Hemostasis following Inoculation and during Spreading of Colon Carcinoma in the Rat

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ABSTRACT

Platelet function following inoculation of chemically induced carcinoma was evaluated in the rat. The original line of tumor (NGW1) was obtained using N-methyl-N-nitrosoguanidine. After trypsin homogenation a cell suspension of \(0.3 \times 10^6\) viable tumor cells was injected subserosally in the cecum of each animal. Controls received injections of equal volumes of 0.9% NaCl solution or trypsin. The animals were subjected to laparotomy 2, 4, and 6 weeks after inoculation. Platelet function was assessed in vivo by measuring bleeding time and blood loss during mesenteric vessel transection or liver resection upon laparotomy. Hemoglobin, hematocrit, platelet count, activated partial thromboplastin time, platelet aggregation, thromboxane B2, platelet factor 4, and fibrinogen levels were evaluated after sacrifice by exsanguination. Significant decrease in bleeding time and blood loss was observed in animals with local primary tumors as well as in rats with lymph node metastases. Hemoglobin and hematocrit were decreased in the presence of metastases. Platelet count was not changed. Activated partial thromboplastin time was not affected by the presence of tumor. Platelet aggregation in vitro was accelerated in the presence of primary tumor or lymph node metastases, as well as following addition of tumor cells to platelet suspensions. No changes in thromboxane B2 or platelet factor 4 could be registered. Fibrinogen levels were decreased in the presence of liver metastases. Enhancement of primary hemostasis and platelet function in the presence of colon carcinoma in the rat was demonstrated both in vivo and in vitro. Direct or indirect interaction of the tumor cell with thrombocytes may play a role in determining the metastatic potential of the neoplasm.

INTRODUCTION

The role of platelets in the pathogenesis of tumor dissemination has been the subject of extensive research in recent years. Several observations have indicated that activation of the hemostatic process by tumor cells entering the circulation might affect metastasis formation (1).

Malignancy has been associated with increased frequency of thromboembolic episodes or disseminated intravascular coagulation (2). Patients with metastatic cancers have been demonstrated to have decreased platelet survival and increased platelet consumption (3).

Procoagulant activity has been shown to exist in extracts of tumor cells (4) and mucus produced by adenocarcinomas (5). Infusion of tumor cells in experimental animals resulted in thrombocytopenia, while production of platelet-aggregating material was demonstrated in the presence of malignant cells (6, 7). In most experimental models, tumor dissemination was evaluated after i.v. injection of tumor cell suspension. The aim of the present study was to investigate the effect of a spontaneously metastasizing colon tumor on the hemostatic mechanism in the rat.

MATERIALS AND METHODS

Male inbred Wistar rats weighing 250–300 g were used. The original line of tumor (NGW1) was obtained in rats by chemical induction using N-methyl-N-nitrosoguanidine. The tumor was kindly supplied by Professor H. O. Sjögren, the Wallenberg Laboratory, Lund, Sweden. The tumor line used in this experiment was continuously transplanted on rat kidneys every 10th day, the number of passages before the utilized generation being 20. This tumor was shown to be immunogenic in syngeneic hosts. Undifferentiated morphology was a feature of the primary tumor; however, a degree of selection leading to cells with low differentiation was also present (8). After homogenization of the tumor by trypsin treatment, a cell suspension containing \(30 \times 10^6\) viable tumor cells/ml was prepared. In vitro studies cells were cultured in Waymouth's NB 752 medium supplemented with 20% fetal bovine serum (Flow Laboratories). Near confluence, tumor cells were harvested with 0.25% trypsin-1% EDTA in Dulbecco's phosphate-buffered saline, calcium and magnesium free. The cells were washed twice with the Dulbecco's and resuspended in the same medium at a concentration of \(10^6\) tumor cells/ml. In the same cell suspension 100 fibroblasts/ml were present. In the in vivo studies 0.01 ml of the initial cell suspension (30 \(\times 10^6\) tumor cells/ml) was injected in the area of apical lymphoid follicles in the wall of the cecum, in order to obtain a primary colon tumor site.

Two groups of 30 animals each, serving as controls, were given injections of equal volumes of 0.9% NaCl solution or trypsin, respectively. Two, 4, and 6 weeks after inoculation the animals underwent laparotomy, tumor status was carefully evaluated, and primary hemostasis was tested.

The animals were assigned to 6 groups of 30 rats according to the status of the tumor and the operating procedure: group 1, controls given 0.9% NaCl solution injections, sacrificed after 6 weeks; Group 2, trypsin-treated, given trypsin injections, sacrificed after 6 weeks; Group 3, T0N0Mo3 (tumor did not develop in 15% of the inoculated animals); Group 4, T0N0Mo5 (animals in this group harvested 2 weeks after tumor inoculation; tumor size, 0.5–1 cm); Group 5, T0N1Mo5 (rats in this group harvested 4 weeks after inoculation); Group 6, T0N1Mo5 (multiple metastases present in the liver 6 weeks after tumor inoculation).

Measurement of Primary Hemostasis. Primary hemostasis was evaluated in vivo by measuring bleeding time and blood loss after transection of mesenteric vessels in eight rats of each group. Through a midline incision, the distal part of the ileum was exteriorized over a preweighed aluminum cup. The vessels in the transparent part of the mesentery were observed through an operating microscope at \(\times 20\), the vessel type and diameter (300–350 µm) being identified prior to transection. Transection of a pair of parallel microvessels (arteriole and venule) was carried out by corneal scissors (T 270 Teufel; Liptingen, Federal Republic of Germany). Bleeding time was recorded and blood loss was collected in the underlying cup and weighed.

Determination of primary hemostasis was also performed during standardized liver resection in eight rats in each group. The liver was exposed via a midline incision and a preweighed aluminum cup was placed under the left anterior liver lobe. Thereafter a standardized resection of the tip of the lobe was performed with scissors, with 3 ± 0.05% (SE) of the liver weight being excised. Bleeding time was recorded by observing the bleeding surface through an operating microscope at \(\times 10\). Thus, bleeding time was determined as the time from trauma until arrest of hemorrhage at the resected liver surface. Extravasated blood was collected in the aluminum cup and weighed. All operative procedures took place under light ether anesthesia (10).

Measurement of Hematological Parameters. Eight animals in each group were sacrificed by exsanguination after puncture of the aorta at times corresponding to mesenteric vessel transection or liver resection. 3 The abbreviations used are: TNM, tumor-node-metastasis classification; APT time, activated partial thromboplastin time; TXB2, thromboxane B2; PGE2, platelet factor 4; PGD2, prostaglandin D2.

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2 To whom requests for reprints should be addressed.
The obtained blood was utilized for the determination of hemoglobin, hematocrit, platelet count, APT time, and platelet aggregation. Hemoglobin was determined photometrically according to the method of van Kampen and Zijlstra (11). Hematocrit was measured by the microhematocrit method. Platelet count was determined in a Bürchner chamber using phase-contrast microscopy. APT time was counted using a commercial kit for automated APT time (General Diagnostic, Morris Plains, NJ). Platelet aggregation induced by ADP or collagen was studied according to the method of Born and Cross (12). ADP (Sigma Plains, NJ). Platelet aggregation induced by ADP or collagen was measured by calculating the slope of the ascending part of the curve corresponding to the first 30 s of reaction.

In vitro, tumor cell suspension prepared as above was added to plasma containing 750 x 10^6 platelets/liter. Platelet aggregation was determined by calculating the slope of the curve and the onset of aggregation.
Table 6 Collagen-induced platelet aggregation in control and tumor-bearing animals

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Platelet aggregation* at following collagen concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 mg/liter</td>
</tr>
<tr>
<td>1. Controls</td>
<td>8</td>
<td>16.1 ± 1.5</td>
</tr>
<tr>
<td>2. Trypsin-treated</td>
<td>8</td>
<td>18.9 ± 1.0</td>
</tr>
<tr>
<td>3. T,N,M</td>
<td>8</td>
<td>19.2 ± 1.5</td>
</tr>
<tr>
<td>4. T,N,M</td>
<td>8</td>
<td>27.4 ± 2.6</td>
</tr>
<tr>
<td>5. T,N,M</td>
<td>8</td>
<td>24.5 ± 1.2</td>
</tr>
<tr>
<td>6. T,N,M</td>
<td>8</td>
<td>20.1 ± 2.5</td>
</tr>
</tbody>
</table>

* Arbitrary units (cm/min) measuring aggregation velocity in the first 60 s for each concentration of collagen.

Table 7 Fibrinogen levels in controls and tumor-bearing animals

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Fibrinogen (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Controls</td>
<td>6</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>2. Trypsin-treated</td>
<td>6</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>3. T,N,M</td>
<td>6</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>4. T,N,M</td>
<td>6</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>5. T,N,M</td>
<td>6</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>6. T,N,M</td>
<td>6</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

Further indication of thrombocyte involvement was offered by ultrastructural studies, which after i.v. injection of malignant cells revealed the existence of platelets surrounding tumor cells attached to the vascular endothelium. Platelets seemed to stabilize the initial adhesion of malignant cells to the vascular lining (18, 19). Tumor cells adhered readily to damaged endothelium and thereafter invaded the vessel wall (20). Platelet-tumor cell coaggregation might be one of the mechanisms underlying overt endothelial damage, thus facilitating metastasis formation. Yet direct evidence that spontaneous cancer cell dissemination was associated with in vivo enhancement of platelet function has been scant.

The present experiment demonstrated in vivo acceleration of the hemostatic process in the presence of colon carcinoma in the rat. In this model the metastatic process could be studied during the formation of spontaneous metastases via dissemination from a primary tumor inoculated in the cecum. In the course of tumor growth and dissemination decreases in hemoglobin and hematocrit were observed. However, no change in platelet count could be registered. Earlier studies showed that during spontaneous metastases to the lung of i.m. implanted Lewis lung carcinoma cells in the rat, thrombocytopenia, hemolytic microangiopathic anemia, and decreased fibrinogen survival were noted. These changes did not occur when lung metastases developed following i.v. injection of the same cells (14). Gasic et al. (6) and Hilgard et al. (15) observed that injection of fibrosarcoma, B16 melanoma, or anaplastic mammary adenocarcinoma in mice and rats resulted in thrombocytopenia, which was correlated to the number of injected tumor cells. Isotope studies using labeled platelet and fibrinogen showed the entrapment of thrombocytes in the lung, where metastases formed. Prior treatment with neuraminidase or antiplatelet antisera, rendering the animals thrombocytopenic, diminished the development of lung metastases. Inhibition of platelet function by PGI2 had a similar effect, while inhibition of endogenous PGI2 production enhanced metastatic tumor formation (16). These findings were thus considered to indicate the involvement of platelets in tumor dissemination. However, i.v. bolus injection of malignant cells resulted in the formation of tumor emboli, which were arrested in the lung, rather than real metastases. It did not exactly simulate in vivo metastases, during which a slow constant release of cells into the circulation was proved more common. Furthermore, the animals usually did not demonstrate the biological changes associated with growth of the primary tumor. The early stages of the metastatic process from a primary location have been considered to be of major importance for the successful spontaneous development of secondary growths. The metastatic potential of various malignant tumors has been directly related to the number and the behavior of circulating tumor cells (17).

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Transsection of mesenteric vessels and standardized liver trauma were proved to be sensitive and closely correlated methods for the investigation of microcirculation and platelet function during primary hemostasis (10, 21). The size of the transsected mesenteric vessels could be easily determined and standardized by using an operating microscope.

Standardization of the size of the resected liver section was possible by the use of the tip of the left anterior lobe and, as
the small standard error of the mean (0.05) indicated, easy to apply in all animals. It is possible that anesthesia and stress during laparotomy affected bleeding parameters. However, controls were subjected to identical treatment so that the observed changes in bleeding time and blood loss were not due to these factors. Application of the above methods enabled us to study in vivo the hemostatic changes in the course of tumor growth, demonstrating decrease in bleeding time and blood loss in the presence of a primary tumor, as well as lymph node metastases. The lack of similar results upon metastases to the liver could be due to the reduction of functional hepatic parenchyma. The hematological changes appearing during cirrhosis, hepatectomy as well as liver exclusion (e.g., portacaval shunt), included thrombocytopenia and impairment of platelet aggregation, which were associated with the decrease in plasma proteins and fibrinogen (22, 23). It has been postulated that such alterations modified the number and structure of platelet membrane binding sites to fibrinogen thus inhibiting thrombocyte activity (23, 24). A decrease in plasma fibrinogen levels was registered during hepatic metastases of the experimental tumor supporting this hypothesis (Table 7). Because fibrinogen values in T1N0M0 and T1N1M0 groups were normal, the decrease upon liver dissemination could not be attributed to the metastatic process per se.

The intrinsic coagulation system, which could have been affected by aberrations in plasma proteins or platelet function, did not show any changes in the present study. Al-Mondhiry et al. (25) demonstrated that human melanoma and breast adenocarcinoma cells possessed procoagulant activity. Melanoma cells activated the coagulation system by indirect activation of factor VII, while breast tumor cells activated directly factor X. Simultaneous platelet activation by the tumor cells took place, making available a platelet-derived procoagulant activity necessary for the clotting process. Conversion of fibrinogen to fibrin was impaired in the absence of activated thrombocytes. Tissue factor-like activity, indirectly activating factor VII, was also observed in leukemic promyelocytes, monocytic tumor cells, and colon carcinoma, while several experimental tumors could activate factor X (26). However, these properties were not unique to malignant cells because even normal cell lines have been shown to affect hemostasis in a similar manner (27).

Platelet aggregation was found to be accelerated when the animals carried a local tumor in the ecocum or in the presence of lymph node metastases. The lack of enhancement during liver metastases was attributed to the decrease in functional parenchyma as earlier related.

Up to now, a considerable number of investigations have demonstrated the activation of thrombocytes by tumor cells in vitro. Gasic et al. (6, 28) showed that several human and murine tumor cells as well as plasma membrane vesicles, could, after incubation with platelet-rich plasma, aggregate platelets. Some tumors induced the serotonin release reaction. These results were confirmed in further experiments by Pearlstein et al. (5, 29) and Hará et al. (30) using murine and human tumor cell lines. According to the report of Pearlstein et al. (5) polyoma-induced PW 20 rat renal tumor cells induced the serotonin release reaction. These results were confirmed in further experiments by Pearlstein et al. (5) polyoma-induced PW 20 rat renal sarcoma cells had platelet-aggregating properties. The platelet-aggregating potential of these cells correlated with the sialic acid content of the cell membrane as well as with the metastatic potential of the tumor. In virally transformed 3T3 mouse fibroblasts the platelet-aggregating substance appeared to be a sedimentable sialolipoprotein, which was activated in the presence of divalent cations and plasma components believed to be constituents of the alternate complement pathway (31). Tumor cell-induced platelet aggregation was detected in several rodent tumor lines. This aggregation was successfully inhibited by PGI2. Prostacyclin was able to arrest tumor cell-induced aggregation after initiation, resulting in dispersal of platelet aggregates (7). Donati et al. (32) reported that tumor lines from murine sarcoma producing PGI2 had a low metastatic potential while these producing TXA2 were highly metastatic. It was postulated that in highly metastatic cells, the balance between PGI2 and TXA2 was shifted to a proaggregatory condition. The above findings led to the speculation that platelets stimulated by tumor cells in vivo enhanced malignant spreading. Still not all tumors had platelet-stimulating properties and direct evidence for the ability of malignancy to increase thrombocyte aggregation in vivo was not demonstrated. In the present in vivo experiments we could not demonstrate alterations in TXB2 and PF4 values during tumor progression. Changes in these parameters at cell level, untraceable by the utilized techniques, could not be excluded, however.

According to the report of Estrada and Nicolson (33) the metastatic potential of mammary adenocarcinoma tumor cell clones did not correlate to the in vitro platelet aggregation activity of the tumor cells. In the present study in vitro addition of tumor cells to platelets at a ratio of 1 tumor cell to 3000 platelets resulted in the initiation of thrombocyte activation, thus verifying the in vivo aggregating potential of the utilized tumor cells.

It is not likely that the observed platelet activation was due to the presence of fibroblasts inasmuch as the number of fibroblasts required for such a reaction by far exceeded the number present in the tested samples as controls showed.

The present study demonstrated that in the presence of colon adenocarcinoma platelet aggregability was enhanced in vivo and in vitro. The general significance of these findings, however, remains to be substantiated by further experiments using several malignant tumors.

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