Cooperative Interaction between Interleukin 10 and Galectin-3 against Liver Ischemia-Reperfusion Injury

Yong J. Lee and Young K. Song
Department of Pharmacology, Cancer Institute, University of Pittsburgh, Pennsylvania 15213

ABSTRACT

Purpose and Experimental Design: To investigate a possible interaction between interleukin 10 (IL-10) and galectin-3 for protection of human breast carcinoma BT549 cells against liver ischemia-reperfusion-induced cytotoxicity, we used a liver/tumor coculture system. We also used IL-10-deficient C57BL/6 [IL-10(−/−)] mice and their wild-type C57BL/6 [IL-10(+/+)] mice to examine these interactions.

Results: More than 90% of galectin-3 cDNA-transfected BT549 cells (BT549-Gal3) survived after 24-h coculture with C57BL/6 [IL-10(+/+)] liver fragments isolated after ischemia. In contrast, ∼70% of control vector-transfected BT549 cells (BT549-Neo) showed metabolic death after culture with liver fragment. However, when the ischemic liver from IL-10(−/−) mice was used, BT549-Gal3 did not exhibit enhanced survival against ischemia-reperfusion-induced cytotoxicity.

Conclusions: These data suggest that IL-10 and galectin-3 cooperatively interact to protect cells from ischemia-reperfusion injury.

INTRODUCTION

Recent studies have demonstrated that a majority of circulating tumor cells arrest in the hepatic sinusoid (composed of endothelial and Kupffer cells) because of size restriction (1). The arrested tumor cells occlude blood flow, and microscopic infarcts develop within the liver (2). The resultant ischemia leads to production of reactive oxygen and nitrogen radicals (i.e., superoxide anion, NO) during reperfusion by both parenchymal and nonparenchymal cells (2). NO is produced from L-arginine, which is converted into L-citrulline by NO synthase. iNOS that is located in sinusoidal cells and hepatocytes generates high levels of NO during ischemia-reperfusion injury (3). NO and superoxide anion (O₂⁻) react to form peroxynitrite (ONOO⁻), a powerful oxidant. Peroxynitrite is a major cytotoxic mediator, responsible for the destruction of tumor cells passing through capillary beds (2). The production of endogenous peroxynitrite may be associated with apoptosis of tumorigenic cells and tumor metastasis (4). Recent studies have revealed that both IL-10 and galectin-3 can protect tissue from ischemia-reperfusion injury (5–7). Although these studies suggest that these protective effects are attributable to direct or indirect inhibition of iNOS (5) or antiapoptotic activity (8–11), the mechanism of protection still remains obscure. In this study, we hypothesize that IL-10 and galectin-3 cooperatively interact to protect cells from ischemia-reperfusion injury. This report demonstrates that both IL-10 and galectin-3 are required to protect human breast carcinoma BT549 cells from ischemia-reperfusion injury.

MATERIALS AND METHODS

Cell Culture. The human breast carcinoma BT549 cell line was obtained from Dr. Hyeong-Rae C. Kim (Wayne State University, Detroit, MI). Galectin-3 was stably transfected by introducing an expression vector containing human galectin-3 cDNA as described previously (9). Galectin-3 transfected BT549 cells (BT549-Gal3) and neo-resistant control vector transfected BT549 cells (BT549-Neo) were grown in DMEM/nutrient mixture F-12 (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 200 μg/ml genetin, and 2 mM L-glutamine in a 95% air and 5% CO₂ incubator at 37°C.

Animals. C57BL/6 mice (C57BL/6NCrlBR) were purchased from Charles River (Wilmington, MA). IL-10-deficient C57BL/6 [IL-10(−/−)] mice were obtained from Dr. John Milburn Jessup (University of Texas San Antonio, San Antonio, TX).

Cell Labeling with Fluorescence Dye. For the measurement of cell viability, BT549 cells were labeled with two fluororescing reagents, Rd-Dx 10s (Sigma Chemical Co., St. Louis, MO), with maximum excitation/emission at 530/590 nm, and calcein-AM (Molecular Probes, Eugene, OR), with maximum/emission at 485/530 nm according to the method described previously with slight modifications (2). The human cells, which were prelabeled with Rd-Dx, are easily distinguished from mouse liver cells. Calcein AM was used to track viability, because it produces a fluorescence signal only in live cells with ATP-dependent esterase activity. Briefly, cells (5 × 10⁶) were suspended in 20 mg/ml Rd-Dx in PBS, and received two electrical pulses (capacity: 330 μF and 300 V; load resistance: high Ω; charge rate: fast) using Cell-Portator Electroporation System I (Life Technologies, Inc.). The Rd-Dx-loaded cells were incubated for 16 h at 37°C in complete tissue culture medium, and then adherent cells were recovered by trypsin. The cells were

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1 Supported by NIH-National Cancer Institute Grant CA-48000.
2 To whom requests for reprints should be addressed, at Department of Pharmacology, University of Pittsburgh, E1056 BST, 200 Lothrop Street, Pittsburgh, PA 15213. Phone: (412) 648-8086; Fax: (412) 624-7737; E-mail: Leeyj@msx.upmc.edu.
3 The abbreviations used are: NO, nitric oxide; IL, interleukin; Rd-Dx, rhodamine B-isothiocyanate dextran; RWV, rotating wall vessel; iNOS, inducible nitric oxide synthase.
suspended in PBS and incubated with calcein-AM at a final concentration of 4 μM for 30 min at 37°C.

**Induction of Liver Ischemia.** To induce liver ischemia, mice were anesthetized by i.p. injection of 2,2,2-tribromoethanol (200 mg/Kg), and the abdomen was aseptically opened. The portal vein and hepatic artery were clamped separately with a hemostat for 20 min.

**Mouse Liver/Tumor Cell Coculture.** The effect of liver tissue on human breast carcinoma cells was studied using a coculture method (12). Briefly, cells prelabeled with Rd-Dx and calcein AM were added to a 55-ml RWV (Systechon, Houston, TX), which had been filled with the coculture medium (an 1:1 mixture of HepatoZYME-SFM (Life Technologies, Inc.) and DMEM/F-12). The liver was dissected into 1–3 mm fragments, washed once with ice-cold PBS, and then loaded into the RWV. The RWV rotates cells around the horizontal axis under low shear stress. An air pump was connected to the RWV to test the role of reoxygenation after liver ischemia. The whole coculture system was placed in a 37°C incubator with 5% CO₂ and rotated at 15 rpm for 24 h. Samples were taken during incubation, and the numbers of Rd-Dx and calcein AM-labeled cells were determined with a Nikon upright microscope equipped for epi-fluorescence (Nikon Microphot-FXL, Tokyo, Japan). Digital images were captured with a 3CCD color video camera.

**RESULTS AND DISCUSSION**

To determine the role of galectin-3 in tumor cell death during liver ischemia-reperfusion, wild-type galectin-3 was stably expressed in galectin-3-null human breast carcinoma BT549 cells (Fig. 1). After stable transfection, we cultured BT549-Gal3 or control vector transfected BT549 (BT549-Neo) cells in a rotating suspension culture system containing mouse liver fragments from normal or ischemic liver. This coculture system maintains the architecture and viability of the liver for at least 24 h (12). As shown in Fig. 2 and Table 1, 96% or 90% of the human breast carcinoma BT549-Neo or BT549-Gal3 cells, respectively, remained viable after 24 h of coculture with normal C57BL/6 mouse liver fragments. When BT549-Neo cells were cocultured with liver fragments from an ischemic animal, only 32% of the cells survived for 24 h. In contrast, 99% of BT549-Gal3 cells survived 24 h of coculture with ischemic liver fragments. These results imply that galectin-3 protects human breast carcinoma cells against cell death during liver ischemia-reperfusion. We also examined whether BT-549 and IL-10 cooperatively play a role in the protection from ischemia-reperfusion injury. To examine this possibility, we used IL-10-deficient C57BL/6 mice [IL-10 (−/−)]. Data from Fig. 2 and Table 1 show that 96% or 99% of BT549-Neo or BT549-Gal3 cells, respectively, were viable after 24 h of coculture with normal IL-10 (−/−) mouse liver fragments. Interestingly, only 36% or 41% of BT-549-Neo or BT549-Gal3 cells, respectively, remained viable when they were cocultured with ischemic IL-10 (−/−) mouse liver fragments. These studies suggest that IL-10 and galectin-3 cooperatively interact to protect cells from ischemia-reperfusion injury.

Liver metastasis is a process that requires a sequence of several steps, including: (a) invasion from the primary breast tumor into the local host breast tissues; (b) intravasation into the circulation; (c) arrest within the microcirculation of a potential site for metastasis; (d) extravasation into the liver organ intersitium; and (e) proliferation within the parenchyma of the metastatic site. The process is relatively inefficient, and a majority of circulating tumor cells arrest in the hepatic sinusoid (endothelial and Kupffer cells) because of size restriction (1). As tumor cells arrest in the hepatic sinusoid, blood flow stops, and microscopic infarcts develop within the liver (2). Ischemia induced during tumor cell arrest in hepatic sinusoids leads to reactive oxygen and nitrogen radical (i.e., superoxide anion, NO) formation during reperfusion by both parenchymal and nonparenchymal cells (2). The toxic oxygen and nitrogen radicals kill circulating breast cancer cells with low but not high metastatic potential. Previous studies showed that metastatic potential is proportional to the number of tumor cells that survive the first 24 h of arrest in the hepatic sinusoid (2). Highly metastatic breast tumor cells are resistant to toxic molecules. In this study, our results suggest that protection from reactive oxygen and nitrogen radicals as well as inhibition of the production of these radicals play an important role against liver ischemia-reperfusion injury, perhaps even breast tumor metastasis in the liver.

IL-10 is an important anti-inflammatory cytokine that attenuates the severity of various disease states (13). Recent studies suggest that IL-10 protects from ischemia-reperfusion injury by inhibiting the activity of iNOS (5). IL-10 deficiency may augment the production of iNOS-derived NO and subsequently peroxynitrite formation. However, Jones et al. (6) reported that the protective effect of IL-10 is not dependent on the presence or absence of iNOS. Thus, this discrepancy needs to be clarified. Galectin-3 (also known as Mac-2, CBP-35, IgEBP, CBP-30, RL-29, L-29, hL-31, and LBL) is a Mr, 31,000 carbohydrate-binding protein with affinity for β-galactosides (14). The protein plays a role in cell-cell and...
studies. The models proposed here provide a framework for future cooperatively protect cells from ischemia-reperfusion injury, we are far from understanding how IL-10 and galectin-3 protects cells from NO-induced cytotoxicity. Although presses NO production by inhibiting iNOS, whereas galec-
tin-3 protects BT549 cells iNOS-induced cytotoxicity in homeostasis (11). We also observed that overexpression of the caspase pathway (9) or by maintaining mitochondrial splicing (16), cell cycle regulation (17), and angiogenesis cell-matrix interactions (10, 15), induction of pre-mRNA splicing (16), cell cycle regulation (17), and angiogenesis (18). Recent studies also demonstrate that overexpression of galectin-3 protects cells from apoptotic death by inhibiting the caspase pathway (9) or by maintaining mitochondrial homeostasis (11). We also observed that overexpression of galectin-3 protects BT549 cells iNOS-induced cytotoxicity in coculture system as well as peroxynitrite-induced cytotoxicity in vitro (7). Previous studies demonstrate that administration of iNOS inhibitor or addition of exogenous superoxide dismutase during coculture indeed blocks the killing of tumor cells (2). Literature and our results suggest that IL-10 suppresses NO production by inhibiting iNOS, whereas galectin-3 protects cells from NO-induced cytotoxicity. Although we are far from understanding how IL-10 and galectin-3 cooperatively protect cells from ischemia-reperfusion injury, the models proposed here provide a framework for future studies.

Table 1  Role of galectin-3 in ischemic liver toxicity in wild-type C57BL/6 [IL-10 (+/−)] mice or IL-10-deficient C57BL/6 [IL-10 (−/−)] mice∗

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Liver fragments</th>
<th>Treatment</th>
<th>% viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT549-Neo</td>
<td>IL-10 (+/+)</td>
<td>Normal</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>BT549-Neo</td>
<td>IL-10 (+/+)</td>
<td>Ischemia</td>
<td>26 ± 10</td>
</tr>
<tr>
<td>BT549-Gal3</td>
<td>IL-10 (+/+)</td>
<td>Normal</td>
<td>90 ± 9</td>
</tr>
<tr>
<td>BT549-Gal3</td>
<td>IL-10 (+/+)</td>
<td>Ischemia</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>BT549-Neo</td>
<td>IL-10 (−/−)</td>
<td>Normal</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>BT549-Neo</td>
<td>IL-10 (−/−)</td>
<td>Ischemia</td>
<td>34 ± 12</td>
</tr>
<tr>
<td>BT549-Gal3</td>
<td>IL-10 (−/−)</td>
<td>Normal</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>BT549-Gal3</td>
<td>IL-10 (−/−)</td>
<td>Ischemia</td>
<td>40 ± 5</td>
</tr>
</tbody>
</table>

∗Results are presented as the mean ± SE of triplicates.

Fig. 2  Survival of BT549 tumor cells after the coculture with the liver fragments from wild-type C57BL/6 mice (A–D) or IL-10-deficient C57BL/6 [IL-10 (−/−)] mice (E–H). Rd-Dx and calcein AM-labeled BT549-Neo and BT549-Gal3 cells were cocultured with the liver fragments isolated from control mouse liver (Normal Liver) or ischemic liver (Ischemic Liver). Viability of BT549 cells was determined by observation under a fluorescence microscope after 24 h. Arrowheads point out the dead cells.

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