ABSTRACT

Radiolabeled guinea pig fibrinogen (GPF) was used to measure fibrinogen influx and fibrin accumulation in line 1 and line 10 hepatocarcinomas growing in the s.c. space of syngeneic strain 2 guinea pigs over the course of 7 days following transplant, an interval of growth uncomplicated by immunological tumor rejection or by significant tumor necrosis. Earlier immunofluorescence studies revealed fibrin deposits in both tumors with line 1 and line 10. In accord with these data, GPF accumulated in both tumors in amounts that matched or exceeded plasma fibrinogen levels. Line 1 tumor GPF content was 4-fold greater than that of line 10 tumors and 11- to 33-fold that of normal s.c. tissue. The composition of tumor fibrinogen-fibrin was investigated by aqueous and urea extraction. The fraction of total accumulated GPF that was urea insoluble, and therefore presumably cross-linked fibrin, was constant over time but strikingly different for line 1 (65%) and line 10 (48%) tumors, as compared with control s.c. tissue (18%). By 7 days, line 1 tumors (mean weight, 0.77 g) contained nearly 2 mg of fibrinogen-fibrin, and line 10 tumors (mean weight, 0.62 g) contained nearly 0.5 mg. Influx of GPF and initial clotting were constant over time and equivalent for the two tumors. Hence, the large differences in GPF accumulation observed between these tumors apparently reflect differences in fibrinolysis, not in fibrinogen influx or coagulation. The data presented indicate substantial traffic of plasma fibrinogen into and out of both tumors, as compared with control tissues, equivalent to nearly 10 and 7 ml of plasma over 7 days of growth for line 1 and line 10 tumors, respectively; comparable values for normal s.c. tissues were 1.0 and 1.4 ml plasma fibrinogen. Even in line 1 tumors with their abundant fibrin gel, only 6.3% of GPF entering tumors over 7 days was retained, as compared with 2% for line 10 tumors and ~1% for control tissue.

INTRODUCTION

An association between coagulation and malignant disease was recognized by Trousseau more than a century ago and has been substantiated since by numerous reports of clinical and laboratory abnormalities of clotting in cancer patients (see Refs. 11 and 25). About 25 years ago, O'Meara and Jackson (22) reported “fibrils” that “occur in the form of a network” deposited on a variety of carcinoma cells. They proposed that these fibrils represented fibrin; but the experimental approach used and the methodologies then generally available were inadequate to make this point convincingly. Subsequent studies by Pressman’s group (6, 16, 17) and others (20, 28), however, showed that both radiolabeled fibrinogen and antibodies directed against fibrinogen were selectively localized to tumor sites. More recently, careful electron microscopic and immunofluorescence studies have demonstrated fibrillar material with the characteristic periodicity of fibrin and with specific reactivity to anti-fibrinogen antibodies in and around a wide variety of animal and human tumors (7-11, 14).

The biological significance of fibrin deposition in tumors has engendered considerable speculation (see Ref. 11) but little hard data. Fibrin and its degradation products may participate in a number of processes critical to tumor growth, survival, and metastasis, including altered microvascular permeability, dampening of the immune response, and angiogenesis. Fibrin deposits analogous to those in tumors are characteristic of wound healing, suggesting that fibrin may have a role in tumor stroma formation and desmoplasia. Also, fibrin gels could serve as a diffusion barrier, limiting tumor antigen egress or the entry of immunoglobulins or other macromolecules.

If fibrin or its degradation products are to exert any of these potentially important effects, it must be shown first that they are present in tumors in amounts compatible with conducting such functions. Unfortunately, virtually nothing is known regarding the quantity or biochemical nature of fibrin deposition in tumors, nor its rate of accumulation and turnover. The present investigation was therefore undertaken to address these matters. We report here quantitative data concerning the influx of circulating fibrinogen and the accumulation of cross-linked fibrin in 2 well-characterized, transplantable hepatocarcinomas, the line 1 and line 10 carcinomas that are syngeneic in inbred Sewall-Wright strain 2 guinea pigs (4, 8, 9, 24). Line 1 and line 10 tumors were particularly attractive for this type of study because they differ significantly in fibrin content as measured both by immunohistochemistry and by ultrastructure (8). Care was taken to confine our study to a period, the first 7 days following transplant, when, according to prior experience, the results obtained would not be complicated by confounding factors such as tumor necrosis or immunological rejection.

MATERIALS AND METHODS

Tumor Cells

Ascites variants of line 1 and line 10 diethylnitrosamine-induced hepatocarcinomas were used in these studies (4, 8, 9, 24). Tumor cells (1 to 3 x 10^6) were passaged in the peritoneal cavities of syngeneic male Sewall-Wright strain 2 inbred guinea pigs at 7- to 10-day intervals and recovered from the peritoneal cavity by injection of 20 ml of Hanks’ balanced salt solution sometimes supplemented with 200 units of heparin (Liquaemin; Organon, Inc., West Orange, NJ). Tumor cells were washed 3 times in Hanks’ balanced salt solution, counted, and viability (≥97%) checked with trypan blue dye. Cells were then diluted to a final concentration of 1.5 x 10^6 cells/ml, and 0.2 ml (3 x 10^6)
cells) were injected s.c. in strain 2 guinea pigs, where they grew as solid tumors (Chart 1). Animals were placed on drinking water supplemented to 1% with saturated KI at least 24 hr before injection of iodinated tracer proteins.

Radioactively Labeled Fibrinogen

Strain 2 GPF was purified from pooled, frozen, PPP by successive adsorption with aluminum hydroxide and precipitation (twice) with 25% saturated ammonium sulfate (3). Clottability was 85% and recovery 87%. GPF was iodinated with either $^{125}$I or $^{131}$I (NEZ-033 or NEZ-035, respectively; New England Nuclear, Boston, MA) by the solid-phase lactoperoxidase-glucose oxidase method (18) to a specific activity of ~0.005 mol iodine/mol fibrinogen. Clottability was 75 to 85% in different iodinated preparations. However, clottability of circulating iodinated fibrinogen became 94 to 97% within 5 min after i.v. injection, as nonclottable iodinated protein was rapidly cleared by the reticuloendothelial system. When $^{125}$I-GPF was electrophoresed on reduced 7.5% sodium dodecyl sulfate-polyacrylamide gels, autoradiographs revealed clear A-, B-, and γ-bands and no other significant radioabeled proteins. Recrystallized human serum albumin (HSA, Miles Laboratories, Elkhart, IN) was iodinated by either the lactoperoxidase method or by the ICI method of Helminkamp et al. (15) to a specific activity of ~0.01 mol iodine/mol albumin and was used as a control tracer. More than 97% of both iodinated proteins were precipitable with 10% trichloroacetic acid. As in other species, iodinated GPF and HSA were cleared from guinea pig blood plasma according to 2 exponential functions (Refs. 2 and 30; Chart 2).

Experimental Design

Flux of radiolabeled GPF into tumors, and accumulation of this tracer in tumors over time, were measured in 3 experiments which differed primarily in the length of time between i.v. injection of iodinated GPF and tumor harvest. In some experiments, iodinated HSA was included as a second tracer for comparison with GPF.

Influx. Strain 2 guinea pigs of either sex weighing 300 to 400 g were given injections of $3 \times 10^6$ tumors s.c. Line 1 tumor cells were implanted at 2 separate sites in one flank and line 10 cells at 2 sites in the other flank. Two, 4, or 7 days later animals were given i.v. injections of $^{125}$I- or $^{131}$I-GPF (5 x 10$^6$ cpm) and tumor and control tissue sites were then harvested 20 min later. Twenty min was chosen because preliminary experiments showed that at this time interval, efflux (loss of extravasated tracers from tumor or control tissues) was insignificant, i.e., at 20 min only influx was measured. At 20 min, animals received i.v. 0.5 ml of an anticoagulant-antifibrinolytic mixture containing 1000 units heparin, 100 units hirudin (Grade IV: Sigma Chemical Co.) or 250 μg diphenylalanyl-L-prolyl-L-arginine chloromethyl ketone (Calbiochem-Behring, San Diego, CA), 25 mg e-aminoacapric acid, and 700 units Trasylol, all in 0.15 M NaCl. Immediately thereafter, animals were anesthetized with ether and exsanguinated; blood samples, which had an infinite clotting time, were centrifuged at 10,000 x g for 20 min to prepare PPP for radioactive counting.

Tumors and roughly equivalent amounts of normal s.c. tissue at a distance from tumors were dissected out and placed in tared tubes in 2 ml ice-cold 0.01 M phosphate buffer, pH 7.5, containing a mixture of proteolytic inhibitors [heparin (10 units/ml), hirudin (2 units/ml) or diphenylalanyl-L-prolyl-L-arginine chloromethyl ketone (5 μg/ml), EDTA (2 mg/ml), 0.1 M e-aminoacapric acid, Trasylol (10 units/ml), 2 mM phenylmethanesulfonfluoride, 2 mM iodoacetate, and 2 mM N-ethylmaleimide]. All experiments with tissue samples were done in this tissue buffer. Wet weights of tumors and control tissues were determined. Radioactivity was measured in a Mark III Tracer gamma spectrometer. The radioactivity measured in the tissue (cpm/g wet tissue) was divided by that in the PPP (cpm/μl plasma) to give a value for influx of radioiodinated GPF as μl of PPP that had entered each g of tumor or control tissue (1, 5). The values so calculated represented the sum of extravasated radioactive tracer plus tracer remaining within blood vessel lumens. Since it was only the former value that was of interest, experiments were performed to determine the amount of tracer remaining inside vessels after animals had been exsanguinated. For this purpose, tumor and control tissue sites were harvested as above on exsanguinated animals but at 5 instead of at 20 min after injection of radioactive GPF. At 5 min, virtually all radioactivity detected was associated with intravascular tracers (27, 29). Therefore, 5-min values (μl/μg at 5 min) served as blanks which were subtracted from the 20-min influx values (μl/μg at 20 min) to give corrected values for total isotope influx over a 15-min interval (μl/μg/15 min). Subtracting a 5-min blank had the additional theoretical virtue of removing from consideration any nonclottable, radioactively labeled tracer GPF that became localized in tumors or control tissues instead of being cleared by the reticuloendothelial system.

In summary, influx of radioiodinated GPF was measured as μl of PPP that had entered each g of tumor or control tissue:

$$\text{Influx}_{\text{t}_{\text{SS}}} = \frac{\text{μl}}{\text{g wet tissue}} = \frac{\text{cpm/g wet tissue}}{\text{cpm/μl PPP}} = \frac{\text{cpm/g wet tissue}}{\text{cpm/μl PPP}} = \frac{\text{cpm/g wet tissue}}{\text{cpm/μl PPP}} = \frac{\text{cpm/g wet tissue}}{\text{cpm/μl PPP}}$$

24-Hr Accumulation. Accumulation of radioiodinated GPF and HSA

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was measured over an 18-hr period in s.c. tumors and control tissues, and the values were normalized to 24 hr. Line 1 and line 10 s.c. tumors were planted in strain 2 guinea pigs, as in the influx experiments. At 1, 3, and 6 days after tumor cell implant, animals were given injections i.v. of 125I-GPF and 131I-HSA (5 x 10^6 cpm, each). Animals were sacrificed 18 hr thereafter, i.e., on days 2, 4, and 7 after tumor cell implant. Anticoagulant-antifibrinolytic buffer was administered and animals were anesthetized, exsanguinated, and tumor and control s.c. tissues were harvested exactly as in the influx experiments in tared tubes containing 2 ml cold tissue buffer. Accumulation of tracers in tumors or normal control s.c. tissues was calculated as for influx measurements by dividing the specific radioactivity of the tissue (cpm/g wet weight) by the radioactivity present in PPP (cpm/μl). Tissue and plasma radioactivity was measured using standard double-isotope counting technology. Five-min GPF and HSA blanks were also subtracted. However, accumulation experiments differed from influx experiments by virtue of the relatively long time interval (18 hr) between tracer injection and termination. Because plasma levels of tracers were falling exponentially throughout this interval (Chart 2), while tumor weight was simultaneously increasing (Chart 1), the tumor weights and plasma radioactivity values measured at the time of sacrifice were not representative of the average values that prevailed throughout the experiment and hence could not be used directly. Using the values of tumor weight and plasma radioactivity measured at the time of tumor harvest, we extrapolated on the appropriate tumor growth (Chart 1) or tracer plasma clearance (Chart 2) curve to obtain the mean values that prevailed over the 18-hr period of experimentation. Use of these corrected values, rather than tumor weight and plasma radioactivity values measured at experiment termination, introduced certain correction factors (−14 to −36% for GPF; −5 to −29% for HSA). Finally, the tracer accumulation values measured over an 18-hr period were multiplied by 1.33 to give 24-hr values.

Accumulation of 125I-GPF over 2, 4, or 7 Days. We wished to determine particularly the actual amount of GPF that accumulated in tumors (or control tissues) over a multiple-day period, starting from the time of tumor transplant. Initially, we attempted to perform these experiments as above, introducing isotope as a single i.v. injection just prior to tumor implant and harvesting tissues at 2, 4, or 7 days later. However, this approach was not successful in that plasma tracer values fell to levels sufficiently low (<20, <10, and <1% of starting values at 2, 4, and 7 days, respectively) that small counting errors and contribution of potential error of >20-fold. To circumvent these problems, we chose an alternate approach, that of maintaining plasma levels of 125I-GPF relatively constant throughout the 2, 4, or 7 days of the experiment. Ideally, this would have been accomplished by continuous i.v. infusion, which was impractical in guinea pigs. An adequate compromise was achieved by giving guinea pigs i.v. injections of suitable amounts of 125I-GPF shortly before tumor implant and at 24-hr intervals thereafter, so that plasma tracer levels were kept within a relatively narrow range throughout the multiple-day period of tumor growth. Plasma radioactivity was monitored by twice-daily ear bleedings, just before and 1 hr following each i.v. injection of 125I-GPF. Plasma tracer levels at the high and low daily extremes were averaged to calculate values that set lower and upper limits, respectively, on 125I-GPF accumulation. Moreover, knowing the pattern of 125I-GPF clearance from the plasma, it was possible to generate mean plasma tracer values for each 24-hr period, just as was done in the 24-hr accumulation experiments. Averaging these 24-hr mean values, we calculated a best estimate of 125I-GPF accumulation at 2, 4, and 7 days of tumor growth.

Estimation of Autologous Fibrinogen and Albumin Influx and Accumulation

Lightly labeled, autologous, or heterologous plasma proteins are known to behave substantially as do their unlabeled autologous counterparts (21, 31). Therefore, amounts of guinea pig fibrinogen and albumin that entered and accumulated in tumors or control tissues could be calculated if plasma levels for these proteins were known and remained constant over the interval of experimentation. To obtain such values (μg/g), tracer influx or accumulation values (μg/g) were multiplied by the plasma concentration (μg/μl) of the corresponding autologous protein. By radial immunodiffusion (19) with rabbit antibodies to GPF and HSA, the following plasma levels were measured in normal strain 2 guinea pigs: fibrinogen, 3.34 ± 0.12 (S.E.) mg/ml; albumin, 26.47 ± 1.45 mg/ml. The plasma levels of these proteins did not change significantly over 7 days in animals bearing line 1 and line 10 solid tumors injected as described.

Extraction of Tumors and Control Tissues

Experiments were also carried out to characterize the radioactive GPF deposited in each tissue sample (5). Tumors and control tissues were promptly minced into fine fragments in 2 ml of cold tissue buffer containing protease inhibitors as described above; they were then extracted in buffer for 18 hr at 4°C. Each tube was centrifuged (1000 x g for 20 min), and the supernatant was decanted. The pellet, composed of tissue fragments, was resuspended vigorously in an additional 2 ml cold tissue buffer and was again centrifuged. The supernatant was aspirated and pooled with the earlier extract for radioactive counting (aqueous extract). Pelleted tissue fragments were suspended and extracted for 2 hr at 37°C in 2 ml freshly prepared 3 μM urea. Finally, tubes were centrifuged again (1000 x g for 20 min), and the urea-soluble GPF supernatant and the urea-insoluble GPF pellet were counted for radioactivity. Total GPF in the tumors should consist of a mixture of fibrinogen, fibrin, and fibrinogen-fibrin degradation products. We anticipated that HSA and fibrinogen, fibrin monomers, and solubilized fibrin degradation products would appear in the aqueous extract, that fibrin polymers that had not been cross-linked by Factor XIII, and perhaps some partly degraded cross-linked fibrin, would appear in the urea-solubilized GPF extract and that cross-linked fibrin as well as some early cross-linked fibrin degradation fragments would remain in the urea-insoluble GPF fraction (32). Indeed, this distribution was observed when fibrinogen and cross-linked or noncross-linked fibrin gels prepared in vitro were subjected to this extraction scheme and the resulting fractions analyzed on reduced sodium dodecyl sulfate-polyacrylamide gels. With even minimal γ chain cross-linking, >70% of cpm were urea insoluble; however, if clotting occurred in the presence of EDTA so as to prevent cross-linking, <3% of cpm were found to be urea insoluble. More than 97% of HSA appeared in the aqueous extract after clotting with or without cross-linking and was similarly distributed in tumor extracts when administered as a radioactive tracer along with iodinated GPF.

RESULTS

Growth Patterns of Line 1 and Line 10 Tumors Planted in the S.C. Space. Line 1 tumors appeared as largely translucent, gelatinous papules comprised of an extensive fibrin gel (~80% of tumor mass) enveloping much smaller, centrally placed, tumor cell clumps (8, 9). Local microvessels were hyperpermeable. Beginning at about 48 hr, vessel sprouts and accompanying fibroblasts began to penetrate the fibrin gel from without. At 5 to 6 days, numerous fibroblasts and new blood vessels had entered the fibrin gel. Thereafter, substantial collagen was laid down, so that by Day 7 the tumors assumed the appearance of scirrhous carcinomas in which tumor cell clumps were scattered in a cellular connective tissue matrix. After Day 8, lymphocytes, basophils, and other inflammatory cells accumulated, leading to tumor rejection by Days 13 to 15.

The growth pattern of line 10 tumors differed significantly from that of line 1. Though inexorably malignant, line 10 tumors grew...
more slowly initially than did line 1 tumors (Chart 1) and were demarcated less clearly from the surrounding host tissue. Line 10 tumors maintained a relatively constant histological pattern at all stages of growth. Permeable blood vessels were evident from the earliest time intervals studied. The fibrin investment was small when compared to that of line 1 tumors, accounting for ~10% of the tumor mass. The angiogenic response was similar to that of line 1 tumors. Line 10 tumors assumed a "medullary" appearance with little tendency to desmoplasia.

Influx of Radiolabeled Tracer GPF into s.c. Line 1 and Line 10 Tumors at 2, 4, and 7 Days after Tumor Transplant. The data in Chart 3 indicate substantially greater influx of both tracers into line 1 and line 10 tumors as compared with equivalent weights of normal s.c. tissues at all time intervals studied. Total GPF influx was 6-fold greater in tumors than in control s.c. tissue. Ratios of urea-insoluble GPF influx in tumors, as compared with control tissue, were even larger, ~15-fold. Two-day line 10 tumors were small and difficult to separate cleanly from surrounding normal tissues, making estimation of actual weight and radioactivity unreliable. Therefore, data from 2-day line 10 tumors are not included.

For line 1 tumors, influx of GPF did not differ significantly when measured at 2, 4, and 7 days. For this reason "grand mean" influx values (μg/g/15 min) were calculated; total GPF, 31 ± 2.5; and urea-insoluble GPF, 14 ± 1.6. These grand means may be expected to express tracer influx more accurately than do the values calculated at the 2-, 4-, or 7-day intervals singly. Influx rates also did not change at different times in the case of normal s.c. tissue, again permitting calculation of grand means for GPF influx into control tissue: total GPF, 5.0 ± 0.4; and urea-insoluble GPF, 1.0 ± 0.1. As noted, influx of tracers into line 10 tumors could not be accurately determined at 2 days but at 4 and 7 days was comparable to the influx rates into line 1 tumors: total GPF, 33 ± 4.2; and urea-insoluble GPF, 15 ± 2.4.

Even in the short time interval of influx experiments, a substantial fraction of the GPF that had extravasated into tumors, but not into control tissue, became urea insoluble as the result of clotting and cross-linking. In the case of line 1 tumors, the percentage of total GPF taking the form of urea-insoluble GPF was 45%. The urea-insoluble fraction in 4- and 7-day line 10 tumors, the percentage of total GPF that was urea insoluble was also 45%. The urea-insoluble fraction in the control tissue was constant throughout the 7-day period and was 20% of total GPF, significantly lower than in the tumors.

Accumulation of Radiolabeled Tracer GPF and HSA in s.c. Line 1 and Line 10 Tumors over 24-Hr Periods Ending at 2, 4, and 7 Days after Tumor Transplant. The data in Chart 4 indicate substantially greater 24-hr accumulations of tracer GPF both in line 1 and line 10 tumors than in control tissue at all time intervals studied. Line 10 accumulation experiments terminating on Day 2 were subject to the same inaccuracy observed in influx experiments and therefore are not included. In line 1 tumors, total GPF accumulation was 15 to 23 times greater than in control s.c. tissues. In the case of line 10 tumors, total GPF accumulation was 9- to 15-fold greater than in control s.c. tissues at the 4- and 7-day intervals. Ratios of urea-insoluble GPF accumulation in tumors, as compared with control tissue, were even larger than those for total GPF, 56- to 92-fold greater than controls in the case of line 1 tumors, and 25- to 27-fold greater in the case of line 10 tumors. HSA accumulation was also elevated 3- to 6-fold in both tumors as compared with control tissue, a significantly smaller increase than for total GPF or urea-insoluble GPF.

Tumors also differed from normal tissues in the percentage of total accumulated GPF that became urea insoluble (Chart 4). At all time intervals studied, the fraction of accumulated GPF in the urea residue of line 1 tumors (65 ± 2%) significantly exceeded that of line 10 tumors (48 ± 2%), which in turn significantly exceeded that of control tissues (18 ± 1%). For comparison, >95% of tracer HSA accumulation in tumors or control tissues appeared in the aqueous extract and <3% was found in the urea-insoluble fraction, as would be expected.

Of interest was a comparison of GPF and HSA as tracers for measuring accumulation of plasma in tumors and control tissues. Because of its smaller size and other properties, HSA enters normal tissue more readily than does GPF (5). As was expected, therefore, we found experimentally (Chart 4) that in normal control s.c. tissue 24-hr accumulations of plasma, as measured by 125I-HSA, substantially exceeded those measured using 129I-GPF as a tracer (p < 0.01). The relationship between tissue plasma accumulation measured by GPF or HSA may be conveniently expressed as a total GPF/HSA ratio, calculated from the data of Chart 4. This ratio takes into account all factors concerned with the traffic of these tracers in tissues; i.e., rates of entry, retention, and efflux. It thus provides an overall comparative measure of tissue handling of these 2 plasma proteins. In the case of normal s.c. tissue, the total GPF/HSA ratio varied within a narrow range at the time intervals studied. The mean value was 0.37 ± 0.03, indicating that plasma accumulation in normal tissue was nearly 3 times greater when measured with tracer HSA than with tracer GPF. By contrast, the total GPF/HSA ratios for lines 1 and 10 tumors were always significantly larger, mean for line 1, 1.39 ± 0.11 and for line 10, 0.93 ± 0.10. Moreover, total GPF/HSA ratios of line 1 tumors always ex-
ceeded those of line 10 tumors (p < 0.01). The dramatic rise in the total GPF/HSA ratio in tumors provides a quantitative measure of the very different handling of both tracers, but particularly of GPF, in tumors as compared with normal tissues. The relative accumulation in tumors of GPF, vis-à-vis HSA, reflects the fact that tumors clot a substantial fraction of extravasated GPF, thereby retaining this tracer for a longer time than do normal tissues.

Accumulation of $^{125}$I-GPF in s.c. Line 1 and Line 10 Tumors over 2, 4, or 7 Days after Transplant. An important goal of our research was to determine the total amount of fibrinogen-fibrin that accumulated in tumors from the time of transplant to the time of tumor harvest 2, 4, or 7 days later. As discussed in “Materials and Methods,” animals in these experiments received an initial i.v. injection of $^{125}$I-GPF (2.5 x $10^6$ cpm) 1 hr prior to tumor implant and smaller amounts (1.5 x $10^6$ cpm) at 24-hr intervals for 1, 3, or 6 days thereafter. The goal was to maintain plasma $^{125}$I-GPF values within a relatively narrow range. This goal was accomplished reasonably well in that blood $^{125}$I-GPF levels were maintained within a 3-fold range (Chart 5) throughout the experiment (as long as 7 days). Knowing the clearance pattern of $^{125}$I-GPF from the plasma (Chart 2A), the hematocrit, and the plasma tracer levels immediately before and after each daily injection, we were able to calculate the mean plasma radioactivity value for each 24-hr interval of the experiment, as was done in the 24-hr accumulation experiments described earlier. Computer-generated mean values, as well as the “high” and “low” values obtained immediately after and before each daily injection, were averaged over the 7-day interval (see legend to Chart 5). The computer-generated mean values were taken to represent the best estimate of plasma $^{125}$I-GPF throughout the experiment. The high and low values were taken as the range about these mean values.

Using the plasma levels of tracer presented in Chart 5, along with tumor or control tissue radioactivity, we determined total GPF accumulation at 2, 4, and 7 days after line 1 or line 10 tumor implant. It can be seen from Chart 6 that total GPF accumulation per g wet tumor weight increased progressively with tumor growth through 7 days. In contrast to the tumors, total GPF accumulation in control tissues showed no significant change with time (grand mean of 24 ± 1 µg/g at 2, 4, and 7 days combined). At all 3 intervals, total GPF accumulation in line 1 tumors exceeded that of line 10 (p < 0.01), and total GPF accumulation in either tumor exceeded that in control tissues (p < 0.01). Following aqueous and urea extraction, similar but even larger differences were observed between urea-insoluble fractions of tumors and control tissues (Chart 7).

From these data and knowledge of the plasma fibrinogen concentration, it was possible to estimate both the amounts of total fibrinogen-fibrin as well as the amounts of cross-linked fibrin present in line 1 and line 10 tumors at each interval after tumor transplant. For example, at 7 days the total GPF content of line 1 tumors (wet weight, 0.77 g) was 2.02 mg and that of line 10 (wet weight, 0.62 g) was 0.45 mg. Expressed as mg of fibrinogen-fibrin per g wet tissue, the total GPF content of line 1 tumors was therefore equivalent to 80% of that of the fibrinogen levels of normal plasma; for line 10 tumors, total GPF content was 22% of plasma fibrinogen levels. Impressive though these...
values are, they actually underrepresent tumor GPF content. Tumors are comprised of cellular and extracellular compartments, and GPF is confined to the latter. The extracellular space of several rat tumors has been estimated to represent 30 to 60% of total tumor mass (13). Morphometric analysis (8) has demonstrated that tumor cells occupy ~90% of line 10 tumor mass and only ~20% of line 1 tumor mass, the remaining interstitial space consisting of connective tissue, benign cells such as fibroblasts, and blood vessels. Correcting our measured values for actual tumor cell mass, the interstitial GPF content of both line 1 and line 10 tumors matches or exceeds that of fibrinogen levels in normal plasma, the exact value depending on assumptions concerning the fraction of the interstitial space that is extracellular.

Combining the multiday accumulation data with the influx and 24-hr accumulation data presented earlier, and utilizing our knowledge of tumor weights at various stages of growth, it was possible also to estimate the amounts of GPF that enter, leave, and accumulate in line 1 and line 10 tumors over 7 days, compared with control tissue. We estimate that GPF from 9.8 ml of plasma entered individual line 1 tumors over the course of 7 days after transplant, but of that amount, only about 600 μl or 6.3% were retained. Somewhat less (6.8 ml) plasma GPF entered the smaller line 10 tumors over the same time interval and a smaller fraction (2.0%) was retained. Comparable influx values of GPF into control tissues were 1.4 and 1.0 ml of plasma, and in both instances, the retained fraction was only 1.3 to 1.4%.

DISCUSSION

The data presented here provide a quantitative analysis of fibrinogen influx and fibrin accumulation and turnover in line 1 and line 10 carcinomas. Substantial fibrinogen influx and fibrin accumulation were found in both tumors studied, as compared with normal control s.c. tissue. Moreover, fibrin accumulation was selectively greater than that of another plasma protein tracer, HSA, presumably because, once rendered insoluble by clotting, fibrin was cleared more slowly from tumors than were soluble proteins. Our data also provide independent support for earlier immunocytological and ultrastructural evaluations of fibrin deposition in and about human and animal tumors (7–10, 15). Overall accumulation of total GPF per g in line 1 tumors exceeded that of line 10 tumors by a wide and fairly constant (~4-fold) margin (Chart 6) over the 7-day interval of tumor growth studied here. Accumulation of total GPF in line 10 tumors, in turn, exceeded that in control s.c. tissue by a substantial margin (5-fold at 4 days, 9-fold at 7 days). Measured differences in accumulation of urea-insoluble GPF between line 1 and line 10 tumors, between line 1 tumors and control tissue, and between line 10 tumors and control tissue, were even larger: 4- to 6-fold, 48- to 117-fold, and 16- to 28-fold greater, respectively. Similar fibrinogen influx and fibrin accumulation have now been found by quantitative methods in spontaneous lung metastases arising from Lewis lung carcinomas and in mouse breast carcinomas arising from hyperplastic alveolar nodules, indicating that fibrin accumulation is a characteristic feature of tumor growth. Furthermore, this accumulation may be substantial as in the case of line 1 and line 10 carcinomas, so that the fibrinogen-fibrin content of the tumor interstitial space comes to approximate or exceed that of normal plasma.

We were impressed by the extensive traffic of plasma fibrinogen that passes into and through both line 1 and line 10 tumors over a relatively short period of time. From transplant of 3 × 10⁶ tumor cells to growth to a mean size of 0.77 g (line 1) or 0.62 g (line 10) 7 days later, fibrinogen equivalent to that contained in nearly 10 and 7 ml of plasma entered line 1 and line 10 tumors, respectively. Only a small amount of the fibrinogen entering these tumors, however, was retained (6.3% for line 1, 2.0% for line 10), the rest passing through the tumor and back into the plasma, presumably in degraded form. Given mean influx rates for total GPF of 31 and 33 μl PPP/g/15 min for line 1 and line 10 tumors, respectively, the corresponding efflux rates are therefore calculated to be 29 and 32 μl PPP/g/15 min. This corresponds to a turnover rate of approximately 0.4 mg/hr/g of tumor. By contrast, the traffic of plasma fibrinogen into equivalent weights of normal s.c. connective tissue over 7 days was much less (1.0 to 1.4 ml), and only about 1% of the GPF entering was retained.

Also of interest was the progression, at least through 7 days, of fibrin accumulation per g in line 10, and particularly in line 1, tumors; by contrast, no such progression was noted in control tissue (Chart 6). Obviously, an indefinite extension of this progression would lead to impossibly high fibrinogen-fibrin concentrations in tumors; however, such a progression is not apt to continue much beyond 7 days for either tumor. As noted earlier, experimental conditions were chosen to avoid 2 potential complications that could confound or possibly invalidate our results, namely, immunological tumor rejection and tumor necrosis. After 7 days, both tumors exhibited irregular foci of necrosis as tumor growth outstripped blood supply; in addition, line 1 tumors underwent immunological rejection (8). It is therefore possible that the levels of fibrin accumulation measured in 7-day line 1 and line 10 tumors represent near maximal concentrations for these tumors.

Finally, as described in detail elsewhere (11), fibrin accumulation in tumors results from enhanced local permeability of tumor-related blood vessels, leading to extravasation of plasma proteins including fibrinogen, followed by extravascular clotting and cross-linking of extravasated fibrinogen. Senger et al. (26) have purified a tumor-secreted protein (M, ~38,000) active in the range that is apparently responsible for the increased vascular permeability found in a wide variety of solid and ascites tumors of several species, including humans. In addition, a number of authors have described tumor-associated or tumor-shed proagulant activities that are capable of clotting locally extravasated fibrinogen (see Refs. 11, 12, and 25). The present studies provide a quantitative framework as a beginning to dissecting the pathogenesis of tumor fibrin deposition. Requiring explanation, e.g., are not only the substantial fibrin accumulation in tumors but also the large differences in such accumulation observed between tumors such as line 1 and line 10. Influx studies provide a measure of local vascular permeability and indicate a constant and virtually equivalent entry of total GPF into line 1 and line 10 tumors (Chart 3). It would therefore appear that differences in vascular permeability cannot explain the striking differences in fibrin deposition observed between these tumors in both 24-hr (Chart 4) and in multiday (Chart 6) accumulation studies. Such differences also cannot be attributed to differences in initial coagulation of extravasated fibrinogen. The fraction of GPF entering line 1 and line 10 tumors that became urea insoluble within 15 min was identical, 45%. Hence, by a process of

4 H. F. Dvorak, unpublished data.
1 I. Goldberg and H. F. Dvorak, unpublished data.
8 B. Asch and H. F. Dvorak, unpublished data.
elimination, differences in fibrinolysis emerge as the most probable explanation for the differences in fibrin accumulation observed; added support for this possibility comes from in vitro studies indicating that viable line 10 cells express (and secrete) substantially more plasminogen activator than do their line 1 counterparts (10, 23).4

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Quantitation of Fibrinogen Influx and Fibrin Deposition and Turnover in Line 1 and Line 10 Guinea Pig Carcinomas

Harold F. Dvorak, V. Susan Harvey and Jan McDonagh

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