Plasminogen activator inhibitor-1 in the pathogenesis of delayed radiation damage in rat spinal cord in vivo

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The pathophysiology of radiation-induced damage to the central nervous system (CNS) is poorly understood. Preliminary data suggest that fibrinolytic inhibitors are involved in the development of necrosis. In this study, cervical spinal cord irradiation was studied in 90 rats by measuring plasminogen activator inhibitor (PAI)-1 on Days 2, 7, 30, 60, 90, 120, 130, or 145 after irradiation. Paralysis due to radiation necrosis developed in all animals kept alive for 140 to 150 days. Assay of PAI-1 was by Western blot, enzyme-linked immunosorbent assay (ELISA), and complex formation with 125I-labeled urokinase. No PAI-1 was detected in normal spinal cord tissue or in irradiated spinal cord up to Day 90. However, PAI-1 was detected at Day 120 and was marked by elevated ELISA levels at the time of paralysis. Western blot showed detectable PAI-1 (51 kD) at Day 120 and very significant levels at the time of paralysis. Complex formation with 125I-labeled urokinase was also detected at Day 120 with similar results. Immunohistochemical studies showed that PAI-1 was highly concentrated within and immediately adjacent to zones of necrosis at 145 days and was absent in normal tissue. This study adds considerable weight to the proposal that PAI-1 is closely associated with the pathogenesis of CNS radiation necrosis.

KEY WORDS: plasminogen activator inhibitor, fibrinolytic enzymes, radiation necrosis, rat

Radiation necrosis is a serious complication of radiotherapy for brain tumors and other malignancies, but its pathogenesis is still unclear. The initial delayed syndrome is mainly restricted to the white matter and is characterized by demyelination and white matter necrosis, whereas a later syndrome mainly exhibits pathological vascular changes in both white and gray matter. The development of white matter necrosis is attributed to a glial origin, resulting from damage to progenitors of oligodendrocytes and to vascular endothelial cells.

The study of the effect of radiation on the fibrinolytic enzyme system, which maintains blood vessel patency by dissolving blood clots, may provide crucial information on the molecular mechanisms of cerebral radiation necrosis. Endothelial cells play an important role in fibrinolysis. They secrete tissue-type plasminogen activator (tPA), which has high affinity for fibrin and converts inactive plasminogen into its active form, plasmin. Plasmin degrades the insoluble fibrin mesh of thrombi and hemostatic plugs. Endothelial cells also synthesize and secrete plasminogen activator inhibitor (PAI)-1, a strong inhibitor of both tPA and urokinase plasminogen activator (uPA). Plasminogen activator inhibitor, which is also produced by vascular smooth muscle cells and certain types of tumor cells, is secreted in an active form but is inactivated rapidly in solution. Latent PAI-1 can be converted to the active form by exposure to denaturing agents such as sodium dodecyl sulfate (SDS), and it remains stable and active through specific binding to extracellular matrix constituents. It is presumably vitronectin. It has been reported that fibrin-bound PAI-1 is capable of forming SDS-stable, 1:1 molar complexes with tPA and uPA. The balance between tPA and PAI-1 is essential for regulation of intravascular fibrinolysis, but irradiation can change the production and secretion of these molecules.

Rat cervical spinal cord has been used as a model for investigating such changes. When irradiated, rat cervical cord develops white matter necrosis, increased vascularity, and telangiectasia, depending on the radiation dose and the duration of the postirradiation period. In this study, we determined the levels
of PAI-1 in irradiated rat cervical spinal cord in vivo in relation to the development of radiation necrosis. We found that levels of PAI-1 were markedly increased in the latter stages of the radiation effects, immediately preceding the histological recognition of tissue necrosis.

Materials and Methods

Animal Preparation

After proper anesthesia was induced, 90 female Fischer rats were placed in a supine position, fixed between styloforms, and given a 24-Gy radiation dose in a single exposure to the cervical spinal cord (2 cm in length). Ten rats were sacrificed on each of Days 2, 7, 30, 60, 90, 120, and 130 after irradiation. The rats remaining alive after Day 140 developed progressive limb weakness due to necrosis. They were sacrificed when they developed clear paresis. Three or four control rats were also sacrificed at each time point; in total, 30 control animals were used.

Cervical Cord Examination

The entire length of the cervical cord was dissected via laminectomy. The middle portion of the cervical cord (2 mm) was cut and processed into formalin-fixed paraffin-embedded sections; the rest of the sample was kept at -80°C until needed for chemical analysis. At that point the tissue was thawed, weighed, homogenized in Tris buffer (50 mM Tris-HCl at pH 9.5 containing 0.75 mM ethylenediamine tetra-acetic acid, and 75 mM NaCl), and centrifuged at 5000 g for 30 minutes. The pellet was discarded and the supernatant was divided and stored at -80°C.

The protein content of these samples was estimated using Coomassie brilliant blue-G as the color indicator.\(^*\)

Western Blot Analysis

Normal and irradiated rat cervical spinal cord extracts (50 μg) were electrophoresed in 10% SDS-polyacrylamide gel and the proteins were transferred onto nitrocellulose paper according to the method of Towbin, et al.\(^*\) The nitrocellulose paper was incubated in blocking buffer (1.5% bovine serum albumin (BSA), 0.15 M NaCl, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Tris-HCl at pH 7.6) for 2 hours at room temperature and washed with antibody buffer (0.3% BSA, 0.15 M NaCl, 20 mM Tris-HCl at pH 7.6) three times at intervals of 10 minutes. Strips of the paper were then incubated with anti-rat PAI-1 antibody (1:200) for 2 hours at room temperature and washed as described above, then incubated with peroxidase conjugated goat anti-rabbit immunoglobulin (IgG) (1:500) for 2 hours at room temperature.\(^*\) The strips were then washed with antibody buffer and Tris-HCl buffers as described above and incubated with the substrate 2,4-chloronaphthol; the color was developed for 15 to 30 minutes in the dark.

Enzyme-Linked Immunosorbent Assay

In the enzyme-linked immunosorbent assay (ELISA) studies, the normal and irradiated spinal cord extracts or buffer containing standard PAI-1 (as control) were mixed with phosphate buffer and incubated overnight. The wells were washed with phosphate-buffered saline (PBS) three times at 10-minute intervals and incubated with anti-PAI-1 antibody at room temperature for 2 hours. After washing, the wells were incubated with a second antibody, an alkaline phosphatase conjugate; the color was developed with p-nitrophenyl phosphate. The concentrations of PAI-1 antigen in the normal and irradiated cervical spinal cords were estimated by reference to the PAI-1 standard curve.

Radioiodination of Proteins

Purified human uPA was iodinated using a method described previously.\(^*\) Briefly, 100 μg protease and 1 mCi 125I-Na in 0.3 ml of iodination buffer containing 0.7 M NaCl and 0.2 M potassium phosphate at PH 7.0 were incubated for 10 minutes at 37°C in a test tube coated with 45 μg diphenyl glycoluril (Iodogen). The reaction mixture was then passed down a Sephadex G-25 column (a small siliconized Pasteur pipette) that had been equilibrated with iodination buffer just before the separation of the iodinated enzyme from the free 125I-Na. The fractions containing radioiodinated proteins were pooled and dialyzed against 1 liter of iodination buffer for 18 hours at 3°C. After dialysis, 50 μl of the solution was precipitated by trichloroacetic acid to determine the extent of incorporation of 125I. The remainder of the radioiodinated protein preparation was stored frozen at -80°C.

Complex Formation Studies

One mg/ml of tissue homogenate from normal or irradiated rat cervical cord was incubated with 1.7 mM SDS (or buffer only) at 37°C for 30 minutes\(^*\) and dialyzed against PBS containing 0.01% Tween-80 and 0.02% sodium azide at 4°C for 18 hours. The SDS-treated samples (100 μg) were incubated with 125I-labeled uPA in the presence or absence of nonlabeled urokinase at 37°C for 30 minutes. The reaction was stopped by the addition of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer containing β-mercaptoethanol. The samples were boiled at 105°C for 3 minutes and loaded on 10% gels. Labeled 125I-protease-inhibitor complex was detected by SDS-PAGE and analyzed by autoradiography. To further quantify the levels of PAI-1 in these samples, the complex band (78 kD) was excised and counted in a scintillation counter.

Immunohistochemical Studies

Serial histological sections 4 μm thick were cut from normal and irradiated cervical spinal cords from Day 145 and mounted on silane-coated slides. Immunohistochemical analysis was based on the avidin-biotin complex (ABC) immunoperoxidase procedure. Paraffin-embedded sections were deparaffinized, rehydrated, and digested with 0.4% pepsin in 0.1 N HCl for 30 minutes at room temperature. After nonspecific binding of the antibodies was blocked by preincubation with a solution of fat-free skim milk (0.5% in buffer) for 30 minutes at room temperature, sections were incubated overnight at 4°C with primary rabbit anti-rat PAI-1 antibody. Irrelevant rabbit antibody at the same protein concentration was used as a negative control. The sections were incubated with biotinylated goat anti-rabbit IgG for 60 minutes at 37°C and then with 0.3% hydrogen peroxidase in methanol to inhibit endogenous peroxidase activity.\(^*\) After

\(^*\) Anti-rat PAI-1 antibody obtained from American Diagnostica, New York, New York; peroxidase conjugated goat anti-rabbit IgG obtained from Sigma Chemical Co., St. Louis, Missouri.

\(^*\) Alkaline phosphatase conjugate obtained from Sigma Chemical Co., St. Louis, Missouri.

\(^*\) 125I-Na obtained from Amersham Corp., Arlington Heights, Illinois; Iodogen (diphenyl glycoluril) obtained from Pierce Chemical Co., Rockford, Illinois.

\(^\dagger\) Primary rabbit anti-rat PAI-1 antibody obtained from American Diagnostica, New York, New York; biotinylated goat anti-rabbit IgG obtained from Amersham Corp., Arlington Heights, Illinois.

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**Results**

**Western Blot Analysis**

The levels of PAI-1 in normal and irradiated rat cervical spinal cord at different time intervals were first analyzed qualitatively by Western blot. Figure 1 shows that the PAI-1 antibody recognized the 51-kD band in both standard PAI-1 (lane 2) and irradiated spinal cord samples from Days 120, 130, and 145 (lanes 9–11). However, PAI-1 was not detectable in normal or irradiated rat spinal cord samples from Days 2, 7, 30, 60, and 90 after irradiation. Plasminogen activator inhibitor-1 was most prominent in the samples from Day 145, when the animals became paretic (lane 11).

**ELISA Studies**

Quantitative levels of PAI-1 in normal and irradiated rat spinal cord samples were estimated by ELISA using specific rat PAI-1 antibodies. Figure 2 shows no detectable levels of PAI-1 in either normal or irradiated spinal cord at Days 2, 7, 30, 60, and 90, but high levels of PAI-1 in irradiated spinal cord was noted at Days 120, 130, and 145. Significantly higher levels of PAI-1 were present in the paretic animals at Day 145.

**Complex Formation With Labeled Urokinase**

It has been previously reported that PAI-1 forms complexes with uPA and tPA but not thrombin, and that an excess of cold plasminogen activator inhibits complex formation. It was found that sodium dodecyl sulfate activates latent PAI-1 activity but inhibits protease nexin I activity. Figure 3 shows that the PAI-1 complex at the 78-kD band in reduced gels was present only in the irradiated samples at Days 120, 130, and 145 (lanes 7, 9, and 11) and that this complex band was inhibited by an excess of nonlabeled urokinase (lanes 8, 10, and 12). No PAI-1 complex was detected in normal or irradiated spinal cord at Days 2, 7, 30, 60, and 90.

**Immunohistochemical Localization**

To further quantitate the levels of this serpin in these samples, the complex band at 78 kD was excised and analyzed in a scintillation counter. Figure 4 shows that there were no detectable levels of PAI-1 in either normal or irradiated spinal cord at Days 2, 7, 30, 60, and 90, but very high levels of PAI-1 complex were found in irradiated samples from Days 120, 130, and 145. Levels of PAI-1 were significantly increased at Day 145, when the animals were paretic.
like spaces, along rims of remnant stromas in small necrotic foci, or in the periphery of large zones of necrosis. The blood vessels near the necrotic foci tended to exhibit strong PAI-1 reactivity in the vessel walls. No positive reaction was observed in the normal spinal cord. The detailed immunohistochemical localization of PAI-1 in rat spinal cord at different time intervals is the subject of a separate study (Kono, et al., unpublished data).

Discussion

Increased levels of PAI-1 activity have been demonstrated in a number of clinical conditions associated with thrombotic events, including myocardial infarction, coronary heart disease, and deep venous thrombosis. Plasma PAI-1 activity was found to be increased in patients with a recent episode of thrombosis and in a variety of human renal disorders, including the nephrotic and hemolytic uremic syndromes.

Delayed radiation necrosis is a serious complication of radiotherapy for malignant tumors in or around the central nervous system (CNS). Its pathogenesis has not yet been clarified, but its pathology is well characterized by demyelination, coagulative necrosis, and vascular changes. Rat cervical spinal cord has been used to model radiation necrosis and it is now known that the type of lesion is dose- and time-dependent. Two distinct hypotheses have been proposed. The first suggests that white matter necrosis is caused by damage to the glial cells and, more specifically, to the cells responsible for myelination in the CNS, namely the progenitors of oligodendrocytes. The second theory suggests that increased vascular permeability causes perivascular edema, which exerts pressure on the vessels and reduces blood flow. It has also been reported...
that abnormal proliferation of endothelial cells causes narrowing of the vessels.\textsuperscript{27} Radiation effects on the fibrinolytic activity of endothelial cells have been extensively studied in rat lungs,\textsuperscript{28} but no researchers have reported the levels of PAI-1 in irradiated CNS tissue. Our studies demonstrated the presence of PAI-1 approximately 30 days prior to the development of symptoms. Biochemical and immunostaining studies of irradiated rat cervical spinal cord at different time intervals correlated with the presence of PAI-1. The increased levels of PAI-1 in paralyzed animals at Day 145 (Figs. 2 and 5) are strikingly different from normal samples.

It has been reported that levels of several growth factors, including basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-\(\beta\), as well as levels of cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF) are elevated in irradiated endothelial cells and other cell types.\textsuperscript{2,12,39} Levels of PAI-1 in cultured endothelial cells have been reported to increase with the addition of bFGF, TGF-\(\beta\), IL-1, and TNF.\textsuperscript{18,30,42,43} The association between growth factors and cytokines and the regulation of PAI-1 activity is not only connected with cell growth but may also play a role in thrombotic diseases. Increased amounts of PAI-1 in the circulation have been suggested to increase the likelihood of thrombosis formation.\textsuperscript{46} During the thrombotic process, prothrombin generation is involved in the amplification of \(\alpha\)-thrombin, which activates platelets, modifies surface properties of endothelium, and subsequently converts fibrinogen into clottable fibrin. Alpha-thrombin becomes actively incorporated into clots, along with other plasma proteins and various blood cells, during fibrin deposition and thrombus formation. It has been reported\textsuperscript{57} that \(\alpha\)-thrombin is a storage reservoir for thrombin that can subsequently be released. The release of thrombin stimulates endothelial or other cells to secrete plasminogen activators, PAI's, or other substances.\textsuperscript{22} Thrombin directly causes the degranulation of the \(\alpha\)-granules of platelets and releases platelet-derived growth factor (PDGF) and TGF-\(\beta\). The levels of PAI-1 depend upon the release of PDGF and on TGF-\(\beta\)-activated smooth-muscle cells.\textsuperscript{41}

Several investigators\textsuperscript{41,57} have reported that platelets secrete both active and latent forms of PAI-1 and that fibrin stabilization occurs only when a relatively large number of platelets have been activated. Fibrin has both low- and high-affinity binding sites for PAI-1, and only active PAI-1 can bind both sites, whereas the latent form binds only the low-affinity sites and is incapable of protecting fibrin from tPA-mediated dissolution.\textsuperscript{40} The presence of PAI-1 in a blood clot protects it from endogenous fibrinolysis.\textsuperscript{20,36,38} Our results also indicated that blood vessels can be stained with PAI-1 antibody (Fig. 5). Thus, the delayed increase in PAI-1 levels following irradiation may play a role in radiation necrosis by protecting thrombi from premature dissolution and impairing microvascular patency. This study in itself, however, does not demonstrate a direct role for PAI-1 in the generation of radiation necrosis, and additional studies will be required to further analyze this clinically important area of research.

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