The Role of Lymphatic Obstruction in the Formation of Ascites in a Murine Ovarian Carcinoma

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SUMMARY

In normal mice, 60 to 70% of $^{51}$Cr-labeled erythrocytes injected i.p. appear in the peripheral bloodstream within 5 hr. In mice previously inoculated with a transplantable, ascites-producing ovarian tumor, this egress of labeled erythrocytes from the abdominal cavity is significantly impaired before ascites develops. Diaphragmatic lymphatic obstruction is demonstrated histologically. Since red cells leave the peritoneal cavity primarily by way of these lymphatic channels, these observations suggest that lymphatic obstruction by tumor cells is probably of pathogenetic importance in the accumulation of ascitic fluid.

INTRODUCTION

In 1953, Holm-Nielsen (8) suggested that ascites accompanying peritoneal carcinomatosis might be caused by the obstruction of lymphatics draining the peritoneal cavity by tumor cells. In normal mice, the rapid egress of i.p.-injected India ink from the abdominal cavity through lymphatics is well documented (9, 13). Holm-Nielsen found that in mice rendered ascitic by tumor virtually no ink was transported through the lymphatics draining the abdominal cavity. Moreover, he presented micrographs from these ascitic animals which appeared to demonstrate occlusion of lymphatic channels by tumor cells. These studies did not determine whether the lymphatic obstruction precedes the formation of ascites and therefore is of pathogenetic significance in the formation, or whether the obstruction was merely an accompaniment of the ascites.

Red blood cells that are free in the abdominal cavity enter the peripheral circulation only via the lymphatics (2, 13). Therefore, measurement of the appearance in the peripheral blood of $^{51}$Cr-labeled erythrocytes injected i.p. allows a quantitative determination of the egress of erythrocytes through the lymphatics draining the abdominal cavity. Courtice et al. (1) found that 70% of labeled erythrocytes left the abdominal cavity of normal rats within 5 hr after they were given the injection.

In our laboratory, a transplantable ovarian embryonal cell carcinoma is maintained by serial i.p. transfers in C3HeB/FeJ female mice. This tumor induces ascites in recipient mice in a reproducible time course.

For an evaluation of the importance of lymphatic obstruction in tumor-caused ascites, this study compares the rate of egress of labeled erythrocytes from the abdominal cavity of normal mice to that of mice previously inoculated with tumor.

MATERIALS AND METHODS

Animals. All experiments were conducted with 3- to 5-month-old female C3HeB/FeJ mice, weighing 18 to 23 g (The Jackson Laboratories, Bar Harbor, Maine).

Tumor. A spontaneous ovarian carcinoma originating in a C3H female mouse at The Jackson Laboratories was maintained by serial transplantation (5). From an initially frozen sample of cells, this cell line has been carried in our laboratory through 15 serial i.p. transplantations. Cells from the 10th to 12th passage were used in these experiments.

Development of Ascites. For a demonstration of the typical time course of the development of ascites, 5 mice were given injections of $1 \times 10^6$ tumor cells and were weighed and observed for ascites at frequent intervals. The 1st animal was noted to have ascites on Day 7, and all had clinical evidence of ascites by Day 10. Chart 1 plots the weights of these animals.

As a test of whether egress of erythrocytes from the abdominal cavity is blocked before ascites begins to accumulate, radioactively labeled erythrocytes were injected i.p. both into normal mice and into mice previously inoculated with tumor cells.

Labeling of Erythrocytes. Sterile sodium chromate-$^{51}$Cr in aqueous solution containing 0.00254 mg $^{51}$Cr per 10 ml with an activity of 394,000 mCi/g $^{51}$Cr was used to label cells. Three to 5 ml of heart blood were withdrawn into sterile, heparinized syringes from several donor mice and deposited into 1 ml sterile 0.9% NaCl solution with several drops of a 10 units/ml solution of heparin. After 5 min of centrifugation at 5000 rpm, the supernatant was removed and replaced with an equal volume of 0.9% NaCl solution. Sterile sodium chromate-$^{51}$Cr solution, 0.4 ml, was added, and the mixture was incubated at room temperature for 1 hr. Centrifugation was then repeated, and the supernatant was removed and discarded. The remaining labeled erythrocytes were diluted with 0.9% NaCl solution to obtain an appropriate volume (4). Less than 0.04 mCi was present in each preparation of labeled erythrocytes.
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There was prompt egress of cells from the abdomen, with about 65% of the cells appearing in the peripheral blood at 4 hr. This rate of egress seems to be independent of the volume of the initial injected material over the range of 0.2 to 1.25 ml.

Chart 1. Daily weights of animals given injections of \(1 \times 10^6\) tumor cells and weighed at the intervals indicated. Each point is indicated, ± S.E. (vertical bars).

Animals were used in groups of 4 to 7, so each animal received less than 0.01 mCi.

Injection i.p. of Labeled Cells. The erythrocyte suspensions thus prepared were injected i.p. through 27-gauge sterile disposable needles. Each mouse received a volume of injection ranging from 0.2 to 1.25 ml, as indicated in "Results."

Sampling Peripheral Blood. At various times after the injection of labeled cells i.p., samples of tail-vein blood were drawn into 0.05-ml micropipets. These 0.05-ml samples were counted on a well scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Total radioactivity in the peripheral bloodstream was calculated as (activity of tail-vein sample) \(\div\) (blood volume of mouse) \(\times\) 0.05 ml. Mouse blood volume can be taken to be a linear function of body weight (6). A conversion factor of 0.778 ml blood per 10 g body weight was used (3). Although the accuracy of this conversion factor may be questioned (10), the data based upon it were used only for purposes of comparisons between groups. Quantitative errors would not invalidate observations of relative differences between groups.

Implantation of Tumor Cells i.p. In these experiments, fluid was withdrawn from the abdomen of an ascitic mouse through a 20-gauge needle into a sterile syringe. A cell count was determined, the ascitic fluid was appropriately diluted with sterile 0.9% NaCl solution, and \(1 \times 10^6\) cells were injected i.p. into test animals through a 27-gauge needle in a bolus of 0.1 to 0.3 ml.

Histology. The diaphragms obtained from mice on the 2nd and 5th day after i.p. tumor transplantation were mounted flat. Serial sections were cut and stained with hematoxylin and eosin.

RESULTS

Egress of Labeled Erythrocytes from the Abdominal Cavity of Normal Mice. Labeled erythrocytes were injected into the peritoneal cavity of normal mice. Chart 2 shows the time course of the appearance of these cells in the peripheral bloodstream. Each group received the red cells in a different volume of injected material, ranging from 0.2 to 1.25 ml. There was prompt egress of cells from the abdomen, with about 65% of the cells appearing in the peripheral blood at 4 hr. This rate of egress seems to be independent of the volume of the initial injected material over the range of 0.2 to 1.25 ml.

Chart 2. Fraction of i.p.-injected radioactivity present in the peripheral blood in normal mice. Each group of 4 to 7 normal mice received a different volume of injected material i.p. Each point is indicated, ± S.E. (vertical bars); n, number of animals in group.

Chart 3. Fraction of i.p.-injected radioactivity present in the peripheral blood in mice inoculated with tumor 1, 3, 4, and 6 days previously. Volume of labeled erythrocyte, i.p. injected material is 0.4 ml. Each point is indicated, ± S.E. (vertical bars); n, number of animals in group.
The same basic data are treated in an alternative fashion to derive the "normal mice" curve of Chart 3. A smooth curve was drawn between the data points for each of the 36 test animals. Values for fraction of injected activity in the peripheral blood were found by interpolation for t = 1, 1.5, 2, 2.5, 3, 4, 5, and 6 hr. These interpolated values were averaged and plotted as the normal mice curve in Chart 3. This maneuver allowed calculation of a standard error of the mean at each of the indicated times, depicted in Chart 3 by brackets.

**Egress of Labeled Erythrocytes from the Abdominal Cavity of Mice Previously Inoculated with Tumor.** Chart 3 depicts erythrocyte egress in groups of mice measured 1, 3, 4, and 6 days after tumor injection. In none of these animals did gross inspection or daily weights indicate evidence of ascites. The chart demonstrates the fact that transport of erythrocytes out of the abdominal cavity 1 day after tumor inoculation was significantly lower than that seen in normal mice. Four days after tumor inoculation, only 2 of 6 animals showed any significant peripheral blood activity after the injection i.p. of labeled red cells. Six days after tumor inoculation, none of 8 animals demonstrated peripheral activity greater than 2% of the i.p.-injected activity, indicating an almost total failure of injected erythrocytes to reach the peripheral circulation in these animals.

**Histological Examination of Lymphatics.** Histological examination of the diaphragms obtained from mice on the 3rd and 5th days after tumor inoculation i.p. revealed 2 pertinent findings. Ovarian tumor cells were adherent to the surface of...
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the diaphragm. Examination at greater depths in the diaphragmatic musculature demonstrated a linear arrangement of tumor cells within the endothelial lined lymphatics (Figs. 1 and 2). These findings were consistent with the physiological data demonstrating lymphatic obstruction prior to ascites formation and were present as early as the 2nd day.

DISCUSSION

In a dynamic system, the accumulation of fluid may be due to increased production, decreased removal, or a combination of both. In cirrhosis, there is substantial evidence that elevated portal pressure shifts the hydrodynamic balance toward an increased flow of fluid out of the intravascular space (11, 12). A recent report pertaining to ascites associated with peritoneal carcinomatosis also indicates that increased rates of formation of ascitic fluid are of primary pathogenetic significance (7). However, regardless of the increase in rate of formation of ascitic fluid, ascites will not accumulate unless its rate of formation exceeds its rate of removal. Increased production alone does not explain ascites formation. A concomitant failure of homeostatic mechanisms to clear the excess fluid must be assumed.

The i.p. fluid reenters the vascular space by 2 routes: Route 1, directly back into peritoneal capillaries; and Route 2, into large veins via lymphatic pathways. Capillary permeability and hydrostatic and oncotic pressures within and outside vascular capillaries regulate fluid movement along the 1st route. The factors that regulate movement along the 2nd are more obscure. However, it is clearly possible that mechanical obstruction of lymphatics might significantly reduce the return of fluid to the intravascular space.

The data presented in this study indicate that the obstruction of lymphatics by tumor cells does occur in murine ovarian carcinomatosis. Egress of labeled erythrocytes from the peritoneal cavity gradually decreases with time after tumor inoculation. This decreased egress begins within 1 day after tumor inoculation, long before ascites becomes evident. By the time ascitic fluid begins to accumulate, egress of erythrocytes from the peritoneal cavity is totally blocked.

Abnormal accumulation of fluid in any body compartment is the result of an altered dynamic balance of flow rates. The lymphatics are an important factor on one side of the balance equation. It is conceivable that, even in such conditions as cirrhotic ascites or pulmonary edema, where increased flow into an area is easily appreciated, relative failure of lymphatic drainage of protein-containing fluid is an unsuspectedly significant pathogenetic factor.

The prominence of obstruction in the pathogenesis of malignant ascites has important treatment implications. For example, the efficacy of locally instilled radioactive colloidal substances or alkylating agents may relate not to destruction of tumor cells on serosal surface but, rather, to the nature of their removal via the lymphatics. Successful treatment, therefore, might depend on whether lymphatic flow was great enough to allow the therapeutic agents to enter involved lymphatics and to destroy the tumor cells residing there. Further, the destruction of cells on the peritoneal surface may delay recurrence of lymphatic obstruction. The lack of success with these agents may be due to their failure to reach cells in the obstructed lymphatics. Perhaps direct treatment of lymphatics in the diaphragm and mediastinum by external radiation, alone or in combination with intracavitary treatment, would be more efficacious in light of the data presented. This laboratory model allows study of these various therapeutic alternatives, and they are currently being investigated.

Malignant ascites formation is a complicated pathophysiological event. However, it seems clear from these studies that obstruction of lymphatics by ovarian tumor cells in our experimental model precedes and is causally related to the accumulation of ascitic fluid.

REFERENCES

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