Selective Thrombosis of Tumor Blood Vessels in Mammary Adenocarcinoma Implants in Rats

Michael K. Samoszuk,* Min-Ying Su,[†]
Ahmad Najafi,[‡] and Orhan Nalcioglu[†]

From the Pathology Department,* the Health Sciences Research Imaging Center,[†] and the Brain Imaging Center,[‡] University of California, Irvine, Irvine, California

Adenocarcinomas in rats and humans frequently contain perivascular, degranulating mast cells that release heparin. Protamine is a low-molecular weight, cationic polypeptide that binds avidly to heparin and neutralizes its anticoagulant properties. We hypothesized that mast-cell heparin functions as a localized anticoagulant that modulates hemostasis and blood perfusion in tumors. Consequently, systemically administered protamine should be able to neutralize the endogenous heparin within tumors, thereby inducing selective thrombosis of blood vessels within tumors. Here we demonstrate with magnetic resonance imaging (MRI) that an intravenous dose of protamine labeled with gadolinium accumulated within the parenchyma of subcutaneous implants of a mammary adenocarcinoma in Fischer 344 rats. Moreover, we show with dynamic contrast enhanced MRI that sequential intravenous doses of protamine in 12 tumor-bearing rats resulted in significantly decreased signal enhancement kinetics (blood perfusion) of the tumor. This functional impairment of MRI signal enhancement was accompanied by histological evidence of thrombosis in the blood vessels within the tumor. There was no histological evidence of thrombosis within normal liver, kidney, lung, spleen, or adjacent muscle of tumor-bearing animals that received protamine treatment or in the tumors of animals that had not been pretreated with protamine. On the basis of these results, we conclude that protamine accumulates within adenocarcinoma implants and induces selective thrombosis of blood vessels within the tumor, probably by neutralizing the endogenous heparin within tumors. (*Am J Pathol* 2001, 159:245–251)

Mast cells are involved in the development of type I hypersensitivity reactions and are widely distributed in tissues. Under normal conditions, they are predominantly located next to blood vessels and nerves and in subepithelial sites. Many types of human and animal carcinomas, however, have been shown to accumulate large numbers of degranulating mast cells at the edges of the

tumor and in the fibrovascular stroma within the tumor mass.^{1–5} Mast cell infiltration is significantly greater within human breast cancers, for example, than in benign breast lesions.^{1,2} The biological significance of the mast cell degranulation within tumors has not been established.

The cytoplasm of mast cells contains membrane-bound granules composed of biologically active mediators such as histamine, tryptase, heparin, leukotriene B₄, and platelet-activating factor.⁵ Tryptase, which accounts for 23% of the total cellular protein in mast cells, is stabilized within mast cell granules by heparin,^{6–8} a highly anionic glycosaminoglycan. On degranulation of the mast cell, tryptase and heparin are both released, along with the other biological mediators, into the extracellular space.⁹ Although tryptase activates single-chain, urinary-type plasminogen activator,⁹ the biological effects of tryptase and heparin on tumor growth remain undefined.

Protamines are low-molecular weight proteins that are rich in arginine and strongly cationic. In the presence of heparin, which is strongly acidic, protamines and heparin rapidly form a stable salt in which the anticoagulant activity of heparin is neutralized.¹⁰ *In vivo*, protamines are rapidly cleared from the circulation, with a plasma half-life estimated to be ~5 minutes.¹⁰ Heparin-protamine complexes are also believed to be metabolized by fibrinolysins.

Because of the extensive degranulation of mast cells with deposition of heparin in adenocarcinomas, we hypothesized that mast cell heparin might function as an anticoagulant that modulates hemostasis and, therefore, blood perfusion in tumors. If this hypothesis were correct, protamine should bind to and neutralize the heparin within mammary adenocarcinomas, thereby inducing thrombosis. Here we present data that indicate that protamine accumulates within mammary adenocarcinomas in rats and induces selective thrombosis of blood vessels within the tumor.

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Address reprint requests to Michael K. Samoszuk, Oncology Center, Quest Diagnostics, Inc., 33608 Ortega Highway, San Juan Capistrano, CA. 92690. E-mail samoszum@questdiagnostics.com.

Materials and Methods

Synthesis of Protamine-Gadolinium (Gd)-Diethylenetriamine Pentaacetic Acid

Protamine sulfate (250 mg) was obtained from Sigma Chemical Co. (St. Louis, MO) and was dissolved in 6 ml of 0.3 mol/L sodium acetate buffer, pH 6.0. The solution was vortexed for 5 minutes and then allowed to sit for 30 minutes before being vortexed again to help dissolve all of the protamine.

After the protamine was completely dissolved, 60 mg of anhydrous DTPA (Sigma Chemical Co.) was added to the protamine solution, vortexed again, and allowed to sit at room temperature for 30 minutes. Gadolinium chloride (50 mg) was then dissolved in 1 ml of 0.3 mol/L citric acid solution, and the pH was adjusted to 11.0. The final product (protamine-Gd-DTPA) was then formed by adding 0.5 ml of the protamine-DTPA solution to 6.5 ml of sodium acetate buffer, and 0.32 ml of the gadolinium chloride in citric acid. The final pH of the solution ranged from 6.5 to 8.0.

Characterization of Protamine-Gd-DTPA

To determine the purity and efficiency of Gd labeling of the protamine, high pressure liquid chromatography (HPLC) analysis was performed using a system from Waters Associates (Milford, Massachusetts), which included two 510 pumps, a U6K injector, a variable length UV detector operating at 210 nm, and a computer that controlled the system and recorded the data. The procedure used tracer doses of radioactive Gd and has been previously described in detail.¹¹ A radioactivity flow detector was also used in conjunction with this system, and the data were tabulated by the computer for accurate analysis of the peaks. A 7.5 × 300 mm Protein-Pak 300 SW column (Waters Associates) was used for HPLC, and acetate buffer (0.01 mol/L) was the isocratic solvent running at 2.0 ml/minute.

The approximate size of the protamine-DTPA conjugate was assessed by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a 4% stacking gel and a 15% protein separating gel on a BioRad Protean II electrophoresis apparatus (BioRad, Richmond, CA). Low-molecular weight markers (BioRad, Richmond, CA) were used as standards to determine the sizes of the conjugate and unreacted protamine. Gels were stained with Coomassie blue R-250 dye after being run at 200 V for 45 minutes.

Molar relaxivity is an index of the ability of Gd ions in the synthesized compound to generate contrast in the images of tumor-bearing rats. The T1 relaxivities (ie, the inverse of the T1 relaxation time) of the compound and the buffer solution were measured, then the molar relaxivity was calculated as their difference divided by the Gd ion concentration of the synthesized compound. Similar measurements were also performed on Gd-DTPA alone.

Animal Studies

The tumor model that we selected for this study used R3230 AC, a rat mammary adenocarcinoma, implanted into the left thighs of Fischer 344 rats. Details regarding the origin, passage, and growth properties of this tumor have been previously described.¹² This tumor was selected for this study because of its histological similarities to human breast (including extensive mast cell infiltration), relatively rapid growth rate *in vivo*, and our extensive previous experience with this tumor using other types of magnetic resonance imaging (MRI) contrast agents.¹²

The imaging studies were performed on a GE Signa 1.5 T scanner. R3230 AC adenocarcinoma was implanted into female Fischer 344 rats subcutaneously. The initial study was intended to demonstrate that protamine could accumulate within the tumor and was conducted on five rats when the tumors reached 2 cm in diameter. The weight of the rats at that time was 190 ± 10 g. The animals were anesthetized with 50 mg/kg ketamine mixed with 5 mg/kg rompun. A 25-gauge butterfly needle was inserted into the tail vein for injection of contrast agents. T2-weighted images were acquired as a localizer, then six slices from liver, kidney, and tumor were selected for dynamic imaging studies. A spin-echo pulse sequence with a TR of 200 ms and a TE of 12 ms was applied to take images continuously before and after injection of contrast agents. Gd-DTPA (0.1 mmol Gd/kg; Magnevist/E, Berlex Lab, Monteville, NJ) was injected first. Three hours later the animals were brought back to the scanner and injected this time with protamine-Gd-DTPA (0.023 mmol Gd/kg). The injection was completed in 1 minute. The dose was equivalent to 2.4 mg protamine per animal, which was well tolerated by these rats. All rats (*n* = 5) survived after this experiment and recovered in good condition.

A region of interest (ROI) was manually outlined to cover the tumor regions excluding the edematous and necrotic areas. The kinetics of Gd-DTPA and protamine-Gd-DTPA were measured from the series of images taken before and after injection of each contrast agent. The kinetics in the liver and kidney were also measured by placing a ROI in the liver parenchyma and a ROI to cover the kidney. The enhancement in the signal intensity measured from each ROI paralleled the concentration of the contrast agent in tissues within that ROI. The baselines for the signal enhancement in all studies were automatically corrected.

Thrombosis Studies

These experiments were designed to study the effects of protamine on blood perfusion and thrombosis within tumors. R3230 AC adenocarcinoma was inoculated subcutaneously into the thigh of 12 rats. The study was performed as described above, except that T2-weighted images were first acquired as a localizer, then six slices from liver, kidney, tumor, and muscle were selected for dynamic studies. Dynamic T1-weighted images were taken with a spin-echo pulse sequence with TR/TE of

Figure 1. Time course of interventions and observations in the thrombosis experiment.

200/12 ms. All curves were automatically baseline-corrected.

The timing and sequence of interventions and observations in this experiment are illustrated in Figure 1. To characterize the baseline blood perfusion of the tumors, Gd-DTPA (0.1 mmol/kg) was first injected into the animals, and the kinetics were acquired for 13 minutes. Thirty minutes after injection of Gd-DTPA, 3 mg of protamine was injected. Two more doses of 2.5 mg of protamine were injected into the rat at intervals of 20 minutes. Twenty minutes after injection of the third dose, another dose of Gd-DTPA (0.1 mmol/kg) was injected, and its kinetics were again measured for 13 minutes. Immediately after the MRI study was completed, the 12 animals were sacrificed and necropsied for histological assessment of the tumor and normal tissues (liver, kidney, spleen, muscle adjacent to tumor, heart, and lungs). For comparative purposes, tumors from three untreated animals were also removed and examined by microscopy as described below.

The tissues from the 12 rats were fixed in formalin and then sectioned at 5-mm intervals. Three or four representative sections of each tumor and a single section of each normal tissue were submitted for paraffin-embedding and routine staining with hematoxylin and eosin. To assess the degree of thrombosis in the tumors and normal tissues, each section was carefully and completely scanned by microscopy at $\times 20$ power by an experienced pathologist without previous knowledge of the treatment status or tumor enhancement kinetics of the tumor-bearing animals. The following scale was used to assess the degree of thrombosis in the tissues: grade 0, no thrombosis evident; grade 1, thrombosis in $<10\%$ of vessels; grade 2, thrombosis in 10 to 50% of blood vessels; and grade 3, thrombosis in $>50\%$ of blood vessels.

In this analysis, we assessed only recent thrombosis and not pre-existing, organized thrombosis. Recent thrombosis was defined as occlusion of small blood vessels by a coalescent mass of red blood cells with indistinguishable cell borders. Pre-existing organized thrombosis was identified by its granular pink appearance (sometimes with laminated lines of Zahn) adjacent to the wall of or completely occluding the lumen of blood vessels that were recognizable by a thin layer of endothelial cells. This distinction between recent and pre-existing thrombosis was necessary to distinguish between an organized thrombus that presumably preceded treat-

Figure 2. From **left to right** and **top to bottom**, the T2-weighted, precontrast 1, 3, 8, 13, 18, and 23 minutes after protamine-Gd-DTPA-enhanced images. Tumor was readily evident in the T2-weighted image but not apparent in the precontrast image. Perfusion of the protamine-Gd-DTPA began in the periphery of the tumor and extended centrally. Contrast material was still evident even at 23 minutes after injection.

ment with protamine and more recent occlusion of blood vessels that might be temporally related to protamine administration.

Results

Synthesis and Characterization of Protamine-Gd-DTPA

The HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies (data not shown) demonstrated that we could achieve stable binding with the ratio of one protamine to three DTPA molecules and to nine Gd ions. Native protamine migrated as a broad smear centered at a molecular weight of ~ 17 kd, thus indicating a somewhat heterogeneous compound. The size of the conjugate protamine (broad smear centered at ~ 21 kd) was slightly larger than the size of native compound, and there were no low molecular weight, breakdown products in the synthetic conjugate.

The molar relaxivity of the synthesized compound was 12.5 (s-1/mmol/L Gd) at room temperature when measured with the 64 mol/L Hz scanner. By comparison, the molar relaxivity of Gd-DTPA measured under the same conditions was ~ 4 s-1/mmol/L Gd. The intrasample variation, intersample variation, and the longitudinal variation were all $<3\%$, which indicated that the synthesis procedure produced consistent and stable products.

Animal Localization Studies

Figure 2 shows the images taken from a slice of a representative tumor in the initial protamine-Gd-DTPA study. The tumor had a high water content; thus, it could be easily differentiated as the bright region at the top of the T2-weighted image. In the precontrast T1-weighted image, the tumor could not be differentiated. After injection of contrast agent, the peripheral region lit up first, and then the contrast agent proceeded to the center of the tumor with the passage of time.

Figure 3A shows the enhancement kinetics of protamine-Gd-DTPA in the tumor slice, liver, and kidney of a single representative animal. The protamine accumulated to a lesser extent in the tumor than in the liver or

Figure 3. Signal enhancement kinetics. In **A**, the signal enhancement kinetics of Gd-DTPA-protamine in tumor, liver, and kidney of a single representative animal are shown. There was sustained retention of the protamine conjugate in the tumor and a rapid clearance from the liver. In **B**, **C**, and **D**, the mean signal enhancement values are shown along with SE bars for 12 rats that were treated as illustrated in Figure 1. In all tissues, protamine treatment caused a decrease in the initial slopes of the curves compared to pretreatment values and a decrease in the peak signal enhancements. Although the liver and muscle quickly recovered to pretreatment levels, there was a sustained and significant decrease in the perfusion of the tumors throughout the period of measurement.

kidney, and the clearance rate of protamine-Gd-DTPA in the tumor was much slower, which indicated some degree of retention of protamine in the tumor. As expected, the compound also induced high signal enhancement in the liver and kidney. In the liver, it showed a bi-exponential decay pattern, and in the kidney it showed a sustained enhancement pattern.

Thrombosis Studies

All 12 animals survived the study and showed similar results. Figure 3, B and D, shows the mean signal enhancement kinetics ($n = 12$, with SE bars) of liver, muscle, and tumor before protamine treatment and after protamine treatment. In all three tissues, there was a delay in the rising phase and a decreased peak enhancement after the treatment with protamine. There was a significant difference ($P < 0.05$) between the pretreatment and posttreatment signal enhancement in the liver for the first 2 minutes after injection. In the skeletal muscle immediately adjacent to the tumor, there was a significant difference ($P < 0.05$) between the pretreatment and posttreat-

ment signal enhancement for the first 4.5 minutes after injection. After 2 and 4.5 minutes, respectively, the post-treatment signal enhancement in liver and muscle was not significantly different from the pretreatment level, indicating that the contrast agent eventually reached the entire extracellular space of these tissues.

By contrast, the delay in signal enhancement in the tumors of the treated rats was much more pronounced than in untreated rats, and the final peak magnitude of enhancement was much lower than the pretreatment level. The mean signal enhancement in the tumors of the treated rats was also significantly lower ($P < 0.05$) than the signal enhancement of the untreated rats throughout the entire experiment.

The percentage reductions of the initial slope and signal enhancement between the pretreated and post-treated animals at 3 and 13 minutes after injection were calculated and are listed in Table 1. At 13 minutes after injection, there was significantly ($P < 0.05$) lower post-treatment signal enhancement only in the tumor and not in the liver or skeletal muscle immediately adjacent to tumor. This finding is consistent with a selective and persistent reduction of blood flow to the tumor compared to normal tissues in protamine-treated rats.

Histological studies of the animals indicated that the 8-mg dose of protamine produced grade 2 or grade 3 recent thrombosis in the tumors of all (100%) of the treated animals (Figure 4; A, B, and C). These recent thrombi were predominantly located in small blood vessels near the edges of the tumor.

It is notable that there was no evidence of necrosis or infarction adjacent to the occluded blood vessels, thereby confirming the recent onset of the vascular occlusion. Recent thrombosis was not observed in any of the tumors of untreated animals (Figure 4D) or in any normal tissues (Figure 4E). The central regions of the tumors in treated and untreated animals frequently contained necrotic regions and evidence of organizing thrombi in blood vessels, suggesting infarction as the tumor outgrew its blood supply.

Discussion

Our imaging studies indicate that intravenously administered protamine accumulated in tumors despite its rapid clearance from the blood by the kidney and liver. Moreover, sequential injections of protamine caused a functional decline in the blood perfusion of tumors that was accompanied by selective thrombosis of small blood ves-

Table 1. Percentage Reduction in Various Parameters Induced by Protamine Treatment

Parameter	Tumor reduction	Liver reduction	Muscle reduction
Initial slope (0 to 1 minute)	75 ± 25% $P < 0.005$	22 ± 21% $P < 0.05$	44 ± 26% $P < 0.01$
3 Minutes enhancement	63 ± 31% $P < 0.01$	ns	22 ± 24% $P < 0.05$
13 Minutes enhancement	46 ± 40% $P < 0.05$	ns	ns

ns, Nonsignificant, mean ± standard deviation ($n = 12$).

Figure 4. Histological studies of thrombosis experiment described in Figure 1. Representative sections of tumors from three rats treated with protamine are shown in **A**, **B**, and **C**. The small blood vessels had lumens that were nearly occluded by a coalescent mass of red blood cells with indistinguishable cell borders. Significantly, there was no evidence of necrosis or apoptosis adjacent to the occluded blood vessels, suggesting that the occlusion was of relatively recent onset. Blood vessels in tumors from animals that were not treated with protamine had open blood vessels with individually identifiable red blood cells in the lumen (**D**). There was no evidence of thrombosis in the blood vessels or sinusoids of any normal tissues from animals treated with protamine (liver shown in **E**). Original magnifications: $\times 60$ (**B–E**); $\times 40$ (**A**). Routine H&E stain.

sels within the tumor. Taken together, these data are consistent with protamine causing thrombosis that inhibited blood perfusion into the interstitium of the tumor. Thus, our findings support the hypothesis that the heparin from mast cells within tumors functions as a localized anticoagulant that maintains blood perfusion into tumors by modulating hemostasis.

It should be noted that protamine injections are known to cause hypotension and bradycardia,¹⁰ systemic alterations that theoretically could have accounted for some of the findings that we observed in the present study. Although we did not measure cardiac output, blood pressure, and tissue perfusion in our experiments, we believe (for the reasons outlined below) that the systemic effects

of protamine on blood pressure, tissue perfusion, and cardiac output alone would not fully explain the differential effects that we observed in tumors. Instead, it is more likely that protamine induced a differential, tumor-specific effect in addition to its systemic effect.

For example, we have previously reported the results of our studies on the effects of various vasomodulators on tumor perfusion.¹³ Those studies clearly showed that angiotensin II (a vasoconstrictor), hydralazine (a vasodilator), and histamine (a permeability modulator) produced only systemic effects on blood perfusion that were nearly identical in normal tissues and tumors. There was never any evidence of a differential, tumor-specific effect that was similar to that produced by protamine.

Moreover, in the current study, the skeletal muscle that was immediately adjacent to the tumors showed a very different response to protamine than did the nearby tumor. A systemic effect of protamine presumably would have acted in a similar manner on both tissues because they probably shared some common blood supply, but this was not observed. Finally, the normal tissues and tumor all demonstrated an initial decline in signal enhancement and slope after the injections with protamine, consistent with a transient systemic effect. The normal tissues, however, quickly recovered to their pretreatment values, and only the tumors failed to recover blood perfusion. This evidence strongly supports a differential, tumor-specific effect of protamine in addition to its systemic effects on blood pressure and perfusion.

Hemostasis itself is a complex physiological mechanism that maintains blood and plasma in a fluid state within blood vessels and interstitial spaces. The coagulation of blood is normally controlled by many cellular components such as tissue factor and platelets and by soluble components such as heparin, fibrinogen, and other clotting factors. In addition, there are vascular bed-specific factors that also influence hemostasis within various tissues.¹⁴

In breast cancer and other cancers but not in benign tissues, for example, fibrinogen has been shown to be abundantly present within the connective tissues.¹⁵ Thrombin-catalyzed, cross-linked fibrin deposition is also a characteristic histopathological finding in many human and experimental tumors.¹⁶ Although the pathogenesis of tumor-associated fibrin deposition is not completely understood, several tumor procoagulants have been described as likely candidates for the generation of thrombin and cross-linked fibrin in the tumor microenvironment. One of these procoagulants is tissue factor, which has been shown to have a distribution similar to cross-linked fibrin in the vascular endothelial cells of human breast cancer.¹⁷

Microvascular hyperpermeability also seems to play a role in the extravasation of plasma proteins such as fibrinogen into the interstitium of tumors.¹⁸ In aggregate, these previously published observations suggest that vascular hyperpermeability in tumors results in fibrinogen extravasation followed by activation of clotting by tissue factor and then deposition of fibrin thrombi.¹⁹ Given the abundance of procoagulant factors within tumors, it is therefore reasonable to propose that endogenous hepa-

rin from mast cells might play an important role in maintaining the fluid state of blood within tumors by acting as a localized anticoagulant. The data from our study support such a role for mast cells in tumors.

It remains unclear if such anticoagulant activity promotes or inhibits the growth of the tumor. Clinical and animal studies have suggested that heparin and other anticoagulants can inhibit the formation of metastatic tumors,²⁰ possibly by inhibiting clot formation that favors growth of micrometastases. Because clotting, fibrinolysis, and angiogenesis are such closely linked phenomena, however, it is likely that hemostasis within tumors will have complex and paradoxical effects on tumor growth and metastasis that will depend on the size of the tumor and other factors such as the presence of mast cells and heparin.

In this regard, it is notable that many previous studies have investigated the role of mast cells in the growth of various tumors in animals and in humans. Roche²¹ demonstrated that mast cells enhance tumor cell proliferation. Subsequent studies demonstrated that mast cell-stabilizing compounds inhibit tumor growth.⁵

Related to their anticoagulant properties, mast cells may also play a role in promoting angiogenesis within tumors. Starkey and colleagues²² showed that mast cell-deficient W/W^v mice exhibited a decreased rate of tumor angiogenesis. This finding was confirmed by Dethlefsen and colleagues²³ who showed that mast cell-deficient mice injected with tumor cells had a decreased number of capillaries at the tumor periphery, reduced tumor size, and absence of metastases. Clinical studies in humans have yielded similar results. For example, there seems to be a direct correlation between the numbers of mast cells and tumor angiogenesis in patients with lung cancer²⁴ and the survival of patients with colorectal cancer.²⁵

Clearly, additional studies are warranted to determine whether protamine can modulate the effects of mast cell heparin on tumor growth, angiogenesis, and metastases. To date, there has been relatively little work in this area. Arrieta and colleagues²⁶ have shown that protamine inhibits the angiogenesis and growth of a C6 rat glioma. Similarly, Trabucchi and colleagues²⁷ have demonstrated that protamine inhibits heparin-mediated angiogenesis in wounded tissue. Ongoing studies in our laboratory are intended to investigate this subject in greater detail.

Finally, our findings are important because heparin-binding factors have been identified in a broad variety of cancers, including bladder cancer,²⁸ hepatocellular carcinoma,²⁹ pancreatic cancer,³⁰ prostate cancer,³¹ and breast cancer.³² Among the heparin-binding factors that have been purified from these cancers are vascular endothelial growth factor, fibroblast growth factor, and pleiotrophin.³³⁻³⁸ All of these heparin binding factors seem to promote angiogenesis to some extent, a property that may account for an earlier observation that heparin derived from mast cells promotes endothelial cell proliferation.³⁹ Thus, the findings in our present report also suggest that protamine should be considered as a possible experimental agent for modulating the effects of heparin on growth factors within a variety of different tumors.

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