Detectable Inhibition of Heparin-Binding Growth Factor Activity in Sera From Patients Treated With Pentosan Polysulfate

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Background: Previous studies indicate that the heparinoid pentosan polysulfate (PPS) can inhibit heparin-binding growth factors (HBGFs) released from tumor cells and thus block tumor growth in animal models. However, because of its heparin-like activity, the major toxic effect expected for PPS is its inhibition of coagulation. Purpose: Our purpose was to determine if anti-HBGF activity could be achieved in patients without causing complications from anticoagulation. Methods: We initiated a phase I trial in cancer patients and developed a cell proliferation assay to detect PPS in human serum based on its anti-growth factor activity. Blood samples from six healthy volunteers were collected in tubes containing different concentrations of PPS (FIBREZYM®; concentration range, 0-10 μg/mL). Additional samples were obtained from four patients in the phase I trial before and after subcutaneous treatment with 15 mg/m² of PPS. The activated partial thromboplastin time (aPTT), which is associated with coagulation, was measured in all blood samples. Serum prepared from the blood samples was heat inactivated and then incubated for 4-5 days with proliferating SW-13 cells, allowing determination of antigrowth factor activity. Results: PPS added to blood samples increased aPTT only at concentrations above 1 μg/mL, whereas HBGF-dependent proliferation was inhibited at less than 0.1 μg/mL. Sera obtained from patients up to 4 hours after PPS treatment specifically inhibited HBGF-dependent cell proliferation by more than 65% even at a 1:10 dilution. At the same time, the aPTT was not altered in these patients, indicating no significant effect on coagulation by this dose of the heparinoid. Conclusions: HBGF-inhibitory concentrations of PPS can be achieved in patients’ sera without significant effects on coagulation. Implication: The assay presented here could be useful to determine doses and scheduling of treatment in studies evaluating PPS as an antitumor agent. [J Natl Cancer Inst 85:1068-1073, 1993]

Tumor cells secrete polypeptide growth factors that may induce autocrine, paracrine, and endocrine effects (1). The autocrine stimulus supports tumor cell proliferation, whereas the paracrine loop allows a growing tumor mass to recruit new blood vessels for its nourishment. This tumor angiogenesis is not only essential for the growth of the local tumor—it also provides the route for distant metastasis of tumor cells (2). Data from different solid tumors (e.g., breast, prostate, or lung) have supported this principle, and the rate of metastasis in patients has been associated directly with the number of capillaries detectable in a given primary tumor (3,4).

This direct role of polypeptide growth factors in local tumor angiogenesis, as well as an indirect role in tumor metastasis, makes these growth factors promising targets for a drug treatment that selectively disrupts the tumor homeostasis (5,6). This approach becomes even more tempting when one considers that in the adult, angiogenesis is absent with a very few well-defined exceptions (e.g., menstrual cycle, placenta growth, or wound healing) (5,7). A number of polypeptide growth factors, in particular endothelial cell growth factors, have marked affinity for the glycosaminoglycan heparin and are commonly known as heparin-binding growth factors (HBGFs) (8). This common feature of

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different growth factors (9) has prompted the use of polysulfated compounds to try to block HBGFs (10). One of the early compounds used was the polysulfated aromatic compound suramin (11). However, because of systemic toxic effects and an extremely long half-life of more than 2 months, this drug is difficult to use (11), and we sought to find a less toxic compound with a similar mechanism of action. Earlier, we reported that the polysulfated sugar pentosan polysulfate (PPS) is very effective in vitro against HBGFs released from tumors and in vivo to inhibit growth in animal tumor models (12-14).

The preclinical data described above prompted a phase I study in cancer patients, and here, we report on a bioassay that we have developed to detect PPS-induced anti-HBGF activity in human serum samples. Furthermore, we show the biological activity of this drug, using sera from some patients in this study. PPS is used as an anticoagulant in Europe because of its heparin-like activity, and the major toxic effect expected for this drug results from its inhibition of coagulation (15). We have, therefore, also studied the effect of PPS on a coagulation parameter (activated partial thromboplastin time [aPTT]) in vitro and show that high concentrations of biologically active circulating anti-HBGF activity can be achieved at doses of PPS that do not substantially affect coagulation. These studies establish a potential therapeutic window for PPS treatment of patients and provide an in vitro assay of drug activity that should prove useful for monitoring in future studies.

Patients and Methods

Healthy Subjects

Fresh blood samples (10 mL) were donated by six different members of our staff. PPS was added at different concentrations (0-10 μg/mL) into the collection tubes immediately after blood was drawn. One aliquot from each sample was used to assay aPTT in our hospital laboratory (Coag-a-Mate X-2, General Diagnostics, Newark, N.J.). Serum was prepared from the rest of the blood samples by centrifugation. After heat treatment to inactivate the serum (55 °C for 1.5 hours), the samples were used in the cell proliferation assays described below.

Patients

From four of the patients participating in a phase 1 clinical trial with PPS, blood samples were drawn before and up to 4 hours after the first subcutaneous injection of PPS (15 mg/mL). These blood samples were assayed for aPTT, and sera were separated, heat treated (see above), and frozen at −20 °C until further use. The investigations involving patients were performed after obtaining informed consent from each patient and after approval by our institutional review board in accordance with guidelines established by the National Institutes of Health.

Cell Proliferation Assays

The SW-13 (human adrenal carcinoma) cell line was obtained from American Type Culture Collection (Rockville, Md.). SW-13 cells were propagated in Improved Minimal Essential Media (IMEM) (Biofluids, Rockville, Md.) with 10% fetal calf serum (FCS; GIBCO BRL, Gaithersburg, Md.). For in vitro studies, SW-13 cells were plated into 24-well plates at 5000-20000 cells per well in 1 mL of medium or in 96-well plates at 500-1000 cells per well in 100 μL of medium (13,14). Different additions to the growth media (i.e., human serum and drugs) are indicated in the respective figures and in the text. Two to six wells were run for each data point. The cultures were allowed to grow in 5% carbon dioxide for 4-5 days at 37 °C. The cells in the 24-well plates were then detached with trypsin-EDTA (GIBCO BRL), and their number was estimated using a Coulter counter. Cells growing in 96-well plates were stained with Mosmann's tetrazolium dye (16) according to the specifications of the manufacturer (MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay, PROMEGA, Madison, Wis.). The number of vital cells in the plates was estimated using an enzyme-linked immunosorbent assay reader at 570 nm with a reference wavelength of 630 nm.

Data Evaluation

To evaluate data, we calculated analysis of variance via the Newman-Keuls Test using the computer program PHARMCALCS version 4.2 (Pharmacologic Calculations Program, PCS, New York, N.Y.). Unless stated otherwise, mean values plus or minus standard deviation of the mean are shown in the graphs.

Drugs

PPS (FIBREZYM) was a gift from Bene-Chemie (Munich, Germany). The HBGF Kaposi's fibroblast growth factor (K-FGF) was prepared as described earlier (17).

Results

Selectivity of PPS in Vitro

In a first series of in vitro experiments, we compared the effect of different concentrations of PPS (concentration response) on coagulation with that on HBGF-dependent cell proliferation (Figs. 1 and 2). For the coagulation studies, each blood sample drawn from six volunteers was divided among seven tubes that contained different concentrations (0-10 μg/mL) of PPS. The coagulation parameter aPTT was measured in these samples, and the respective data are shown in Fig. 1. A concentration-dependent increase of the aPTT is apparent. Above 1.25 μg/mL of PPS, this increase is statistically significant relative to control values (P<.05). The aPTT values achieved during standard heparin therapy are shown for comparison and are reached at PPS concentrations of approximately 2.5 μg/mL (Fig. 1, shaded area).

In parallel with these in vitro coagulation studies, we established the concentration response of PPS on HBGF-dependent cell proliferation. These assays were carried out using proliferating SW-13 cells in standard growth media (IMEM plus 10% FCS) and had no human serum added (13). SW-13 cells were chosen as indicator cells because their proliferation on dish surfaces depends on the presence of endogenously produced HBGFs (in particular, basic fibroblast growth factor) and is highly sensitive to inhibition by polysulfates such as PPS or suramin (13,17,18). In the experiment shown in Fig. 2, even the lowest concentration of PPS (0.1 μg/mL) inhibited cell proliferation by more than 50%. This inhibition was reversed by addition of excess K-FGF (100 ng/mL) (8), confirming the specificity of the inhibitory action of PPS on cell proliferation. After comparing data from these in vitro studies, we concluded that the PPS concentration at which an effect on HBGF-dependent cell proliferation was observed is less than one tenth the concentration that affected coagulation.

Preparation of Human Serum Samples for Use in Cell Proliferation Assays

In the next series of experiments, we established experimental conditions that allowed use of human serum samples in the cell proliferation assay. PPS (25 μg/mL) was added to a subset of blood samples from different volunteers and...
serum was separated, pretreated as above, and used in the SW-13 cell proliferation assays. Fig. 3 shows cell counts from one of these experiments. Obviously, adult human serum that was heat inactivated for 1.5 hours lacks cytotoxic effects that are associated with complement activity present in nonheated serum. The effect of PPS added to the samples is retained. This result is supported by concentration response curves of PPS using blood samples from six different healthy volunteers as a source of serum (Fig. 4). The IC_{50} (i.e., the concentration that causes 50% inhibition of growth) value of about 0.1 μg/mL agrees closely with results from earlier in vitro studies (13,14). Heat inactivation of serum for up to 6 hours did not affect the result (data not shown), and we used a 1.5-hour heat inactivation of serum as our standard protocol.

Results From Patients Treated With PPS

The above protocol was applied to blood samples obtained from patients receiving the first dose of PPS (15 mg/m², injected subcutaneously). Blood samples were drawn before and up to 4 hours after injection of PPS, and cell proliferation in the presence of the respective samples was measured. In addition, aPTT values were recorded. Raw data from one patient are shown in Fig. 5. The SW-13 cell number increased by fourfold in the presence of the serum sample obtained before onset of the PPS treatment (time = 0 hours; Fig. 5). Addition of an excess of PPS (25 μg/mL) to this sample resulted in a number of cells at or slightly below the number of cells originally plated. In contrast to the pretreatment values, cell proliferation in the presence of serum samples obtained after PPS treatment was inhibited by 65%-75% (time = 1, 2, 3, and 4 hours; Fig. 5). Preliminary studies indicate that growth factor-inhibitory activity can be detected for 6-8 hours after a single dose of PPS and is below detection after 12 hours (data not shown).

Data from four different patients and from separate experiments were pooled after subtracting the number of SW-13 cells plated in the respective experiments. As illustrated in Fig. 6, the cell number drops to about 25%-35% of that observed in the control experiment in the presence of serum samples obtained after PPS treatment (open circles). At the same time, the aPTT values were not substantially affected (asterisks; Fig. 6). When excess PPS
Fig. 3. Effect of human serum on SW-13 cell proliferation. Heat inactivation: 5000 cells per well were grown for 5 days in IMEM with 10% FCS (control group) or with additional 10% human serum that had been heat inactivated (HI) or not heat treated (NHT). Absolute cell numbers per well are shown. Diagonal bars: Addition of PPS (25 μg/mL). Cross-hatched bars: Serum samples from one patient 1 hour after PPS administration (15 mg/m², injected subcutaneously).

Fig. 4. Concentration response of PPS in the presence of human serum: 5000-8000 cells per well were grown for 5 days in the presence of heat-inactivated serum from six different volunteers without or with addition of different concentrations of PPS. Data from independent experiments were pooled by subtracting the number of cells plated and setting the control value without PPS as 100%.

was added to the SW-13 cell assay, the number of cells after the 4-5 days of incubation was still close to or below the number of cells originally plated (closed circles; Fig. 6). Addition of excess K-FGF restored cell proliferation to or above control values (closed squares; Fig. 6).

From these results with samples from PPS-treated patients, we concluded that significant amounts of biologically active PPS (or active metabolites of PPS) circulate in serum after treatment with a dose of PPS that did not affect coagulation. The minimal additional effect of exogenous PPS added to the samples from PPS-treated patients and the reversal of this inhibition by K-FGF support the specificity of the assay for HBGFs (14).

Discussion

Assessment of PPS dosage and timing in previous clinical studies has relied on coagulation parameters because of the drug’s heparin-like activity (15,19). From these studies and from studies with radioactive tracers (19), it appears that PPS behaves in a manner pharmacokinetically similar to that of heparin. Like heparin, it shows poor oral absorption, and the drug is eliminated with a half-life of 2-4 hours (heparin’s half-life is 0.5 hours). In our studies, we wished to avoid the anticoagulant dose range of PPS since we were interested in its antigrowth factor activity. This interest was based on preclinical studies from our laboratories showing efficacy of PPS in animal tumor models (13,14), and it prompted a phase I trial of PPS in cancer patients. Our previous animal studies had shown that the antitumor activity in animals occurs at doses lower than those required to affect coagulation; therefore, we attempted to develop a bioassay based on the anti-HBGF activity of PPS.

The reasons for using a bioassay versus a chemical detection method (e.g., high-pressure liquid chromatography) of PPS were twofold: 1) PPS is a semisynthetic compound that consists of a heterogeneous mixture of polysulfates of different molecular mass. The raw material for PPS is extracted from beechwood shavings and is chemically
modified into a highly sulfated polymer (1.8 sulfates per sugar moiety). It has a molecular mass ranging from 1800 to 9000 d, with an average of 6000 d. This varied chemical composition makes a high-pressure liquid chromatography detection method very difficult—if not impossible—and renders the usual pharmacokinetic analysis useless (20, 21). 2) It is unknown which subspecies of the drug (or potential metabolite or metabolites) contribute to its activity. Chemical detection and analysis of pharmacologically significant subspecies of PPS is, hence, extremely difficult if not impossible (20, 22).

In our present study, we used the coagulation parameter aPTT to define the threshold of the anticoagulation activity of PPS. Parallel cell proliferation studies with the same samples or material established the selectivity of PPS as an anti-HBGF drug. Under the conditions of our initial in vitro studies (Figs. 1 and 2), PPS inhibited SW-13 cell proliferation at less than one tenth the concentration at which it affected aPTT. The later studies, with PPS added to blood samples from volunteers or with serum samples from patients that had been previously treated with PPS, yielded similar results.

The major technical "trick" that allowed us to use serum from volunteers or from patients in our cell proliferation studies was to heat inactivate cytotoxic material (mainly complement) present in this adult serum (Fig. 3). Other methods, such as gel filtration or dialysis, failed to generate usable serum preparations (data not shown). Generally speaking, cytotoxic drugs present in heat-inactivated sera might also be detected in similar cell proliferation assays if appropriately sensitive target cells are used.

The most impressive data were obtained with serum samples from patients after PPS treatment (Fig. 6). Even at the dilution of 1:10 used in these assays, more than 50% of SW-13 cell proliferation was inhibited by drug present in these samples. This result fits well with the in vitro data and with the aPTT measurements observed in parallel, and we wish to highlight this point: No substantial elevation of aPTT

Fig. 5. Time response of PPS in patients. Raw data from one patient: Blood samples were drawn before and at different time points after treatment with PPS (15 mg/m², injected subcutaneously). Activated PTT was measured in one aliquot from each of the samples, and serum was prepared from the rest. After heat inactivation of the remaining serum, 5000 SW-13 cells per well were incubated with addition of 10% of this serum for 5 days in the absence (open circles) or presence (closed symbols) of 25 μg/mL additional PPS. The means of absolute cell numbers (with error bars) from quadruplicate dishes are shown.

Fig. 6. Time response of PPS in patients. Pooled data from four patients: Data were obtained as described in Fig. 5. Cell numbers from separate experiments were pooled after subtracting the number of cells plated and by setting the control value before onset of treatment as 100%. Data obtained with patient sera only (open circles), addition of PPS (25 μg/mL; closed circles), and addition of PPS plus Kaposi's fibroblast growth factor (100 ng/mL; closed squares) are shown. *P<.01; **P<.005; ***P<.001.
was detected in these patients (Fig. 6). Based on our in vitro coagulation studies (Fig. 1), the PPS concentration should thus not exceed 2 µg/mL. The respective serum samples were diluted by 1:10 in the proliferation assay and hence the final concentration would be 0.2 µg/mL at maximum. As Figs. 2 and 3 illustrate, a greater than 50% inhibition of cell proliferation is to be expected at this concentration of PPS, and our experimental data in Fig. 6 validate this prediction.

One potential shortcoming in the above line of reasoning could be the appearance of active metabolites of PPS during chronic treatment. In particular, active metabolites with a longer half-life than the parent compound could tilt the balance in favor of anti-HBG or anticoagulant activities and could increase or reduce the selectivity of the drug with respect to these two effects. Preliminary studies with chronic administration of PPS and monitoring of parameters for both effects indicate that active metabolites do not seem to play a significant role. (Zugmaier G, Wellstein A: unpublished data).

PPS has significant growth-inhibitory effects on HBG-dependent cell proliferation at concentrations that do not affect aPTT. This finding is apparent in vitro assays with PPS added to blood samples from healthy volunteers as well as in blood samples obtained from patients treated with PPS. The assay presented here could be useful to determine doses and scheduling of treatment in studies evaluating PPS as an antitumor agent.

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