Apoptosis: An Early Event in Metastatic Inefficiency

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ABSTRACT

Whereas large numbers of cells from a primary tumor may gain access to the circulation, few of them will give rise to metastases. The mechanism of elimination of these tumor cells, often termed “metastatic inefficiency,” is poorly understood. In this study, we show that apoptosis in the lungs within 1–2 days of introduction of the cells is an important component of metastatic inefficiency. First, we show that death of transformed, metastatic rat embryo cells occurred via apoptosis in the lungs 24–48 h after injection into the circulation. Second, we show that Bcl-2 overexpression in these cells inhibited apoptosis in culture and also conferred resistance to apoptosis in vivo in the lungs 24–48 h after injection. This inhibition of apoptosis led to significantly more macroscopic metastases. Third, comparison between the extent of apoptosis by a poorly metastatic cell line to that by a highly metastatic cell line 24 h after injection in the lungs revealed more apoptosis by the poorly metastatic cell line. These results indicate that apoptosis, which occurs at 24–48 h after hemogenous dissemination in the lungs is an important determinant of metastatic inefficiency. Although prior work has shown an association between apoptosis in culture and metastasis in vivo, this work shows that apoptosis in vivo corresponds to decreased metastasis in vivo.

INTRODUCTION

Metastasis, the dissemination of tumor cells from a primary site to distant sites, is thought to occur by a series of steps in which tumor cells first migrate from the primary tumor, penetrate into the circulation, and eventually colonize distant sites. Evidence exists for each of these steps. Migration from the primary tumor site has been directly observed from tumors in the mammary fat pad, and intravasation from tumors in the chicken chorioallantoic membrane has been demonstrated (1, 2). Entrance of cells into the blood stream can be observed very rapidly after s.c. injections in experimental model systems and can also be documented in human patients (3). Tumor cells can be found in the blood of cancer patients, although their presence often does not predict prognosis (4–6).

Introducing tumor cells i.v. into immune-deficient experimental mice can be used as a model to duplicate the later steps of hematogenous metastasis for a wide variety of tumor cells. When lung colonies develop after i.v. injection, the cell line is regarded as having metastatic potential. In general, lung colonies form only if the cells injected are positive in spontaneous metastasis assays (i.e., if they form metastases after growth as s.c. tumors; Refs. 7 and 8). The injection of metastatic tumor cells into the mouse tail vein usually leads to the formation of lung, pleural, or mediastinal colonies, although colonies at other sites sometimes occur. By harvesting colonies from specific sites and selecting through several cycles of injection, it has been possible to select for cells that preferentially metastasize to these sites, such as the ovary or liver (9). Homing to the lung can be partially attributed to the anatomy of the circulation that obliges all blood to flow through the lungs. Some have proposed that cells lodge mechanically in the pulmonary capillaries due to size constraints, but capillary occlusion cannot be sufficient in itself for growth as a metastasis because not all tumorigenic cells give rise to lung colonies (for review see Refs. 10 and 11). Adhesion factors have been identified that are required for some tumor cells to attach to pulmonary endothelium. For example, adhesion of B16F10 melanoma cells in the lung has been shown to depend on Lu-ECAM-1, whereas dipeptidyl peptidase IV (also known as CD26) is required for the attachment of R3230AC rat mammary and RPC-2 rat prostate carcinoma cells (12–16). Esb cells derived from a murine T-cell lymphoma that metastasizes to the liver after i.v. injection instead form colonies in the muscle when B1 integrin is disrupted, but only rarely form colonies in the lung (17). Furthermore, we have recently directly observed attachment of tumor cells to precapillary arterioles in the lung (18).

Whereas introduction of metastatic tumor cells into the circulation results in colony formation, the majority of the injected tumor cells do not produce colonies. For example, i.v. injection of 5 × 10^5 cells may result in, at most, 200 colonies (19). The failure of the majority of the cells to form colonies has been termed “metastatic inefficiency” (20). Fidler and Nicolson (21) injected highly metastatic tumor cells labeled with ^125I-iodide, a radioactive thymidine analogue, into mice. By measuring radioactivity levels in various organs, they were able to determine where the injected cells migrated to after injection. These experiments indicated that the majority of cells was rapidly cleared from the blood and initially arrested in the lungs (21, 22). They demonstrated that by 24 h, >85% of the cells initially arrested in the lungs were lost. Two cell lines were used, one with a 8–10-fold greater ability to form pulmonary colonies than the other, yet both led to equivalent counts retained in the lungs at 2 min to 1 day (9, 21). These observations have been confirmed with other cell lines and with different methods of radioactive labeling by other groups (23–27). The fate of these cells that fail to metastasize has not previously been demonstrated to be due to death by apoptosis.

To directly examine the fate of tumor cells within the lungs, we labeled metastatic tumor cells with GFP and observed their fate in the first 1–2 days after tail-vein injection. Our observations indicated that many of the injected cells undergo apoptosis within the lungs. These results directly establish that tumor cells die in the lungs after introduction into the circulation through apoptotic processes.

MATERIALS AND METHODS

Cells and Cell Culture. The 2.10.10 and the 3.7 rat embryo fibroblast cell lines are independent ^ras and ^v-myc transformed metastatic lines (28). RA3.1 is a poorly metastatic rat embryo fibroblast cell line transformed by ^ras and E1A (29, 30). Clones overexpressing human Bcl-2 were isolated after transformation of 2.10.10 or 3.7 with pZIP-Bcl2 (kindly provided by John Reed, Burnham Institute, San Diego, CA) using lipofectamine (Life Technologies, Inc., Grand Island, NY), following the manufacturer’s directions. After selection with 200 μg/ml Geneticin (Life Technologies, Inc.), independent clones were isolated using cloning cylinders (2.10.10 Bcl-2 # 3; 2.10.10 Bcl-2 # 9).

3 The abbreviations used are: GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
The cells were cultured at 37°C in 5% CO₂ in DMEM (Life Technologies, Inc.) supplemented with penicillin-streptomycin (Life Technologies, Inc.) and 5% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT). Selection was maintained throughout. 2.10 and HT1080 cells expressing GFP were isolated after transfection of pEGFP-C2 (Clontech, Palo Alto, CA), 2 weeks of selection with Geneticin, followed by sorting in a flow cytometer. The 5% brightest cells were sorted and subcloned. This resulted in constitutively fluorescent clones. GFP-expressing adenovirus was a kind gift from Meenhard Herlyn (Wistar Institute, Philadelphia, PA). B16F10 cells were infected with the virus at a ratio of 20 plaque-forming units/cell, resulting in at least 90% fluorescent cells.

Radiation Treatment. Cells (1 x 10⁶) were plated on 10-cm dishes. The next day, they were subjected to 10 Gy of Cesium 137 irradiation (Shephard Mark 1 Model 68A irradiator). Twenty-four and 48 h following irradiation, cells were trypsinized, pelleted, and resuspended in 200 μl of DMEM. Cells in the medium and attached cells were pooled because apoptotic cells frequently detached. Ten microliters of propidium iodide solution [containing 3.4 mM trisodium citrate, 0.1% NP40, 4.83 mM spermine tetrahydrochloride, 0.5 mM Tris, and 0.62 mM propidium iodide (pH 7.6)] was added to 20 μl of cell suspenion, and the cells were examined under a fluorescent microscope. They were scored for apoptosis as determined by its morphology (ruffled edges, condensed chromatin and nuclear fragmentation, cell shrinkage, and formation of apoptotic bodies).

Tumorigenicity and Experimental Metastasis Assays. Female NCR-nu/nu mice, 4–6 weeks of age, were obtained from Taconic Farms (Germantown, NY) and housed aseptically (laboratory animal facilities, University of Pennsylvania). Cells used for injection were grown to subconfluence, subjected to brief trypsin treatment, washed, and resuspended in serum-free DMEM. For tumorigensis experiments, mice were injected bilaterally s.c. in both flanks with 5 x 10⁶ cells in a single cell suspension in 100 μl. Tumors were measured using vernier calipers for calculation of tumor size. Six tumors were measured for each time point. For experimental metastasis assays, mice were injected with a single cell suspension of 1 x 10⁶ cells in 100 μl into the tail vein. Animals were killed when exhibiting labored breathing or after 19 days. Lungs were fixed in 10% buffered formalin, and a dissecting microscope was used to examine lungs for evidence of metastasis.

Intravital Video Microscopy. This assay was performed as described by Al-Mehdi et al. (18). Briefly, mice were sacrificed by an i.p. overdose of sodium pentobarbital at time points ranging from 30 min to 24 h after injection with cells expressing GFP. The chest was opened, and pulmonary circulation was cleared of blood by gravity flow of perfusate through a cannula inserted in the main pulmonary artery, exiting from the transected left ventricle. The perfusate was Krebs-Ringer bicarbonate solution [118.45 mM NaCl, 4.74 mM KCl, 1.17 mM MgSO₄·7H₂O, 1.27 mM CaCl₂·2H₂O, 1.18 mM KH₂PO₄, 24.87 mM NaHCO₃ (pH 7.4), 10 mM glucose, and 5% dextran]. To visualize lung vasculature, the lungs were infused with DiI-acetylated low-density lipoprotein (Molecular Probes, Eugene, OR). The lungs were removed and examined under an inverted fluorescence microscope.

Fluorescent Microscopy. Ten-micrometer sections were cut from mouse lung frozen in Histo Prep (Fisher Scientific, Fair Lawn, NJ). The sections were fixed in 2% paraformaldehyde and stained with 2.5 μg/ml DAPI (Sigma Chemical Co., St. Louis, MO). Green fluorescent cells were confirmed by overlay with a DAPI-stained nucleus.

Immunohistochemistry. Ten-micrometer sections were cut from mouse lung frozen in Histo Prep (Fisher Scientific). The sections were fixed in 2% paraformaldehyde and postfixed in 2:1 ethanol:acetic acid solution. A Tris NaCl buffer [1 M Tris, 140 M NaCl, and 0.1% Tween 20 (pH 7.6)] was used as rinsing buffer, and the sections were blocked with 5% goat serum. The primary antibody, polyclonal rabbit anti-GFP antibody, was obtained from the University of Alberta, Calgary, Canada (Ref. 31; 1:1500; overnight at 4°C). The alkaline phosphatase-antirabbit IgG complex was from DakoCytomation (Carpinteria, CA; 1:100; 1 h at room temperature). Levamisole (10 mg/ml; Sigma Chemical Co.) blocked any endogenous alkaline phosphatase. The chromogen used to visualize the reaction was Fast Red/Naphthol Phosphate (Research Genetics, Huntsville, AL), and the sections were counterstained with aqueous hematoxylin.

TUNEL Staining. Female nu/nu mice, 4–8 weeks of age, were given injections with a single cell suspension of 2 x 10⁶ cells in 100 μl into the tail vein. After 24 or 48 h, the mice were sacrificed and the lungs were frozen in Histo Prep. Ten-micrometer frozen sections were made, and apoptosis was identified using the ApopTag kit (Intergen, Gaithersburg, MD) according to the manufacturer’s protocol. Briefly, sections were quenched in 2% hydrogen peroxide. The optimal dilution and incubation with the TdT enzyme was 1:54 for 1.5 h at 37°C. We used an antidigoxigenin antibody from Boehringer Mannheim (Indianapolis, IN; 1:1000; 1 h at room temperature) in place of the antibody in the kit. The reaction was visualized by dianaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA) as the chromogen, followed by a methyl green counterstain. Spleen sections were used as a positive control with each preparation. The number of positive cells was determined by light microscopy at ×400 magnification. The slides were scanned using a digital microtome (Microcode II, Boeckelet Instruments, Tucson, AZ) to ensure that all areas were counted only once. To determine the area of the histological sections, the slides were digitally scanned and the area of each section was calculated using Openlab software (Improvision, Coventry, United Kingdom).

Double-labeled apoptosis and anti-GFP sections were first stained with anti-GFP and visualized with Fast Red as described above, rinsed in water, and then stained with the ApopTag kit and visualized by dianaminobenzidine tetrahydrochloride, as described above. The double-labeled sections were counterstained with aqueous hematoxylin.

Immunoblotting. Western blotting was as described by Maniatis et al. (32). Briefly, 10⁶ cells were plated in 10-cm tissue culture dishes. The next day, they were lysed with 200 μl of sample buffer [10% glycerol, 2% SDS, 100 mM DTT, and 50 mM Tris (pH 6.8)]. Protein samples were denatured by boiling for 5 min and run on a 12% SDS-polyacrylamide gel. After transfer onto nitrocellulose membrane (Life Technologies, Inc.), the membrane was blocked overnight in 5% milk-PBS. Bel-2 was detected using a mouse anti-human Bel-2 monoclonal antibody (Calbiochem, San Diego, CA) at a dilution of 1:200 and a 1:5000 dilution of secondary antibody, goat antimouse IgG horseradish peroxidase (Boehringer Mannheim). Bands were visualized using the enhanced chemiluminescence kit (Amersham Pharmacia, Piscataway, NJ).

RESULTS

Observation of GFP-labeled Tumor Cells in the Lungs after i.v. Injection. To directly observe the fate of tumor cells in the lungs after tail-vein injection, we introduced a vector encoding enhanced GFP into two metastatic sarcoma tumor cell lines: 2.10.10, a rat embryo fibroblast transformed by Hras and v-my, and HT 1080, a cell line derived from a human fibrosarcoma (28, 33). Clones 2.10.10-GFP and HT1080-GFP, which express high levels of GFP, were isolated (18). 2.10.10-GFP cells were injected i.v. into nude mice. After 24 h the animals were sacrificed. Green fluorescent cells could readily be visualized in frozen sections of the lungs (Fig. 1A). Counterstaining with DAPI revealed the intact nuclei of the tumor cells surrounded by the nuclei of the cells of the lung (Fig. 1B). We noted that many of the green fluorescent cells appeared to have ragged edges, often with blebbing of the surface, and that the intensity of the fluorescence appeared diminished (Fig. 1C). The nuclei of these cells resembled apoptotic bodies (Fig. 1D). HT1080-GFP cells showed a similar morphology after i.v. injection (data not shown; Ref. 18).

TUNEL staining was performed on the lung sections to determine whether the morphological changes were associated with tumor cell apoptosis. TUNEL-positive cells could readily be detected as brown after staining (Fig. 2A). To show that the apoptotic cells were tumor cells, we used immunohistochemistry with an antibody to GFP to identify the tumor cells. The anti-GFP antibody was visualized using alkaline phosphatase-coupled anti IgG as a secondary antibody. The detection reaction for the alkaline phosphatase gives rise to a red color. GFP-positive cells could readily be detected in the frozen sections from the lungs that had been injected with tumor cells, but not in the un.injected lungs (Fig. 2B). Double-staining revealed that many of the TUNEL-positive cells also contained GFP, confirming that apoptotic cells within the lung were, indeed, tumor cells (Fig. 2C). In similar experiments, other metastatic cell lines, including HT1080 and...
the murine melanoma B16F10, also gave rise to apoptotic cells within 24 h of i.v. injection into nude mice (data not shown).

**Effect of Bcl-2 on Apoptosis and Metastasis.** An expression vector for Bcl-2 was introduced into 2.10.10 and another clone of transformed metastatic rat embryo cells, 3.7. Several clones that expressed Bcl-2 at markedly higher levels than the parental cells were isolated (Fig. 3). These cells proved to be resistant to apoptosis induced by radiation (Table 1). We then asked whether the number of TUNEL-positive cells in the lungs differed after the injection of the parental cells compared with injection with cells overexpressing Bcl-2. We counted the number of apoptotic cells in sections from the lungs harvested 24 or 48 h after i.v. injection of each cell type and computed the area of the sections counted (Table 2). Nine- to 11-fold fewer apoptotic cells were evident in the lungs of mice that received the cells overexpressing Bcl-2 at 24 or 48 h ($P = 0.005$). Thus, Bcl-2 protected cells from apoptosis in the lungs following i.v. injection. The number of macroscopic lung colonies formed, on average, was 5-fold greater ($P \leq 0.001$) after injection of either of two different clones of the 2.10.10 cells expressing Bcl-2 relative to the parent line (Table 3).

Since Pietenpol et al. (34) had found that Bcl-2-overexpressing tumor cells sometimes have diminished tumorigenicity, we asked whether tumorigenicity was affected in these cells. 2.10.10 cells expressing Bcl-2 or 2.10.10 parental cells resulted in equivalent tumor growth after s.c. injection into the flanks of mice (Fig. 4). Thus, alteration in tumorigenicity would not seem to account for these results.

**Difference in Apoptosis in Vivo between Cell Lines of Different Metastatic Potential.** Since the results reported above suggest that the extent of apoptosis in vivo affects the outcome in the lung colonization assay, we determined the numbers of apoptotic cells found in the lungs after injection of two different lines of transformed rat embryo fibroblasts with different metastatic potential. Transformation of rat embryo fibroblasts by $^H$ras with either v- or c-myc or adenovirus E1A as cooperating oncogenes results in tumorigenicity. Cells transformed by ras plus E1A, such as the cell line RA3.1, are either negative or only weakly metastatic in the lung colonization assay. In contrast, rat embryo fibroblasts transformed by $^H$ras and myc, such as 2.10.10, are highly metastatic in this assay (29, 33, 35). We compared both cell lines before injection into nu/nu mice and found that $<1\%$ were apoptotic at the time of injection. Lungs were then harvested 24 and 48 h after injection. Although both cell lines gave rise to apoptotic cells, injection of the poorly metastatic cell line resulted in significantly more apoptotic cells, especially at the 24-h time point ($P = 0.003$; Fig. 5).

**DISCUSSION**

Our data indicate that apoptosis of tumor cells occurs in the lungs within 24 h of i.v. injection and inhibition of that apoptosis can enhance lung colonization. Tumor cells that undergo less apoptosis in the lungs were more likely to successfully establish colonies. This work suggests that apoptosis is an important component of metastatic inefficiency, at early times after initial attachment. Our recent work using intravital microscopy to examine tumor cells in the pulmonary

![Fig. 1. Visualization of GFP-labeled tumor cells in the lungs. 2.10.10-GFP (2 x 10^6) cells were injected i.v. into nu/nu mice. The lung was harvested 24 h later, frozen in Histo Prep, sectioned, and counterstained with DAPI. A and C; DAPI staining. B and D, green fluorescence. A and B show the same section, and C and D show the same section. Arrows, tumor cells.](image1)

![Fig. 2. Apoptotic 2.10.10-GFP cells in the lungs after i.v. injection. 2.10.10-GFP (2 x 10^6) cells were injected i.v. into nu/nu mice. After 24 h, lungs were harvested, sectioned, and stained either for apoptosis with the ApopTag kit (A), or for GFP as described in “Materials and Methods” (B), or for both (C).](image2)

![Fig. 3. Bcl-2 expression in transfected 2.10.10 and 3.7 clones. Lysates (60 μl) from each of the indicated cells were immunoblotted using antihuman Bcl-2.](image3)
circulation, has shown that circulating tumor cells attach to the pulmonary endothelium within 4 h in vivo or minutes in organ culture (18). By that time, all detected tumor cells are attached and no longer blood-borne (9, 18, 22, 36). Thus, the regulation of apoptosis must be influencing metastasis of tumor cells in the early stages after attachment to the pulmonary endothelium. The attachment of tumor cells or normal cells to substratum has been shown in some instances to normal cells to substratum has been shown in some instances to provide protection from apoptosis induced by lack of adhesion (41). Apparently, the majority of the injected tumor cells, even from a metastatic cell line will not encounter the survival signals needed to remain viable and proliferate. Whether survival signals are provided through attachment to the endothelium, to extracellular matrix components on the surface of the endothelial cells, or in exposed basement membrane or through secreted molecules is yet to be established.

Survival in vivo in the circulation may be affected by mechanical factors. Although it has been proposed that cells are destroyed directly through sheer forces in the vasculature, it is also possible that mechanical stresses lead to apoptosis in susceptible cells (42, 43). The effect of the immune system is also unclear. In these experiments, young nude mice were used to reduce the influence of immune reactivity to tumor cells. Furthermore, there is no reason to expect that the immune system would respond within 24 h or that differential effects would be seen between the various isogenic cells used in these experiments.

The expression of genes known to affect apoptosis in culture, such as p53, Bcl-2, fas, integrin α6, or nitric oxide production affects metastasis in experimental assays. Takaoka et al. (44) observed that Bcl-2 overexpression in B16 melanoma cells strongly enhanced pulmonary metastasis, but they did not examine the effect of Bcl-2 on apoptosis in vivo. Del Bufalo et al. (45) hypothesized that Bcl-2 overexpression enhances tumorgenicity and metastatic potential of MCF7ADR cells by inducing metastasis-associated proteins. However, we found no significant differences in tumorgenicity by cells overexpressing Bcl-2 (Fig. 4). There are inverse correlations between the extent of induction of apoptosis in culture and metastases. The question raised by these studies is whether the signals used in culture bear any relationship to the actual signals received in animals. Nikiyorov et al. (46) injected a mixture of two mouse fibroblasts, one expressing Bcl-2 and the other not expressing Bcl-2, into the tail vein of nude mice. They observed a significant enrichment of Bcl-2-
expressing cells recovered 2 h after incubation in the lungs. At the same time, PCR analysis of total lung DNA revealed no change in the ratio of the injected cells. These data suggest that the depletion of non-Bcl-2-expressing cells was due to loss of viability rather than escape from lungs. However, Bcl-2 overexpression failed to alter the frequency of experimental metastasis in this case (46). McConkey et al. (47) also reported that a nonmetastatic prostate carcinoma cell line was more susceptible to apoptosis in tissue culture, when compared with a metastatic prostate carcinoma cell line that expressed twice as much Bcl-2. Owen-Schaub et al. (48) similarly found that melanoma cells resistant to fas-mediated apoptosis were more prone to metastasize, and that fas knockout mice had higher numbers of metastasis. Xie et al. (49) have demonstrated that nitric oxide production results in apoptosis in culture and that there is an inverse correlation between apoptosis in culture and metastasis. In this study, we extend these results examining apoptosis 24–48 h in vivo after injection and show that Bcl-2 overexpression inhibits this early apoptosis. We also showed that the difference in metastatic potential between two paired cell lines correlated with the difference in apoptosis in vivo at 24–48 h after injection. Thus, the effect is in addition to the possible effects on cell proliferation, dormancy, and apoptosis that will determine the rate of nodule growth.

In other studies of metastatic inefficiency, Luzzi et al. (50) observed only 20% cell death of B16F1 after injection into the superior mesenteric vein. They attributed metastatic inefficiency to the failure of dormant solitary cells to initiate growth and failure of early micrometastases to continue growth into macroscopic tumors (50). These results are significantly different with the results obtained after injection of radiolabeled B16F1 in that ~70–80% of radioactivity located in the liver is lost after 24 h, suggesting extensive cell loss (21, 22). Cameron et al. (51) observed only 25% cell loss in the lung of mice 3 days after injection of B16F10 cells into the inferior vena cava, but 75% cell loss after 3 days. Although this correlates with the study by Luzzi et al. (50), their observation regarding cell loss conflicts with the observations of Figure and Nicolson (21), also using B16F10 cells and the same mouse strain, that >90% of cells are lost within 1 day of injection (9, 22, 36, 50).

Additional data linking apoptosis to metastasis include studies examining the effect of CD44. Overexpression of a soluble CD44 fragment in a metastatic murine mammary carcinoma cell line blocked metastasis. The cells secreting this CD44 fragment underwent considerably more apoptosis in the lungs than the parental cells at 24–48 h after injection (52).

The ability of apoptosis to modulate long-term growth of a metastatic tumor colony may be separate from the apoptotic regulation that determines early survival (24–48 h) after attachment to the pulmonary endothelia. These studies indicate that the extent of apoptosis early after pulmonary attachment correlates with the outcome in terms of formation of lung colonies.

In several cases, genes that affect apoptosis and metastasis have been found to affect the number of TUNEL staining cells when the cells were grown as tumors. Kimchi’s (53–55) laboratory isolated a gene, DAP, that bears a death domain that can interact with signals through fas or tumor necrosis factor α and whose expression affects the growth of lung carcinoma cells as tumors or as metastases. Similarly, metastatic breast carcinoma cells in which αvβ1 heterodimerization is prevented grow poorly as tumors with increased apoptosis and have decreased lung colonization (56). In these cases, whether the effect is on the ability of the cells to grow as a tumor or whether survival in the circulation is also affected has not been established. Our methods allow a distinction between survival in the circulation and later growth as a tumor. They could be applied to directly evaluate the effect of specific genes on survival in the circulation.

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