the vascular endothelium, which readily forms a complex with thrombin (Fig. 3) [6]. When a thrombin-TM complex is formed, the activity of protein C is enhanced to more than 1000 times that for thrombin alone (Fig. 3) [5]. Activated protein C, therefore, inactivates clotting factors Va and VIIIa (FVa and FVIIIa) [6]. When the thrombin-TM complex is formed, thrombin-induced activation of platelets and conversion of fibrinogen to fibrin are also inhibited [6]. Although TM is widely distributed among the vascular endothelium of arteries, veins and capillaries in nearly every tissue [19], normal cerebral vessels lack or have only little TM [11, 19, 22]. The present study demonstrated that the endothelial AT-III tended to decrease while TM tended to increase in the microvessels in malignant gliomas. Although both these factors are important in maintaining the antithrombotic activity of the vascular endothelium, the effect of decreased AT-III and increased TM does not seem simple. However, when the expressions of these two factors were evaluated individually in the capillaries and other larger microvessels, we noticed several interesting occurrencies. In several cases of glioblastomas, AT-III was decreased while TM was almost not changed in the capillaries (cases 6, 7 and 9). These capillaries may be more readily thrombosed. In other cases of glioblastomas, AT-III was not changed while TM was increased in the microvessels larger than capillaries (cases 8 to 12). Such microvessels may tend to bleed more easily. Histological evidence of intratumoral hemorrhage or intravascular thrombosis were, however, rarely observed in the limited volume of specimens in the present series of study. The direct correlation between the altered expression of those anti-

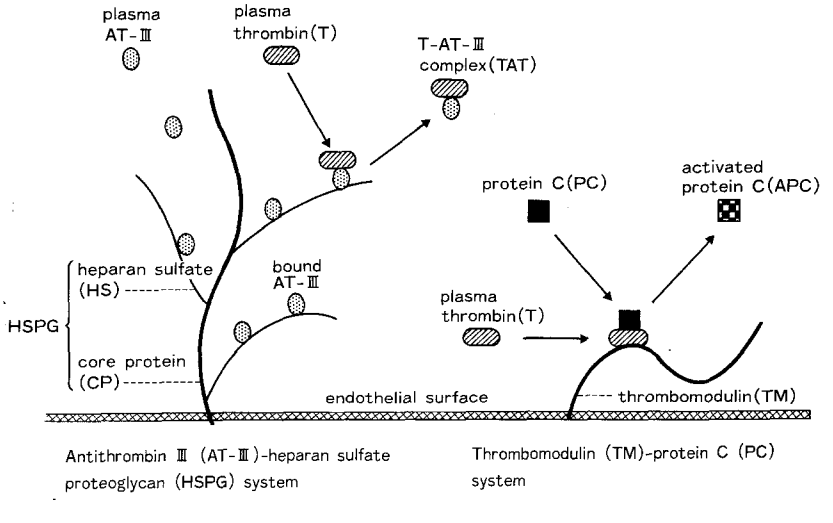

Fig. 3 A schematic illustration of AT-III-HSPG system (*left*) and TM-protein C system (*right*) on the vascular endothelium. HSPG is a complex of a core protein and glycosaminoglycan (HS). When AT-III in the circulating plasma is bound to HS, the thrombotic activity of thombin is significantly inhibited. TM is localized on the endothelial surface which readily forms a complex with thrombin. When thrombin-TM complex is formed, intravascular antithrombotic processes, like activation of protein C, is readily enhanced. AT-III; antithrombin III, HS; heparan sulfate, HSPG; heparan sulfate proteoglycan, TM; thrombomodulin

thrombotic molecules and the actual tendency for hemorrhage or thrombosis was, thus, not evaluated in the present study.

The glial tumor cells were negative for both AT-III and HSPG in the present study. Deschepper et al. [3] have, however, reported that the cultured astroglial cells express AT-III mRNA and AT-III protein. The expression may be different between cultured cells and the original tumor cells. Since they did not describe the nature of antibody they used, a direct comparison of their and our results is, however, difficult. Steck et al. [21] reported the expression of HSPG in glial tumor cells, but we do not. This difference may come from the different nature of antibodies employed. They employed an antibody raised against proteoglycans prepared from the glial cells or tumors. The antibody we employed was raised against the basement membrane of bovine glomeruli. Since a wide variety of constituent core protein and glycosaminoglycan is present in HSPG [9, 10], it seems possible that the two antibodies recognize different species of this macromolecules. Our findings suggest that the vascular endothelial cells in glial tumors, but not glial tumor cells, contain HSPG that shares the same antigenesity of HSPG of the basement membrane of kidney glomeruli.

Although the alterations in the expression of antithrombotic molecules, like AT-III and TM, in malignant gliomas were demonstrated in the present study, many other different factors, like TF, PAI-1, and PN-1, are also involved in the regulation of thrombotic and antithrombotic properties of the vascular endothelium [13]. Overall effects of these factors seem very complex. Further study on the expression of these additional factors may help us to understand more completely the mechanisms for intratumoral hemorrhage and intravascular thrombosis in malignant gliomas.

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