Promotion of metastasis by a specific complex of coagulation factors may be independent of fibrin formation

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Summary Coumarins inhibit metastasis in a number of animal models, but the mechanism of this effect remains unclear. We have investigated the relationship between the coagulation system and metastasis using a new model system, involving i.v. injection of Mtln3 rat mammary carcinoma cells into Fischer 344 rats, and subsequent estimation of pulmonary seeding.

Injection of factors II, VII, IX and X elevated the median number of surface pulmonary seedings per animal to 182, and injection of factors II, IX and X to 181, compared with a median for control animals of 12 (P<0.001). Injection of factor VII alone, or of bovine serum albumin did not significantly affect pulmonary seeding. In a second experiment, arvin defibrination reduced the mean plasma fibrinogen concentration to 76.8 mg dl⁻¹ from a control value of 228 mg dl⁻¹. This degree of defibrination had no significant effects on pulmonary seeding, nor on the enhancing effects of factor complex injection (median numbers of seedings per animal; control 15, arvin 21, arvin plus factors II, VII, IX and X 170, factors II, VII, IX and X only, 157). Factor complex injections did not detectably shorten thrombotest clotting times. In vitro testing suggested that Mtln3 cells contain little or no conventional factor X activating cancer procoagulant.

The complex of coagulation factors II, IX and X appears to contain a component which greatly enhances metastasis in this model. This may explain the previously reported antimitastatic effect of coumarin anticoagulants, which suppress factors II, VII, IX and X. The enhancing effect of the factor complex does not appear to be altered by significant reductions in fibrin forming capacity, and defibrination itself has no effect on metastasis. These findings suggest the possibility that the effect of this factor complex on metastasis may be mediated via mechanisms other than the formation of a fibrin clot.

There is extensive evidence from both clinical and experimental studies for an interaction between the coagulation system and the spread and growth of malignant disease (Wood, 1958; O'Meara, 1968; Hilgard et al., 1977; Zacharski et al., 1979; Dvorak et al., 1981). Patient studies have demonstrated the existence of marked subclinical disturbances of the coagulation system in nearly all cancer patients (Sun et al., 1979; Rickles & Edwards, 1983; Mannucci et al., 1985), whilst animal experiments have suggested that the coagulation system may play an important role in the pathogenesis of blood borne metastasis (Kolke, 1964; Agostino et al., 1966; Brown, 1973; Wood, 1974; Poggi et al., 1978). The most striking and consistent finding in such animal experimentation has been the antimitastatic effect of the coumarin group of anticoagulant drugs in a variety of tumour/host combinations (Ryan et al., 1969; Hilgard & Maat, 1979; Williamson et al., 1980). The coumarins mediate their anticoagulant activity by antagonising the action of vitamin K, an essential cofactor in the hepatic synthesis of the coagulation factors II (prothrombin), VII, IX and X (Stenflo & Sutie, 1977). We have demonstrated that warfarin, a member of the coumarin group, inhibits metastasis in a model system comprising the Mtln3 rat mammary carcinoma clone and the syngeneic Fischer 344 rat, both when intravenous injection of the cells is employed and in the more realistic model involving spontaneous metastasis (McCulloch & George, 1987). In addition, we showed that warfarin has no important cytotoxic effects for these tumour cells, that it inhibits metastasis principally by its effects on the host animal, and that the inhibition of metastasis is reversed by replenishment of the coagulation factors which warfarin suppresses. The exact role of these factors in the metastatic process therefore merited further study. The present studies were designed to determine whether the previously studied factor complex, or parts of it, could enhance metastasis in normal rats, and if so, whether this enhancing effect was dependent on a normal capacity to form fibrin.

Animals and methods

Animals

Female Fischer 344 rats (Olac Limited, Bicester, UK), 6–8 weeks old, mean weight 140 g, were used in all experiments. Animals were fed a standard laboratory diet (CRM diet, Labsure, Cambridge, UK) and tap water with a chlorine content of 7 mg l⁻¹. All animals were healthy according to visual observations, and to the results of routine microbiological testing for infection.

Tumour cells

The tumour cells were a clone of rat mammary carcinoma designated Mtln3, originally derived by Neri and Nicolson (Neri et al., 1982) from the 7,12-dimethylbenz(a)anthracene-induced adenocarcinoma 13762 (Segaloff, 1966). Cells were cultured in 75 cm² tissue culture flasks (Gibco, Paisley, UK) in equal parts of Hams' F10 and Dulbecco's modified Eagles' Medium (F10/DMEM), with 10% foetal calf serum (FCS) but without antibiotics. Cultures were maintained at 37°C in equilibrium with 2% CO₂ in air. Subconfluent cultures were passaged by the use of Ca²⁺ and Mg²⁺ free PBS followed by 0.25% Trypsin (Gibco, Paisley, UK). Subculture was performed by adding 3 × 10⁶ viable cells to further 75 cm² flasks. Cells were passaged a maximum of six times between thawing and use, to minimise problems of phenotypic drift (Neri & Nicolson, 1981). Multiple subcultures of the cell line were stored in liquid nitrogen at −196°C, and fresh cultures were begun from these as required. Inocula of 10⁶ cells from stock cultures injected into the mammary fat pad of Fischer rats at the beginning and at the end of this series of experiments showed no change in the metastatic potential of the line.

Coagulation factor preparations

A heat treated concentrate of human coagulation factors II, IX and X, prepared from pooled plasma by cryoprecipitation and supernatant adsorption with DEAE cellulose, was obtained from Dr R.J. Perry of the Protein Fractionation Centre, Edinburgh, UK. A heat treated concentrate of
human factor VII, prepared by a similar procedure using DEAE sepharose, was obtained from Dr J.K. Smith of the Protein Fractionation Centre, Churchill Hospital, Oxford, UK. The factors were administered according to a regimen which had been shown in previous experiments to reconstitute coagulopathy in the fully warfarinised rat for ~12 h (McCulloch & George, 1987). Each rat was given a dose representing 6 units of factors II and X, 7 units of factor IX and 10 units of factor VII at the time of tumour cell injection, and the dose was repeated after 6 h. The fluid volume of the factor injections totalled 0.6 ml. One unit is sufficient to restore 1 ml of completely depleted human plasma to normal activity for the factor concerned.

**Experimental model of metastasis**

The model of metastasis used involved intravenous injection of Mtln3 tumour cells into F344 rats, with subsequent sacrifice and examination of the lungs for tumour seeding. Mtln3 cells were prepared from subconfluent cultures as described above, then washed twice by centrifugation for 5 min at 200 g in F10/DMEM with FCS, and a third time in F10/DMEM alone. After resuspension in F10/DMEM, cell density was assessed using a Coulter model ZB cell counter, and viability by Trypan Blue exclusion. All cultures used were >90% viable. An injection of 0.2 ml of this cell suspension was made into the lateral tail vein of F344 rats under light ether anaesthesia. Rats were maintained on normal diet and water for 17 days, then killed by cervical dislocation. Full autopsy was performed on all animals, and any tissue suspected of containing tumour deposits was submitted to histological examination. The lungs were prepared for assessment of pulmonary seeding by the method of Wexler (1966). This entails inflation of the lungs via the trachea with a 15% solution of India ink, followed by fixation in Fekete's solution for at least 48 h. Surface pulmonary tumour nodules can then be identified and counted accurately. All specimens were counted on two occasions by a single observer, who was unaware of the treatment given.

**Experiment 1: Enhancement of metastasis by coagulation factors**

Five groups of 10 F344 female rats, 6–8 weeks old, were used. All animals were injected intravenously with 10⁵ Mtln3 cells as described above. At the same time, additional treatments were begun as follows:

**Group A:** These control animals received no form of treatment other than tumour cell injection.

**Group B:** These animals received two i.v. injections of a complex of the coagulation factors II, VII, IX and X, as described above.

**Group C:** These animals received two i.v. injections of factor VII alone.

**Group D:** These animals received two i.v. injections of the complex of factors II, IX and X, but not factor VII.

**Group E:** These animals received two i.v. injections of bovine serum albumin (Sigma, Poole, UK).

No animal received treatment with warfarin or any other anticoagulant. In all groups receiving some form of treatment (Groups B–E) the first intravenous injection was given at the same time as the tumour cells, and the second 6 h later. The dose of bovine serum albumin was used as 30 mg in 0.6 ml of F10/DMEM; this gave the same protein concentration as the factor complex injection in Group B rats. Bovine serum albumin and factor complex preparations were passed through a 0.2 μm filter before injection, for sterilisation and removal of any potentially embolic material. Comparison of the numbers of seedlings per set of lungs in the different groups was performed by Mann–Whitney U test.

**Experiment 2: Role of fibrin formation in enhancement of metastasis**

Four groups of 10 Fischer 344 female rats, 6–8 weeks old, were used. All animals were injected i.v. with 10⁴ Mtln3 cells as described above. At the same time, additional treatments were begun as follows:

**Group A:** These control animals received no form of treatment other than tumour cell injection.

**Group B:** These animals received two i.v. injections of a complex of the factors II, VII, IX and X.

**Group C:** These animals received factor complex injections in the same way as group B animals, but received additional treatment before and at the time of tumour cell injection with arvin.

**Group D:** These animals received treatment with arvin according to the same protocol as group C animals, but did not receive any factor complex injections.

Factor complex injections were given as in experiment 1. Arvin was given i.v. in a dose of 150 units kg⁻¹, 6 h before, and s.c. in the same dose 6 h after injection of tumour cells. The arvin solution was filtered in the same way as the factor complex injections. Sacrifice, autopsy and estimation of pulmonary metastasis was performed exactly as in experiment 1.

**Monitoring of coagulation system activity**

The effects of the various treatments on coagulation were monitored by performing thrombostest (Nyegaard, Oslo, Norway) estimations on 3 animals per group immediately after the first injection. Tail vein blood (50 μl) was used for the assay. In experiment 2 the effects of arvin treatment were monitored by measurement of plasma fibrinogen concentration in samples of tail vein blood at the time of tumour cell injection and again 12 h later. Fibrinogen was measured by the method of Claus (1957), on 0.3 ml plasma samples. Thrombostest and fibrinogen values in different groups were compared where appropriate using the t test.

**Analysis of procoagulant activity of tumour cells**

Mtln3 cells were trypanised and washed in F10/DMEM as described above, resuspended in F10/DMEM (without FCS), counted and adjusted to a cell density of 8 x 10⁶ ml. This suspension (0.1 ml) was added to 0.1 ml of citrated plasma and 0.1 ml of CaCl₂ (0.25 M) at 37°C and the clotting time of the mixture recorded electronically using a Dade fibrinometer. The procedure was carried out in triplicate with normal human plasma and with bovine plasma deficient in factor VII (factor VII DBP) and in both factors VII and X (factor VII and XDBP). Mtln3 cells were compared with two non-neoplastic cell lines. 3T3 (untransformed mouse fibroblast) cells and MDCK (neonatal dog kidney) cells, and with Mtln3 cells exposed for 72 h in vitro to a 10 μM concentration of warfarin.

**Results**

**Experiment 1: Enhancement of metastasis by coagulation factors**

The median number of pulmonary seedlings and semi-interquartile range are recorded for each group in Table I. In the control group (group A), the median number of seedlings per animal was 13, and this was not significantly affected by treatment with factor VII alone (group C, median 11) or with bovine serum albumin (group E, median 23). Treatment with the entire factor complex, or with the component comprising factors II, IX and X produced many more seedlings; the median was 182 in group B and 181 in group D. These results were both very significantly greater than
clotting time in the presence of Mtn3 cells was significantly prolonged in factor VII DPB compared with normal plasma, whilst no clotting occurred in factors VII and X DPB. Similar results were recorded for MDCK and 3T3 cells. Pre-incubation of Mtn3 cells in 10 \mu M warfarin did not affect their ability to promote coagulation of normal or factor deficient plasmas (Table III).

Activities are expressed as a percentage of the clotting time obtained using pooled normal human plasma. Results represent the mean of triplicate assays performed on at least two occasions. Within assay variation was <10%.

**Discussion**

The experiments described in this paper demonstrate contrasting effects on metastasis of two different manipulations of the coagulation system. In order to interpret the results correctly, it is important to understand clearly the effect on coagulation of the treatments used.

The injection of an excess of coagulation factors into animals whose coagulation was already optimal did not appear to produce excessive or supranormal coagulation, as measured by thrombotest estimation. This result demonstrated that the preparations used had no significant content of activated factors. It is therefore unlikely that the striking effect of factor complex injections on metastasis can be explained by a major effect on the activity of the coagulation system.

Arvin removes fibrinogen from the circulation by cleaving fibrinopeptide A from the molecule; this produces an inactive monomer, des-A-fibrinogen, which is rapidly cleared by the reticuloendothelial and fibrinolytic systems (Bell et al., 1978). Arvin effectively reduced plasma fibrinogen concentration of our animals by nearly 70% at the time of tumour cell injection. This is roughly equivalent to a reduction of fibrin forming capacity of the same amount. The dose used was chosen to avoid causing any significant circulatory disturbance. Preliminary experiments showed that rats could tolerate very high doses of arvin, but that above 300 IU kg⁻¹, significant toxic effects could be observed during i.v. injection. Half of this dose was therefore used in the present experiment. The level of defibrination achieved was similar to the target range in humans when the drug is used clinically as an anticoagulant.

The results of our two experiments can now be summarised. First, injection of the coagulation factors II, VII, IX and X into normal rats greatly enhances the metastasis of tumour cells injected at the same time; second, this effect persists if factor VII is omitted from the complex, whilst this factor alone has no discernible effect on metastasis. Third, a very significant reduction in fibrin forming capacity has no discernible effect on metastasis. Finally, this degree of reduction in fibrin forming capacity does not diminish the enhancing effect on metastasis of the II, VII, IX and X factor complex. These findings indicate that some component of the II, IX and X complex is capable of greatly enhancing metastasis. They also suggest that this enhancing effect may not be dependent on the formation of fibrin. These conclusions are consistent with the results of our previous studies, and with reports (Hagmar, 1972; Donati et al., 1978), that defibrinating agents have no consistent effect on metastasis in other models.
Complete defibrination cannot be achieved with arvin in this model, and it is therefore possible that enough fibrin remains after arvin treatment to fulfil a vital role in enhancing metastasis. However, the very marked reduction in metastasis achieved with coumarin anticoagulant treatment (Ryan et al., 1969; Brown, 1973; Hilgard et al., 1976; Poggi et al., 1978; McCulloch & George, 1987) is in striking contrast with the complete absence of any such reduction following arvin treatment. If the formation of a fibrin clot is essential to the metastasis-enhancing process, it is surprising that two major suppressive influences on coagulation should have such contrasting results. Conversely, the marked enhancement of metastasis achieved in normal animals by injecting coagulation factors is unlikely to be due to enhanced coagulation activity, since this could not be detected. Further work is required to confirm our findings, but the evidence of these studies is in favour of a potentiating mechanism which involves specific coagulation factors, rather than coagulation as a whole.

Certain tumour cells have been shown to produce a vitamin K dependent cysteine protease procoagulant (CP) which directly activates factor X (Gordon et al., 1975), and which may be implicated in the process of metastasis (Colucci et al., 1983). Such a molecule might be activated by injections containing factor X, and a modification of the method of Gordon was therefore used to measure the procoagulant capacity of cultured Mtln3 cells. We have previously shown that warfarin treatment of Mtln3 cells prior to injection into a host animal does not affect their metastatic behaviour, and this makes it unlikely that CP plays a major role in the metastatic process in this model. Direct in vitro analysis of the procoagulant properties of Mtln3 cells shows that the component of total procoagulant activity which appears to be dependent on factor X (but not factor VII) is not predominant, and is unaffected by warfarin treatment of the cells. These results are similar to those obtained using non-malignant cell lines, and suggest that Mtln3 cells limit little or no CP.

Our previous results, and those of others (Brown, 1973; McCulloch & George, 1987), suggest that the antimetastatic effect of warfarin probably occurs within the first few hours after tumour cells enter the bloodstream. The model we have adopted allows this part of the metastatic process to be studied closely, whilst eliminating the influence of changes in the primary tumour. It has also enabled us to design useful experiments which would not have been possible in a more complete model of metastasis. The use of a small number of cells from a genuinely metastatic neoplasm minimised the risk, which arises in such models (Poggi et al., 1981) of artefacts caused by effects of the cell injection on the coagulation and immune systems, and on the lung vasculature.

The mechanism by which the factors II, IX and X complex enhances metastasis cannot be deduced from this work. Interactions between certain coagulation factors, notably factor XII and other biological systems such as the complement system, the kinin system, fibrinolysis and platelet activation are known to occur (Zimmerman et al., 1977), and the metastasis-enhancing effect may be mediated via a similar mechanism. Our findings must cast doubt on many of the commonly proposed theories for the interaction of cancer with coagulation, which have assumed that the interaction occurs at the level of fibrin clot formation (Zacharski, 1984). If confirmed by further studies, these results will require the formulation of quite different theories of the cancer/coagulation relationship. The finding that intravenous injection of coagulation factors enhances metastasis may, if it can be shown to extend to the human situation, have implications for the current controversy on the effects of perioperative blood transfusion on survival in cancer patients.

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