Ethyl pyruvate administration inhibits hepatic tumor growth

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ABSTRACT

EP is a potent inhibitor of HMGB1 release that has significant anti-inflammatory activities and exerts a protective effect in animal models of inflammation. As inflammation is linked to cancer growth, we hypothesized that EP would have anti-tumor activity and explored its effects in a liver tumor model. Mice injected intraperitoneally with MC38 colorectal cancer cells led to the growth of visible hepatic tumors within 2 weeks. Pretreatment with EP 30 min prior to infusion of tumor cells and continuing daily for 9 days inhibited tumor growth significantly in a dose-dependent manner, with 80 mg/kg EP achieving >70% reduction in the number of tumor nodules when compared with untreated animals. Delayed treatment with EP also suppressed tumor growth significantly, although to a lesser extent. Tumors had early, marked leukocytic infiltrates, and EP administration decreased innate (NK cells, monocytes) and adaptive (T and B cell lymphocytic) immune cell infiltrates acutely and significantly in the liver. Serum IL-6 and HMGB1 levels, which were elevated following tumor injection, were decreased significantly in EP-treated animals. Tumors showed an increase in apoptosis in EP-treated mice, and tumor cells treated in vitro with EP had marked increases in LC3-II and cleaved PARP, consistent with enhanced autophagic flux and apoptosis. Thus, EP inhibition of tumor growth in the liver was mediated by tumor (induction of apoptosis) and host (decreased inflammation) effects. EP administration may have a therapeutic role in the treatment of cancer in conjunction with other therapeutic agents. J. Leukoc. Biol. 86: 000–000; 2009.

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Abbreviations: ALT=alanine aminotransferase, AST=aspartate aminotransferase, BL=bioimunissence imaging, CCK-8=Cell Counting Kit-8, DAP=-damage-associated molecular pattern, EP=ethyl pyruvate, HMGB1=high-mobility group box 1, HPF=high-power field, IF=immunofluorescence, MGO=methylglyoxal, NPC=nonparenchymal cells, PARP=poly(ADP-ribose) polymerase, PI=propidium iodide, RAGE=receptor for advanced glycation end products, Z-VAD-FMK=benzyloxy_carbonyl-Val-Ala-Asp-fluoromethylketone

Introduction

The liver is the most frequent site of metastasis in patients with colorectal carcinoma, the most common gastrointestinal malignancy and the second-leading cause of cancer-related deaths (over 50,000 patients/year) in the United States [1]. Liver metastasis portends a poor diagnosis, with a 5-year survival of ∼30%, and treatment options are often limited. The molecular and cellular mechanisms involved in the implantation and growth of colorectal metastatic tumors in the liver are poorly characterized, although increased attention to the so-called premetastatic niche suggests complicit involvement of host hematopoietic cells [2]. Murine studies of hepatic metastases have shown that the influx of tumor cells into the liver is accompanied by an early, acute inflammatory process characterized by the release of TNF-α and increased expression of adhesion molecules that may facilitate tumor cell adhesion and eventual growth and invasion [3]. The role of inflammation in inducing and promoting the development and growth of cancers and even tumor metastasis is becoming increasingly evident [4, 5].

Chronic inflammatory diseases such as ulcerative colitis or Crohn’s disease predispose patients to develop colorectal carcinoma, just as chronic hepatitis B or C infection increases the risk for hepatocellular carcinoma [4]. However, as inflammation is such a complex and broad process, it is still unclear as to which aspects of inflammation are important in promoting or combating neoplastic growth [6]. Chronic activation of innate immune cells—macrophages, NK cells, granulocytes, dendritic cells, and the secretion of proinflammatory cytokines and mediators—propagates a milieu that may enhance tumor growth [6, 7], and the release of DAMP molecules such as HMGB1 by stressed tumor cells may also serve as an important catalyst in promoting a pro-growth, inflammatory tumor microenvironment [4, 8]. HMGB1 signals through receptors such as the RAGE or TLR2, TLR4, and TLR9, as well as most recently, CD25/Siglec 10 [9]. Engagement of these receptors results in
the activation of NF-κB and the induction of a wide range of phenotypic changes such as activation of the innate immune system and secretion of proinflammatory cytokines and mediators; activation of endothelial cells and angiogenesis; and stem cell migration and cell motility and proliferation [8]. As a nuclear DNA-binding protein, HMGB1 plays a role in the transcription of several genes that have been implicated in cancer development such as E-selectin [10], TNF-α [11], and melanoma inhibitory activity protein [12]. It also inhibits apoptosis [8], promotes autophagy [13], and interacts with cell surface and nuclear RAGE to mediate invasion, migration, growth, and spread of cancer cells [14, 15]. Numerous studies have suggested that HMGB1 plays a role in metastasis development and thus, links it to poor prognosis in a variety of cancers, including prostate, breast, pancreas, and colon [8]. Collectively, these findings indicate that HMGB1 is an important mediator of chronic inflammatory/reparative responses, which in the setting of cancer, may lead to tumor cell survival, expansion, and metastases. We have hypothesized that inhibiting HMGB1 and other DAMPs may inhibit tumor growth, increasing the efficacy of chemotherapeutic agents [3, 16].

Pyruvate, a key metabolite in cellular energy metabolism, is the end-product of glycolysis and the starting substrate for the tricarboxylic acid cycle, which generates NADH and ATP synthesis during oxidative phosphorylation. Pyruvate has been shown in one study to display anti-inflammatory and antioxidant properties in vivo [17]; however, other studies have demonstrated a lack of such activity in vivo and in vitro [18, 19]. EP improves survival and organ dysfunction in animal models of severe sepsis, ischemia-reperfusion, acute pancreatitis, and stroke by exerting potent, anti-inflammatory effects mediated through the inhibition of NF-κB and the release of cytokines such as TNF-α, IL-1β, and IL-6 [20–23]. EP also inhibits HMGB1 release and facilitates a necrosis-to-apoptosis switch in A549 lung adenocarcinoma cells deprived of glucose (akin to the metabolic conditions found within the tumor microenvironment) via inhibition of HMGB1 [24]. In vitro studies have suggested that the ethyl moiety as well as delivery of the intact EP molecule is required for the anti-inflammatory effects of EP, as the combination of ethanol and pyruvate does not suppress the inflammatory response of endothelial cells [18]. In this study, we explored the effects of EP administration in hepatic tumor growth. We hypothesized that EP may suppress tumor growth in the liver through its anti-inflammatory and apoptotic-inducing properties.

Liver and s.c. tumor models
Intrahepatic tumors were generated by direct portal injection of MC38 tumor cells. Mice were anesthetized with a single i.p. injection of ketamine (50 mg/kg, NLS Animal Health, Owings Mills, MD, USA) and xylazine (10 mg/kg, NLS Animal Health). The portal vein was exposed through a small midline incision, and 200 μl of a suspension of 1 × 10^6 MC38 cells in HBSS was injected. Hemostasis was obtained by gentle compression of the inoculation site with a cotton swab. Mice were randomized and injected i.p. with 40–80 mg/kg EP (Sigma-Aldrich, St. Louis, MO, USA). Two dosing schedules were used: starting 30 min prior to tumor injection and daily up to 9 days and daily from 7 to 10 days after infusion of tumor cells. Control mice were injected with the same amount of PBS at the same schedule. The animals were killed by cervical dislocation 14–17 days later for measurement of tumor size, and liver metastases were counted in a blinded manner without knowledge of the treatment. The livers were collected and weighed, and blood was collected from the tail vein or by direct intracardiac puncture. For the s.c. model, MC38 tumor cells were injected into the right flank and allowed to reach a tumor size of ~5 × 5 mm and 8 × 10 mm prior to treating animals with EP (80–240 mg/kg). Two dosing schedules were used: every other day for 14 days starting 7 days after tumor injection and daily on days 11–15 and 18–22. Mice were killed, and tumors were measured using calipers. There was no evidence of any toxicity in mice administered with these EP doses.

Luciferase transfection of MC38 cells and BLI
Stably transduced MC38 cells expressing the firefly luciferase gene were generated by lentiviral transfection of the pGL4 luciferase reporter vector (Promega, Madison, WI, USA) and selected with puromycin. Growth characteristics and phenotype of the transfected cells were compared with the parental strain in vitro to verify the absence of any effects secondary to retroviral insertion. Prior to imaging, mice were anesthetized by Isoflurane (Wester Veterinary, Sterling, MA, USA) inhalation, followed by i.p. injection of luciferin (300 mg/kg, Caliper Life Sciences, Hopkinton, MA, USA). Eight minutes following administration, to allow proper distribution of luciferin, the mice were placed in the chamber of an IVIS 200 optical imaging system (Xenogen Corp., Hopkinton, MA, USA). Photon emissions in the region of interest over the tumor sites were quantified using Living Image software V.3.0 (Xenogen Corp.). Bioluminescent flux was determined and reported as photon/s/cm²/Sr².

Isolation of NPC and flow cytometry
Mouse livers were perfused in situ with 1% collagenase (Sigma-Aldrich) solution followed by further ex vivo digestion at 37°C for 30 min. To obtain adequate numbers of NPC, five livers were combined from each treatment group. The NPC were then isolated by centrifugation over a Percoll gradient (Sigma-Aldrich). Cell-surface antigen expression was analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA) using FITC- or PE-conjugated mAb against mouse CD11c, CD14, CD19, CD4, CD8, Gr-1, and NK1.1 (all from BD PharMingen, San Diego, CA, USA). Appropriate isotype and species-matched, irrelevant mAbs were used as controls.

Cell proliferation and survival assay
Cell proliferation and survival were measured using a CCK-8 (Dojindo Molecular Technologies Inc., Gaithersburg, MD, USA), according to the manufacturer’s protocol, based on measuring NADH production, resulting from dehydrogenase activity in viable cells. Briefly, 1 × 10^5 cells/100 μl well were added into 96-well flat-bottom culture plates, and individual concentrations of EP (Sigma-Aldrich) were added to the culture. After 48 h, 10 μl/100 μl CCK-8 solution was added for 1–4 h, and absorbance was measured at 450 nm using a microplate reader.

Detection of apoptosis
MC38 tumor cells (2×10^5/ml) were cultured in 24-well plates and treated with EP (10 mM) for 6 h and 24 h in the presence or absence of the

MATERIALS AND METHODS

Animals and tumor cell lines
Female C57BL/6 (B6, H-2b) mice, 8–10 weeks old, were purchased from Taconic (Germantown, NY, USA). Animals were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center (Pittsburgh, PA, USA) and used in accordance with institutional and National Institutes of Health guidelines. MC38 murine colon adenocarcinoma cells (C57BL/6 syngeneic) were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate.
caspase inhibitor Z-VAD-FMK (20 μM; Calbiochem, San Diego, CA, USA). Cells were stained with Annexin V (BD PharMingen) and PI (BD PharMingen), according to the manufacturer’s protocol. Quantitative analysis was performed by flow cytometry, with 10,000 events acquired from each sample. For identification of apoptotic cells in tissue cryostat sections, DNA strand breaks were identified by FITC-conjugated TUNEL staining. Tissues were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate. The TUNEL reaction mixture (cell death detection kit, Roche Diagnostics, Indianapolis, IN, USA) was added, according to the manufacturer’s instructions. Tissues incubated with label solution in the absence of terminal transferase were used as negative controls.

**IF staining**

A part of each lobe of the liver was embedded in OCT compound (Miles, Elkhart, IN, USA), frozen, and stored at −80°C. Cryostat sections (8 μm) were used for IF evaluation. The following primary mAb were used: rat anti-mouse CD45 (BD PharMingen) and rat anti-human HMGB1 (R&D Systems, Minneapolis, MN, USA). Slides were incubated with primary antibody overnight at 4°C. After washes in PBS, the sections were incubated with fluorescent-conjugated secondary antibodies for 45 min followed by Hoechst nuclear staining. Negative controls included staining with the corresponding isotype for each antibody and staining with secondary antibody alone. Positive controls included immunostaining of known positive-staining tissues. For the in vitro studies, MC38 cells were harvested, washed with 2% FCS-PBS twice, and resuspended at a concentration of 106 cells/ml. Each sample (100 μl) was aliquoted quickly into a cytopsin centrifuge and spun at 800 rpm for 5 min, after which, slides were dried and IF performed.

**Western blot analysis**

Western blot analysis was performed in whole-cell lysates, which were resolved on 10% SDS-PAGE gel and transferred to 0.2 μm nitrocellulose membrane. After blocking, membranes were incubated overnight at 4°C with primary antibodies specific for cleaved PARP (Cell Signaling, Danvers, MA, USA) and β-actin (Sigma-Aldrich). After incubation with peroxidase-conjugated secondary antibodies for 1 h at 25°C, membranes were developed with the SuperSignal West Pico chemiluminescent kit (Pierce, Rockford, IL, USA) and exposed to film.

**Serum aminotransferase and cytokine levels**

Blood was collected from the tail vein or by direct intracardiac puncture at different times post-tumor inoculation, and ELISA was used to measure serum IL-6 (R&D Systems), TNF-α (R&D Systems), and HMGB1 (Shino test, Japan) levels. Serum aminotransferase (ALT and AST) was measured according to the manufacturer’s instructions. Tissues incubated with label solution in the absence of terminal transferase were used as negative controls.

**RESULTS**

**EP inhibits tumor growth in the liver**

EP has anti-inflammatory activities and has protective effects when used in animal models of inflammation, sepsis, or ischemia-reperfusion [20–23]. We hypothesized that EP would inhibit tumor growth and explored its effects in vivo in a liver tumor model. Mice were injected with MC38 colorectal tumor cells into the portal vein, leading to the growth of bulky tumors as early as 14 days postinfusion (Fig. 1A), with no evidence of intra-abdominal spread or pulmonary metastases on postmortem examination of animals. Pretreatment of mice with EP 30 min prior to infusion of tumor cells and continuing daily for 9 days inhibited tumor growth in a dose-dependent manner, and a dose of 80 mg/kg EP achieved >70% reduction in the number of tumor nodules (mean tumor number with EP, 36.67 ± 10.4, vs. without EP, 10 ± 7.0; \( P < 0.05 \)), as well as a significant reduction in liver:body-weight ratio (Fig. 1B). To determine whether the anti-tumor effect of EP extended beyond early treatment, mice were injected intraportally with tumor cells, after which, EP (80 mg/kg i.p.) was administered daily for 4 days on the 7th–10th day after tumor injection. Late treatment with EP also suppressed tumor growth significantly (36% decrease; \( P = 0.05 \)) but not to the same extent as earlier and longer treatment (Fig. 1C), suggesting that suppression of early events important for tumor implantation and growth in the liver is critical to maximize the tumor inhibitory effects of EP. BLI of luciferase-labeled tumor cells permitted in vivo tracking of tumor growth and assessment of treatment efficacy (Fig. 1D). BLI showed rapid growth of tumors in the control group and reduction of tumor burden with EP treatment consistent with the pathologic examination, and early treatment displayed better efficacy than late treatment. To determine whether the effect of EP was specific to the liver, we also administered EP to treat s.c. tumors, finding minimal to no antitumor activity in this setting (Fig. 1E).

**EP decreases acute immune cell infiltrates in the liver**

IF staining of mice injected in the portal vein with MC38 cells revealed substantial numbers of infiltrating CD45+ cells, a broad marker for leukocytes, within tumors (Fig. 2A). To begin to characterize the anti-tumor activity of EP, we first examined its effect on intrahepatic leukocytes in mice with liver metastasis. Flow cytometric analysis was performed on NPC isolated from whole livers. Intraportal infusion of MC38 tumor cells resulted in no significant changes in intrahepatic leukocyte numbers 24 h after tumor injection (Fig. 2B). No changes occurred in the number of dendritic cells or neutrophils (not shown); however, there was an increase in NK1.1, CD14+*, CD4+*, CD8+*, and CD19+ cells 3 days after tumor infection, and numbers tended to decrease at the 7-day time-point. EP treatment resulted in decreased innate immune cell (NK cells, monocytes) and T and B cell lymphocytic infiltrates compared with untreated animals 3 days post-tumor cell infusion, demonstrating a significant, anti-inflammatory effect of EP.

**EP inhibits the release of inflammatory mediators**

Liver metastasis is associated with the release of proinflammatory cytokines such as TNF-α [2]. In patients with advanced colorectal cancer, serum IL-6 levels have been found to be elevated in patients with metastasis and increased HMGB1 expression along with enhanced expression of the RAGE, correlating with invasion and metastasis [25]. Mice injected with MC38 tumor cells had undetectable serum TNF-α levels (not shown); however, serum IL-6 levels were elevated significantly compared with control and sham ani-
mals, and levels peaked at 24 h and became undetectable 7 days following tumor cell infusion (Fig. 3, upper). EP administration resulted in a significant decrease of IL-6 levels 3 days post-tumor injection ($P < 0.05$). We also measured HMGB1 levels, as this DAMP molecule plays a central role in the pathogenesis of many inflammatory states, including colon cancer [8, 26]. The anti-inflammatory effects of EP are associated with decreased, systemic HMGB1 levels in mouse models of sepsis or ischemia-reperfusion [20, 22]. EP also inhibits HMGB1 release in glucose-deprived, cultured

Figure 1. EP inhibits hepatic tumor growth. (A) Representative livers removed 14 days after portal vein injection of $1 \times 10^5$ MC38 tumor cells in C57BL/6 mice, which received no treatment or EP (80 mg/kg) administered i.p. from Days 0 to 9 (Early treatment) or from Days 7 to 10 (Late treatment). Arrows point to malignant lesions. (B) Early treatment showing the dose-dependent, anti-tumor effect of EP ($n=5–6$ animals/group). Graphs demonstrate hepatic tumor number and liver:body-weight ratio. Mice were killed 14 days after portal vein infusion of tumor cells, after which, livers were removed and weighed and tumors counted. (*, $P=0.05$; **, $P=0.008$; †, $P=0.001$, by Student’s $t$-test). (C) Late treatment with EP suppressed tumor growth significantly but to a lesser extent than early treatment. *, $P<0.05$; NS = Not significant. (D) Luciferase-labeled MC38 tumor cells infused intraportally allowed in vivo tracking of tumor growth with BLI. Data are representative of three individual experiments. (E) EP does not affect the growth of s.c.-injected tumors. MC38 tumor cells were injected into the right flank of mice and allowed to reach a tumor size of $\sim 8 \times 10$ mm prior to treating animals with EP (80–240 mg/kg) daily on Days 11–15 and 18–22 after tumor injection. Mice were killed, and tumors were measured using calipers. Data are representative of three separate experiments.
A459 tumor cells [24]. Acutely following injection of tumor into the liver, significant increases in serum HMGB1 were detected in comparison with sham animals 24 h following tumor injection, and EP administration decreased these levels significantly (P<0.01; Fig. 3, lower). Curiously, there was also an apparent effect of EP administration on HMGB1 distribution within the liver. IF staining showed that HMGB1 was present predominantly in the nuclei of tumor cells and hepatocytes in untreated animals (Fig. 4). Tumor HMGB1 was not visibly affected by EP. Hepatocytes adjacent to the tumor had noticeably less HMGB1 in their nuclei and more in the cytosol when compared with hepatocytes located further from the tumor, reminiscent of a field effect [27]. These results show that EP not only decreases systemic HMGB1 levels but also may modulate HMGB1 nuclear levels in hepatocytes adjacent to metastatic tumors. Intraportal tumor injection resulted in mild, acute hepatic inflammation, as evidenced by a slight but not significant increase in serum ALT and AST levels 2 days following infusion, which decreased to normal levels by the 6th day (not shown), indicating that portal vein tumor cell infusion was not accompanied by apparent hepatic necrosis.

**EP induces tumor apoptosis in vivo and in vitro**

Apoptosis in cancer cells can be induced by hypoxia, shortage of nutrients, growth factors, γ- or ultraviolet irradiation, or chemotherapy. In A549 tumor cells stressed by glucose deprivation in vitro, EP induces a necrosis-to-apoptosis switch associated with the inhibition of HMGB1 release [24]. TUNEL staining demonstrated an increase in tumor cell apoptosis in vivo in metastatic mice treated with EP (80 mg/kg; Fig. 5A). We next characterized the effect of EP on
the relative levels and degree of apoptosis and autophagy in vitro in MC38 cells. Autophagy is an important and tightly regulated process important in maintaining cellular homeostasis that involves the degradation and recycling of proteins and cell components [28]. The role of autophagy in tumor cell survival has not been elucidated completely, and the relationship between autophagy and apoptosis is complex, but in general, they are regulated reciprocally, with inhibitors of autophagy promoting apoptosis, perhaps by modulating mitochondrial pathways or mitophagy [29]. MC38 tumor cells had a dose-dependent decrease in cell viability in vitro with increasing concentrations of EP (Fig.

Figure 4. EP decreases HMGB1 levels in the nuclei of hepatocytes adjacent to tumors. (A) Livers were removed 14 days after intraportal tumor injection in mice, treated or not treated with EP (80 mg/kg i.p.), and IF was performed using primary rat anti-human HMGB1 (orange) mAb and fluorescent-conjugated secondary antibodies. (B) Low-power (20×) image showing a “field effect” of EP on HMGB1 nuclear distribution in hepatocytes. Data are representative of three individual experiments.

Figure 5. EP induces tumor cell apoptosis. (A) Livers were harvested 14 days following portal vein injection of MC38 tumor cells in mice treated or not treated with EP. Tissues were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate, and TUNEL staining was performed. Mice treated with EP had increased tumor cell apoptosis (green spots). (B) CCK-8 assay demonstrating a dose-dependent decrease in tumor cell viability in response to EP in vitro. *, P < 0.05, compared with untreated cells by Student’s t-test. (C) Cultured MC38 cells treated with EP (40 mM) for 6 h and 24 h were harvested. (Upper) IF of Hoechst (blue), TUNEL (red), and LC3-II (green) staining. Apoptotic cells were quantitated by counting TUNEL+ cells/HPF. (Lower) Annexin V/PI staining by flow cytometry. (D) Western blot analysis for cleaved PARP (C-PARP) demonstrating a dose-dependent increase in apoptosis of MC38 tumor cells treated with EP.
The addition of EP to MC38 cells induced an increase in autophagy, as seen by LC3-II staining (Fig. 5C, upper panels), as well as significant dose- and time-dependent increases in apoptosis, as demonstrated by TUNEL and Annexin V staining (Fig. 5C), and Western blot analysis of cleaved PARP expression (Fig. 5D). The addition of Z-VAD-FMK (20 μM), a broad spectrum of caspase inhibitor, did not inhibit EP-induced apoptosis (not shown), suggesting the involvement of caspase-independent apoptotic pathways.

**DISCUSSION**

Here, we have demonstrated that the administration of EP significantly inhibited the growth of colorectal tumors in the liver. Portal vein infusion of syngeneic MC38 tumor cells in immunocompetent C57BL/6 mice resulted in the growth of liver lesions observable within 2 weeks postinfusion (Fig. 1A). Intraportal tumor injection was associated only with the development of liver tumors, with no pulmonary involvement or intra-abdominal seeding. Portal vein tumor injection has been used frequently [3, 30, 31] to study mechanisms of “metastatic” tumor growth in the liver as well as the treatment of these lesions, and our goal was to determine the effect of EP on the growth of hepatic tumors. BLI, using luciferase-labeled MC38 cells, allowed the tracking and better visualization of in vivo tumor growth and demonstrated excellent correlation with data from pathologic examination and gross measurement of tumor bulk (Fig. 1D). Pretreatment of mice with EP 30 min prior to infusion of tumor cells and continuing daily for 9 days inhibited tumor growth significantly in a dose-dependent manner (>70% reduction in the number of tumor nodules), and delayed treatment on the 7th–10th day following tumor injection also suppressed tumor growth significantly, although not to the same extent as prolonged and earlier treatment. This difference in the degree of tumor growth suppression relative to the time and duration of EP administration strongly suggests that in addition to a more modest inhibitory action in established lesions, the significant anti-tumor effect of EP in this model was associated with marked disruption of early processes and events important for the initial growth of these tumors. By contrast, s.c. tumor growth was not inhibited by EP (Fig. 1E) using doses up to three times more than what was administered to animals with hepatic tumors, demonstrating some specificity to the liver. It is unclear why s.c. tumors did not respond to EP, but the difference may be primarily a result of the timing of administration—EP was administered to animals with large, established tumors, and the lack of anti-tumor activity is consistent with the decreased efficacy seen with late EP administration in the liver tumor model. In addition, the differential distribution of EP into the skin versus the rather better-vascularized liver, differences in the individual tumor microenvironments, or alterations related to the multiple smaller tumor emboli in the portal vein versus a single large tumor at a s.c. site may also have contributed to the discrepant results in the two models.

Accumulating evidence suggests that inflammation and the development and progression of cancer are closely linked [4, 5]. Tumor metastasis into the liver triggers a profound and early (within a few hours) inflammatory cascade that begins with the release of TNF-α and IL-1β by activated Kupffer cells and leads to rapid expression of E-selectin and other vascular adhesion receptors such as ICAM-1 and VCAM-1 on hepatic sinusoidal endothelial cells [3, 30]. These early, initial events facilitate tumor cell transmigration from vessels into the extravascular space, and blockade of TNF-α-mediated E-selectin induction in mice markedly reduces the number of experimental liver metastases formed by colorectal cancer cells [30]. Inhibiting the inflammatory response modulates the premetastatic niche in the liver and therefore, abrogates colorectal tumor engraftment [2]. EP attenuates the inflammatory process significantly, inhibiting TNF-α, IL-1β, IL-6, and HMGB1 release as well as NF-kB activation [18, 23]. It is this potent, anti-inflammatory effect that led us to hypothesize that hepatic tumor growth may be suppressed by EP. There is increasing data that suggest that innate immune cells (macrophages, granulocytes, mast cells) may potentiate tumor growth and development by helping sustain a pro-growth, pro-survival, and pro-angiogenic phenotype in the tumor microenvironment [32, 33]. In the current study, intraportal infusion of tumor cells resulted in a substantial influx of leukocytes into the liver within 3 days after injection, characterized by a broad increase in innate and adaptive immune-infiltrating cells, including macrophage/monocytes (CD14+), NK cells (NK1.1+), T cells (CD4+), and B cells (CD19+), followed by a general reduction in numbers by the 7th day (Fig. 2). EP treatment led to a marked and transient decrease in inflammatory cell numbers, and the inhibitory effect was most apparent 3 days following intraportal tumor cell infusion. Tumor-bearing mice also had significant increases in serum IL-6 and HMGB1 levels 24 h following portal vein infusion and decreasing after 3 days (Fig. 3), whereas no measurable levels of TNF-α were detected (not shown). EP administration lowered IL-6 levels significantly 3 days after tumor injection and also diminished serum HMGB1 levels substantially 24 h post-tumor injection. IL-6 has multiple biologic activities and has been shown to be a marker of hepatic metastasis in patients with colorectal cancer [34, 35], and increased expression of HMGB1 in colon cancer has been demonstrated [27, 36], with one study associating overexpression of HMGB1 and its receptor, RAGE, with metastases and poorer prognosis in patients [36]. Collectively, these data suggest that inhibition of early inflammatory events that occur in the liver following tumor infusion is critical to the anti-tumor effects of EP and may partly explain the more modest activity of EP, when treatment is initiated 1 week after tumor injection.

If demonstrated abundant expression of HMGB1 in the nuclei of metastatic tumors and hepatocytes (Fig. 4). Interestingly, EP treatment resulted in a reduction of HMGB1 nuclear staining in hepatocytes adjacent to tumor, an effect that receded at a distance from the tumor, akin to a field effect phenomenon [27]. Slaughter et al. [37] first described the field effect in oral cancers (and subsequently demonstrated in multiple other cancers including colorectal cancer) in reference to the presence of abnormal epithelium adjacent to malignant tumors. The model proposed to explain the multicentricity of cancers and has been attributed to the wide area that a carci-
nogenic agent affects. The significance of this EP-induced field effect in HMGB1 distribution in hepatocytes is unclear—HMGB1 can be actively secreted by hypoxic hepatocytes or activated macrophages [38, 39] or passively released by necrotic tumor cells or tumor cells killed by cytolysic lymphokine-activated killing cells or T lymphocytes [3, 16]. It has been hypothesized that extracellular HMGB1 in the tumor microenvironment may induce pro- or anti-tumor actions, perhaps dependent on downstream effects of its interaction with cognate receptors such as RAGE or TLR4.

EP likely exerts other significant salutary effects that have not been characterized completely, such as induction of changes in reduction-oxidation status (X. Liang and M. E. de Vera, unpublished results) or in cellular metabolism critical for tumor growth. The anti-inflammatory effects of EP are associated with the inhibition of glyoxalases, enzymes that detoxify MGO, a reactive byproduct of glycolysis, into D-lactate in a two-step reaction using glutathione as cofactor [40]. Accumulation of MGO was speculated to induce reactive oxygen species that reacted with cysteine residues of proteins involved in mediating cytokine suppression. Autophagy is an important mechanism by which eukaryotic cells respond to cellular stress, providing routine housekeeping functions to remove long-lived proteins and dysfunctional organelles as well as the bioenergetic needs of the cell necessary to program cell survival and adapt [28]. Autophagy appears to have a dichotomous role in tumorigenesis and tumor progression [41], and the effect of EP on the process of autophagy in neoplastic cells is not known. Inhibiting autophagy can promote carcinogenesis by encouraging increased levels of protein synthesis and decreased levels of degradation, increasing unrepaird and accumulated mutations, removing the suppressive effects of oncogenes associated with increases in damaged organelles, and producing additional genotoxic stress such as generation of reactive oxygen species and free radicals. By contrast, in response to hypoxia, acidosis, or nutrient deprivation, autophagy may be accelerated in cancer cells, thus enhancing "programmed cell survival," and increased autophagic flux has been observed in human colon cancer [42], breast cancer [43], and hepatoma cell lines [44]. Our other studies indicate that HMGB1 is a critical regulator and promoter of autophagy, translocating from the nucleus to the cytosol in response to autophagic stimuli and limiting apoptotic cell death (M. T. Lotze and H. J. Zeh, manuscript in preparation). Indeed, inhibition of autophagy in Myc-induced lymphoma cells from p53ER<sup>ERTAM</sup> mice has been shown to enhance the ability of p53 activation or alkylating drug therapy to induce apoptosis in these cells [45]. Tumor apoptosis was markedly increased in mice treated with EP (Fig. 5A), and EP treatment of cultured MC38 cells led to a dose-dependent decrease in cell viability (Fig. 5B). EP administration increased autophagy in MC38 cells, as demonstrated by LC3-II staining (Fig. 5C) as well as apoptosis measured by TUNEL, Annexin V staining, and Western blot analysis (Fig. 5, C and D).

This study has shown that EP displays broad effects on host (inflammatory cells) and tumor cells (apoptosis). At present, it is unclear which mechanism of action is predominantly involved in the anti-tumor effects of EP, although the data suggest that both are important. The increase in leukocytic infiltrates is a transient effect and is maximal early after tumor infiltration (Day 3), complementing the finding that significant increases in IL-6 and HMGB1 release also occur early (Days 3 and 1, respectively; Fig. 3) following tumor injection. Early treatment with EP provides the maximum anti-tumor effect compared with late treatment, suggesting that inhibition of acute inflammation that occurs early following portal vein tumor infiltration may be mediating this early effect. It is our contention that the "late" anti-tumor activity of EP may be a result of its apoptotic-inducing properties, as shown by the presence of significant apoptosis in hepatic tumors harvested 2 weeks after tumor injection (Fig. 5A).

In conclusion, EP displayed marked anti-inflammatory activity and apoptotic-inducing properties and inhibited the growth of hepatic tumors significantly. Importantly, EP has been tested in Phase II trials of high-risk cardiac patients undergoing cardiopulmonary bypass and has been shown to be safe and well-tolerated [46]. Although there was no benefit conferred to these patients, EP and its more potent analogs, such as methyl-2-acetamidoacrylate [47], have never been tested in the cancer setting in humans, an area that appears promising for clinical testing.

REFERENCES

Ethyl pyruvate ameliorates host inflammation and induces tumor apoptosis


**KEY WORDS:** metabolism • cancer models • liver metastases • colorectal cancer • inflammation • HMGB1 • apoptosis • autophagy