Bone Marrow Cytolysis Induced by Hepatoma, Teratocarcinoma, and Transformed Fibroblasts

Rita Marie Lysik, Kenneth Cornetta, John F. DiStefano, and Stanley Zucker

ABSTRACT

The purpose of this study was to explore further an in vitro system to evaluate the interaction between cancer cells and normal cells. In the tumor-induced marrow cytotoxicity assay, 59Fe- and 51Cr-labeled bone marrow cells were cocultured with different types of cancer cells. Following an 18-hr incubation, the mixed-cell cultures were centrifuged, and the 59Fe and 51Cr in the supernatant solution (released by cell death) were compared to the total radioactivity and expressed as a release index. In the presence of cocultured Novikoff or Yoshida hepatoma cells, the release of isotopes by rat bone marrow cells increased linearly with increasing tumor cell concentrations. With equal numbers (1 x 10^7/ml) of cancer cells and 59Fe-labeled marrow cells, the marrow release indices with Novikoff and Yoshida hepatoma cells were 36.1 ± 0.1% (S.D.) and 38.6 ± 1.0%, respectively, as compared to a spontaneous marrow release index of 27.4 ± 0.1%. Similar results were obtained when mouse teratocarcinoma cells were cocultured with syngeneic mouse marrow cells. Normal rat spleen cells, blood lymphocytes and fibroblasts were inactive as effector cells in the tumor-induced marrow cytotoxicity assay. Activated macrophages which have demonstrated a cytolytic capacity in other assay systems were able to induce marrow cell lysis. However, phytohemagglutinin-stimulated lymphocytes were not cytotoxic to normal marrow. These experiments suggest that the capacity to induce the release of 59Fe and 51Cr from labeled marrow cells is a common property of transplanted cancer cells. We propose that the tumor-induced marrow cytotoxicity assay will be a useful experimental technique to enhance our understanding of the mechanisms of cancer invasion.

INTRODUCTION

A characteristic aspect of cancer biology is the ability of cancer cells to invade normal tissues. Recent attention has focused on the enzymatic activity of cancer cells as related to fibrinolysis and collagenolytic activity (2, 7, 12). The interaction between normal cells and cancer cells during the invasive process, however, has not been clearly characterized beyond the stage of morphological observation (1, 3, 4, 8). Attempts to delineate the interaction between normal cells and cancer cells have suffered from lack of appropriate experimental models.

To evaluate the cytolytic potential of tumor cells and to explore its possible relationship to cancer invasiveness, we have developed an assay which measures the death of radiolabeled marrow cells when cultured with tumor cells in a mixed-cell suspension (18). Using this assay, we recently reported that cells of W-256* (a highly invasive cancer) were able to induce the lysis of normal bone marrow cells.

In the current study, we have extended our previous observations and have evaluated the cytotoxic potential of 4 other types of cancer cells using the TIMC assay. The results indicate that many types of invasive cancer cells have the capacity to induce marrow cytolysis. We propose that the TIMC assay might be a useful in vitro technique to explore further the mechanism of cancer invasion.

MATERIALS AND METHODS

Tumor Effector Cells. W-256 cells were obtained from Arthur D. Little Co. (Boston, Mass.) under the auspices of the NIH. Tumor cells were harvested from the ascites fluid which accumulated 7 days after the i.p. transplantation of 1 x 10^6 W-256 cells to adult Wistar rats. Tumor cells were separated from contaminant erythrocytes by centrifugation (400 x g) on a sterile gradient of sucrose polymer and diatrizoate salts (lymphocyte separation medium solution, sterile gradients, 1.077 to 1.080; Litton Bionetics, Kensington, Md.). Tumor cells located at the interface were collected, washed twice, and suspended in complete media (70% NCTC 135; Grand Island Biological, Grand Island, N. Y.; 30% fetal calf serum, Colorado Serum Co., Denver, Colo.; 50 units penicillin and 50 units streptomycin per ml; Microbiological Associates, Bethesda, Md.).

Yoshida and Novikoff hepatoma cells in ascitic form were also obtained from Arthur D. Little Co. The tumor was subsequently maintained by transplanting either 5 x 10^6 Yoshida or 1 x 10^6 Novikoff hepatoma cells i.p. into male weanling Sprague-Dawley rats. The preparation of tumor cells for the cytotoxicity assay from the resultant ascites at 7 days is described above.

The mouse teratocarcinoma used in these studies, designated OTT/6050-2033, was kindly provided by Dr. Leroy Stevens (The Jackson Laboratory, Bar Harbor, Maine). The

1 This research was supported by grant funds from the Veterans Administration Medical Center (MVIS 9939) and the National Cancer Institute (CA 15629).
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4 The abbreviations used are: W-256, Walker 256 carcinosarcoma; TIMC, tumor-induced marrow cytotoxicity; NCTC, National Collection Tissue Culture; HBSS, Hanks' balanced salt solution; PHA phytohemagglutinin M; BCG, Bacillus Calmette-Guérin; RI, release index.

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teratocarcinoma was initiated by transplanting a 6-day embryo (removed from the oviduct of a pregnant female) into the testis of a syngeneic adult male mouse (strain 129/Sv). Since host and donor tissues were histocompatible, the embryo grafts grew well, but soon after they were grafted the cells migrated away from the original graft site, and the growth became disorganized. The embryonic mouse cells retained their undifferentiated proliferative state indefinitely and gave rise to progressively growing teratocarcinomas (14). The teratocarcinoma, now primarily composed of primitive neural cells, has been maintained in 120/Sv mice and can be grown as an ascitic tumor. An ascitic tumor develops 3 weeks after the i.p. transplantation of ascites fluid from a carrier mouse. Teratocarcinoma cells were prepared for use in the cytotoxic assay following the procedure described for W-256 cells.

Spontaneously transformed Wistar rat fibroblasts (RF-75) were kindly provided by Dr. Stanley Weiner (Long Island Jewish Hospital, N. Y.). RF-75 fibroblasts have been maintained in vitro for 2 years in Eagle’s minimum essential media with Earle’s salts and 10% fetal calf serum (16). These fibroblasts have a doubling time of 17 hr, grow to high cell density, are not anchorage dependent for growth, and are able to proliferate in low serum concentration (0.5%). Injection of RF-75 fibroblasts (5 x 10^6) into the peritoneal cavity of normal Wistar rats results in an invasive sarcoma with death of the host in 3 weeks. Prior to use in the cytotoxicity assay, the malignant fibroblasts were cultured in vitro and were harvested from Petri dishes by incubation with 0.25% trypsin at 37° for 20 min. The cells were then washed twice in HBSS by centrifugation and suspended in complete media.

**Normal Effector Cells.** Normal heparinized peripheral blood was obtained from rats via cardiac puncture. Lymphocytes were separated from other blood elements by centrifugation on lymphocyte separation medium for 30 min at 400 x g. Lymphocytes, collected at the interphase, were washed twice with HBSS and then resuspended in Roswell Park Memorial Institute Medium 1640 with 20% fetal calf serum. The cell concentration was adjusted to 1 x 10^6 cells/ml, and 1-ml aliquots were dispensed into 10- x 75-mm Falcon friction cap tubes. PHA-stimulated cultures received 50 μl (50 μg) of PHA (Grand Island Biological Co., Grand Island, N. Y.); control cultures received 50 μl of Roswell Park Memorial Institute Medium 1640. Lymphocyte cultures were then incubated for 72 hr at 37° in 5% CO2-95% air. Cultured lymphocytes were washed by centrifugation to remove PHA. Stimulated lymphocytes (1 x 10^6) were then added to radiolabeled marrow cells (1 x 10^6) in 1 ml of complete media, and the cytotoxicity assay proceeded as described above. PHA-stimulated blast transformation was verified by comparing the incorporation of [3H]thymidine (1.0 μCi/culture) into trichloroacetic acid-precipitated DNA in control cultures and PHA-stimulated cultures.

Macrophages were obtained from rats which had received i.p. injections of BCG (manufactured by University of Illinois; 50 IU) 3 weeks previously and then rechallenged with purified protein derivative of tuberculoprotein (50 units) 24 hr before sacrifice. Macrophages were collected by peritoneal lavage with 20 ml of NCTC 135. Macrophages were centrifuged and washed once prior to being resuspended in media at an appropriate cell concentration. Nonactivated rat macrophages were also obtained by peritoneal lavage with NCTC 135.

Normal spleen cells were disassociated from intact spleens by mincing and passage of the cells through a wire mesh. Liberated cells were then washed with HBSS prior to use in the TIMC assay.

**Normal Target Cells.** The preparation of normal rat marrow cells (Wistar or Sprague-Dawley) for suspension cultures has been described in detail elsewhere (18). A similar technique was used to obtain mouse marrow cells (strain 129/Sv: The Jackson Laboratory) except that a 26 gauge needle was used to force the marrow cells from the femur. Marrow cells (1 x 10^6/ml) were radioactively labeled by incubation with 2 μCi transferrin-bound 59Fe per ml for 4 hr at 37°. Marrow cells were simultaneously labeled with 51Cr by the addition of 1 μCi 51Cr per ml for the last 0.5 hr of the 4-hr incubation period. Chromium incorporation was terminated by the addition of 5 mg ascorbic acid per ml cell suspension. Unincorporated isotopes were eliminated by 6 washings of the cells. Marrow cells were then suspended in complete media at a concentration of 1 x 10^6 cells/ml.

Normal rat fibroblasts were propagated from s.c. epidermal tissue. Normal fibroblast cultures (doubling time, ~7 days) which had been grown to confluence were utilized for the cytotoxicity assay. Fibroblasts were removed from culture flasks by trypsinization with 0.25% trypsin for 20 min at 37°. Fibroblasts were washed free of trypsin, and then radioactively labeled with 51Cr as described for marrow cells. After unincorporated 51Cr had been removed by washing, the fibroblasts were suspended in complete media at a concentration of 1 x 10^6 cells/ml.

**TIMC.** Labeled target cells (1 x 10^7 cells in 0.1 ml) were then added to various concentrations of tumor cells. The final volume of all cultures was adjusted to 1.0 ml with complete media and the cell suspensions were mixed thoroughly. Cultures were incubated at 37° in 5% CO2-95% humidified air in 10- x 75-mm Falcon friction cap tubes. At 18 hr of incubation, the cultures were terminated by centrifugation at 400 x g for 10 min at 4°. The supernatant was decanted, and the radioactivity was measured using the dual-window isotope counting technique. Cytotoxicity was expressed as a function of the RI (18):

\[
RI = \frac{\text{Radioactivity released due to cell death (supernatant \times 100)}}{\text{Total radioactivity (supernatant and cell pellet)}}
\]

Significant differences between triplicate cultures of target cells alone (spontaneous release) and target cells cultured with effector cells were determined by Student's t test. (In all experiments, the base-line RI of target cells cultured alone as well as the release induced by effector cells in a 1:1 ratio was determined.) Determination of pH changes were made using an Orion Model 801 digital pH meter.

**RESULTS**

**Hepatoma Cytotoxicity.** The effect of culturing various numbers of Novikoff hepatoma cells with 10 million rat marrow cells is depicted in Chart 1A. For comparative purposes, we have monitored the release of 2 isotopes, 51Cr and 59Fe. The release index for marrow cells cultured...
A. M. Lysik et al.

without tumor was 30.5 ± 0.2% (S.E.) for 51Cr and 27.4 ± 0.1% for 59Fe. (The maximum iron release from freeze-thawed marrow cells was 76.0 ± 1.3%) As the ratio of tumor cells cocultured with marrow cells increased, there was a proportional and significant increase in the release index for both 51Cr and 59Fe. There were no substantial differences in the release of 59Fe as compared to 51Cr.

The release of radioisotope from marrow cells cultured with Yoshida hepatoma is illustrated in Chart 1B. The proportional increase in the release of both 59Fe and 51Cr with increasing tumor cell concentrations parallels that seen with Novikoff hepatoma. Pelleting the tumor:marrow cell suspension (1:1 cell ratio) by centrifugation at 400 x g for 10 min at the initiation of the incubation increased 59Fe release of marrow cells cocultured with tumor cells to 52.4 ± 0.4% as compared to a release index of 40.8 ± 1.2% for cells not pelleted. This emphasizes the requirement for cell-cell contact in the TIMC assay (18).

To determine if an increased marrow cytolysis could be effected by a mixture of Yoshida and Novikoff hepatoma cells, we cultured 10 million marrow cells with either Novikoff cells, Yoshida cells, or a mixture of equal numbers of Yoshida and Novikoff hepatoma cells. The tumor:marrow ratio was maintained at 1:1. The mixture of Yoshida and Novikoff hepatoma cells did not produce increased cytoly-

S Figure 1A-Novikoff Hepatoma Cytotoxicity

S Figure 1B-Yoshida Hepatoma Cytotoxicity

Table 1

Release of 59Fe by bone marrow cells (5 x 10^6/ml) cocultured with teratocarcinoma cells in various effector cell:target cell ratios

<table>
<thead>
<tr>
<th>T:M ratio</th>
<th>Syngeneic mouse marrow</th>
<th>Wistar rat marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5:1M</td>
<td>40.1 ± 1.2^b</td>
<td>24.0 ± 0.2</td>
</tr>
<tr>
<td>1:1M</td>
<td>46.3 ± 0.2^c</td>
<td>45.3 ± 1.9^c</td>
</tr>
<tr>
<td>1:1M (using W-256 cancer cells)</td>
<td>50.4 ± 2.7^c</td>
<td>68.1 ± 1.7^c</td>
</tr>
<tr>
<td>1:1M</td>
<td>47.5 ± 0.7^c</td>
<td>36.3 ± 1.1^c</td>
</tr>
</tbody>
</table>

^a T. teratocarcinoma cells; M, bone marrow cells.

^b Mean ± S.E.

^c Significant difference (p < 0.05) between spontaneous RI (0T:1M) and effector cell-induced RI.
Release of isotope by rat marrow cells (59Fe-labeled) after coinubcation with normal effector cells

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>Spontaneous RI (%)</th>
<th>Effector cell-induced RI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marrow</td>
<td>30.7 ± 0.4</td>
<td>32.6 ± 1.0</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>30.7 ± 0.4</td>
<td>31.0 ± 0.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>30.7 ± 0.4</td>
<td>31.3 ± 0.9</td>
</tr>
<tr>
<td>Lymphocyte (peripheral blood)</td>
<td>30.7 ± 0.4</td>
<td>32.0 ± 0.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Effector cells</th>
<th>Spontaneous RI (%)</th>
<th>Effector cell-induced RI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA-lymphocytes</td>
<td>27.5 ± 2.7</td>
<td>26.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Unstimulated lymphocytes</td>
<td>27.5 ± 2.7</td>
<td>26.0 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 3</th>
<th>Effector cells</th>
<th>Spontaneous RI (%)</th>
<th>Effector cell-induced RI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonactivated macrophages</td>
<td>22.4 ± 0.9</td>
<td>21.7 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Activated macrophages</td>
<td>22.4 ± 0.9</td>
<td>38.8 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference (p < 0.05) between spontaneous RI and effector cell-induced RI.

**Mean ± S.E.**

not increase marrow lysis in the TIMC assay. Blast transformation was monitored by determining DNA synthesis. Lymphocytes stimulated with PHA increased [3H]thymidine incorporation 10 times over control cultures of lymphocytes cultured for 72 hr without PHA.

Cocultivation of BCG-activated peritoneal macrophages with 59Fe-labeled marrow cells resulted in a significant increase in marrow cytotoxicity when compared to marrow cultured alone. Nonactivated macrophages cultured with marrow cells did not increase marrow cell death (Table 2).

To determine if marrow cell cytotoxicity is the result of the depletion of essential nutrients from the media by metabolically active tumor cells or of marked pH changes produced by cell metabolism, the culture conditions were altered as follows. Marrow cells alone or marrow and W-256 cancer cells were cultured in 10 ml of complete media in 12-ml Falcon friction cap tubes. At the initiation of the culture, the cell suspensions were centrifuged for 10 min at 400 x g to ensure cell contact. When 1 x 10^7 marrow cells were cultured in a 10-ml volume of complete media for 18 hr, the spontaneous release index was 25.4 ± 0.6%, whereas marrow cultured with an equal number of W-256 tumor cells had a RI of 38.2 ± 0.6%. After 18 hr of incubation, the pH of the culture fluid was 7.59 ± 0.20 for marrow cultured alone compared to 7.18 ± 0.19 for marrow and tumor cultured in a 10-ml volume. In the standard TIMC assay on which 1-ml cultures are assayed in small Falcon friction cap tubes, the initial pH of marrow cultures is 7.53 ± 0.03. At the termination of the incubation period, the pH for marrow cultured alone was 7.63 ± 0.02 as compared to 7.28 ± 0.05 for marrow cultured with W-256 cells (1:1 ratio).

In another experiment, 59Fe-labeled marrow cells were cultured in the supernatant fluid obtained from W-256 cells that had been cultured overnight in complete media. Tumor cell supernatant fluid was not cytotoxic to normal marrow cells during an 18-hr incubation period (media pH 7.20).

**DISCUSSION**

In this study, we have extended our recent observation that rodent cancer cells (W-256) will induce cytolysis of normal bone marrow cells in mixed-cell cultures (18). Currently, we have evaluated the capacity of other types of transplantable cancer cells (Novikoff hepatoma, Yoshida hepatoma, RF-75 fibroblasts, and teratocarcinoma cells) to induce the release of 59Fe and 51Cr from radiolabeled rodent bone marrow cells.

The Novikoff and Yoshida hepatoma cells were evaluated because they are invasive experimental cancers that have been studied extensively (1, 8). The Novikoff hepatoma which was initially induced by the administration of 4-dimethylaminoazobenzene (11) has been maintained subsequently as either a solid or an ascites tumor transplant. The Yoshida tumor was derived from a hepatic cancer which was induced by dimethylaminoazobenzene (17). In our studies, both Yoshida and Novikoff hepatoma cells were able to induce rat marrow cytolysis. Furthermore, there was a linear relationship between isotope release and effector cell number. The RI for 51Cr which nonspecifically binds to the proteins of all marrow cells (13) closely parallels the RI of 59Fe, which is specifically incorporated into heme proteins found primarily in erythroid tissue (15).

To determine whether cytolysis would occur in a system in which both target and effector cells were syngeneic, we assayed the cytolytic capability of transplantable teratocarcinoma cells derived from undifferentiated embryonic cells (14). Our studies indicate that mouse teratocarcinoma cells can induce marrow cytolysis when normal syngeneic mouse cells were used as target cells. In addition, mouse teratocarcinoma cells were able to induce cytolysis of rat marrow target cells. In this study, the high spontaneous release of isotope by mouse marrow target cells (40%) can probably be attributed to the fact that our culture system was devised for rat marrow cells and may not have been ideal for maintaining mouse cells.

To explore further the possibility that TIMC was the result of a transplantation phenomenon, normal mouse spleen cells were cultured with rat marrow target cells. The lack of a significant increase in rat marrow cell lysis in the presence of cocultured mouse spleen cells also suggests that tumor-induced marrow cytolysis is not a transplantation phenomenon.

RF-75 fibroblasts were evaluated because they represent a spontaneously transformed and rapidly growing cell line (16) which could be used as effector cells against normal fibroblast target cells as well as normal marrow cells. The results indicate that both RF-75 fibroblasts and W-256 cells are capable of inducing the cytolysis of normal target cells, either 59Fe-labeled marrow or 51Cr-labeled normal fibroblasts. Marrow cells appear to be more susceptible to cytolysis in the assay system used.

In this study, we considered the possibility that marrow cytotoxicity induced by tumor cells was an artifact of an in vitro system in which a relatively quiescent target cell was cultured with a metabolically active effector cell. To ensure
that marrow cell death was not due to nutrient depletion by the more active tumor cells, TIMC was assayed in a larger volume of complete media. Increasing the volume of culture media from 1 to 10 ml did not alter the ability of a constant number of tumor cells to kill marrow cells. Also, the pH change which occurs during coincubation of cancer and marrow cells was monitored and was not found to be excessive. Furthermore, when radiolabeled marrow cells were cultured in cell-free W-256-conditioned media, there was no increase in marrow cytolysis. It therefore seems unlikely that marrow cytolysis can be attributed to pH changes, nutrient depletion by tumor cells, or release of tumor degradative products.

The ability of normal cells to induce marrow cytotoxicity was evaluated. As anticipated, neither normal fibroblasts nor normal marrow cells were able to induce significant cytolysis when cocultured with normal target cells. In addition, spleen cells, normal peripheral blood lymphocytes, or nonactivated peritoneal macrophages did not induce marrow cytolysis. However, it is known that cytolytic activity is a physiological function of certain normal cells such as activated macrophages (5) and lymphocytes (6). BCG-activated peritoneal macrophages were capable of inducing the cytolysis of normal marrow cells. However, actively dividing rat lymphocytes stimulated for 3 days with PHA did not demonstrate a cytolytic capacity directed towards marrow cells. These studies indicate that marrow cytolysis is not a general property of metabolically active or rapidly dividing normal cells but is a specific function of cancer cells and activated macrophages.

We previously reported that W-256 cells were also able to lyse RBC target cells (18). Muchmore et al. (10) also reported that PHA-stimulated mastocytoma cells, mouse L-cells, and embryonic fibroblasts were able to lyse peripheral RBC.

Further experimentation with human cancer cells will be of great interest. It is important to emphasize that the demonstration of target cell lysis is dependent on the type of target cells used and the assay conditions. Using 125I-labeled fragments of embryonic chick heart and mesonephros as target tissues, Mareel et al. (9) were unable to detect the release of 125I from target cells cocultured with HeLa cells, although invasion was demonstrated morphologically.

At present it is premature to attempt to correlate TIMC with cancer invasiveness. This easily performed assay system, however, should lend itself to more definitive experiments. Furthermore, it would be of interest to correlate the enzymatic activity of cancer cells (activation of plasminogen, collagenase, etc.) with an ability to lyse normal cells.

REFERENCES

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