Role of Heparanase in Radiation-Enhanced Invasiveness of Pancreatic Carcinoma

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Abstract

Pancreatic cancer is characterized by very low survival rates because of high intrinsic resistance to conventional therapies. Ionizing radiation (IR)-enhanced tumor invasiveness is emerging as one mechanism responsible for the limited benefit of radiotherapy in pancreatic cancer. In this study, we establish the role of heparanase—the only known mammalian endoglycosidase that cleaves heparan sulfate—in modulating the response of pancreatic cancer to radiotherapy. We found that clinically relevant doses of IR augment the invasive capability of pancreatic carcinoma cells in vitro and in vivo by upregulating heparanase. Changes in the levels of the transcription factor Egr-1 occurred in pancreatic cancer cells following radiation, underlying the stimulatory effect of IR on heparanase expression. Importantly, the specific heparanase inhibitor STS0001 abolished IR-enhanced invasiveness of pancreatic carcinoma cells in vitro, whereas combined treatment with STS0001 and IR, but not IR alone, attenuated the spread of orthotopic pancreatic tumors in vivo. Taken together, our results suggest that combining radiotherapy with heparanase inhibition is an effective strategy to prevent tumor resistance and dissemination, observed in many IR-treated pancreatic cancer patients. Further, the molecular mechanism underlying heparanase upregulation in pancreatic cancer that we identified in response to IR may help identify patients in which radiotherapeutic intervention may confer increased risk of metastatic spread, where antiheparanase therapy may be particularly beneficial. Cancer Res; 71(7): 2772–80. ©2011 AACR.

Introduction

Pancreatic cancer is one of the most aggressive neoplasms with an extremely low 5-year survival rate (1–3). Currently, pancreaticoduodenectomy is the only curative form of treatment, however approximately 90% of pancreatic cancer patients miss the opportunity for complete surgical resection at the time of diagnosis (3, 4). Thus, radiotherapy remains a major component of treatment modalities for controlling pancreatic tumor progression (5). However, pancreatic cancer often shows resistance to radiation, and randomized trials could not demonstrate benefit from radiation, revealing rather conflicting results (6, 7). Although the high resistance of pancreatic tumors to radiation treatment is likely multifactorial, ionizing radiation (IR)-induced increase in carcinoma cell aggressiveness is emerging as one of the important mechanisms responsible for the limited benefit of radiotherapy in pancreatic cancer (8, 9). Indeed, accumulating preclinical and clinical data suggest that under some circumstances IR may stimulate tumor aggressiveness (8–14), although the identity of downstream effectors acting at the cell or tissue levels and responsible for this effect, remains poorly investigated. Here we report that IR promotes pancreatic cancer aggressiveness through upregulation of the heparanase gene. Heparanase is a single mammalian endoglycosidase capable of degrading heparan sulfate (HS), the main polysaccharide component of the basement membrane and other types of extracellular matrix (ECM; refs. 15–17). HS is a key element participating in the self-assembly, insolubility, and barrier properties of the ECM (18–20). In addition, HS moieties in the ECM are responsible for binding of heparin-binding growth factors (i.e., bFGF, VEGF, HGF; refs. 20, 21), which are thereby protected, stabilized, and sequestered from their site of action, but upon enzymatic degradation of HS can be readily mobilized to induce growth factor–dependent processes (i.e., neovascularization, tissue repair, tumor progression; refs. 22–24). Thus, cleavage of HS by heparanase leads to disassembly of extracellular barriers, enables cell invasion (15, 16), releases HS–bound angiogenic and growth factors from the ECM depots, and generates bioactive HS fragments which promote growth factor-receptor binding, dimerization, and signaling (18, 22, 24). Direct evidence for a causal role of heparanase in tumor progression was provided by demonstration of an accelerated...
primary tumor growth (25–29) and increased metastatic ability of melanoma, lymphoma, and prostate carcinoma cells (26, 30) following overexpression of the heparanase gene, as well as by a marked decrease in the tumorigenic/metastatic potential of cells following heparanase silencing (26, 31, 32). The role of heparanase in sustaining the pathology of malignant tumors was further confirmed by numerous reports, demonstrating preferential overexpression of the enzyme in numerous cancers (reviewed in ref. 15). Causal involvement of heparanase in pancreatic carcinoma progression is particularly well-documented. There was a 30-fold increase in heparanase mRNA in pancreatic cancer tissue samples, in comparison to normal pancreatic tissue (33). Moreover, elevated levels of the enzyme have been found in body fluids of patients with active pancreatic cancer disease (34) as compared with healthy donors. Pancreatic cancer patients whose tumors exhibit high levels of the heparanase mRNA had a significantly shorter postoperative survival time than patients whose tumors contained relatively low levels of heparanase (33, 35). Cultured pancreatic cancer cells overexpressing the heparanase enzyme displayed enhanced invasiveness in vitro (33, 35). Recent finding that heparanase is a highly significant independent variable for lymph node metastasis in pancreatic carcinoma patients further demonstrate a crucial role of the enzyme in the aggressiveness of pancreatic cancer (36).

In the present study we investigated heparanase involvement in radiation-enhanced aggressiveness of pancreatic cancer. We found that clinically relevant doses of IR augment invasive ability of pancreatic carcinoma cells in vitro and in vivo through upregulation of heparanase expression, and revealed that the molecular mechanism responsible for IR-induced heparanase transcription involves the Early growth response 1 (Egr-1) transcription factor. These observations may assist in defining a subset of patients in which radiotherapeutic intervention may confer increased risk of tumor growth and metastatic spread. Moreover, compound SST0001 (nonanticoagulant N-acetylated, glycol split heparin100NA, RO-H) a specific heparanase inhibitor (37), abolished IR-enhanced invasiveness of pancreatic carcinoma cells in vitro, whereas combined treatment with SST0001and IR, but not IR alone, attenuated orthotopic pancreatic tumor spread in vivo. Taken together, our results suggest that combination of radiotherapy with a specific heparanase inhibitor as an effective strategy to prevent tumor resistance and progression, observed in many IR-treated pancreatic cancer patients.

Materials and Methods

Cell culture, irradiation, and immunostaining

PANC1 and AsPc-1 human pancreatic carcinoma cells (ATCC) were routinely maintained in DMEM supplemented with 1 mmol/L glutamine, 50 µg/mL streptomycin, 50 U/mL penicillin, and 10% fetal calf serum (FCS; Biological Industries) at 37°C and 7.5% CO2. Prior to IR treatment, cells were maintained for 16 hours in serum-free medium and then irradiated with doses indicated in Results, using a 60Co Picker unit irradiator (1.56 Gy/minute). Cells were then lysed and processed for RNA/protein extraction and assessment of heparanase enzymatic activity. In some experiments, cells were cultured on glass coverslips (12 mm; Carolina Biological Supply Company), fixed with 100% ice-cold methanol and processed for immunofluorescent staining with 1:500 diluted antiheparanase monoclonal antibody H100 (38, 39), kindly provided by Dr. P. Kusse (ImClone Systems Inc.).

Orthotopic injection of pancreatic carcinoma cells

Six weeks old male severe combined immunodeficient (SCID) mice were anesthetized, a small abdominal flank incision was made and the spleen exteriorized. The luciferase expressing PANC1 (PANC1-Luc) cells (0.5 × 106) were resuspended in 0.02 mL PBS and injected subcapsularly to the pancreas. A successful subcapsular intrapancreatic injection of tumor cells was verified by the appearance of a fluid bleb without i/p leakage. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee.

In vivo imaging

To monitor in vivo progression of Luc-expressing tumors, the cooled-charge-coupled device (CCCD) camera model LN/CCD-1300EB equipped with ST-133 controller and a 50-mm Nikon lens (Roper Scientific; Princeton Instrument), supported with appropriate software was used for light detection, as described (26, 40). In all experiments animals were anesthetized before light detection. The exposure conditions (including exposure time, distance of lens from the object, and time after injection of luciferin) were kept identical. Ten minutes before monitoring light emission, the animals were injected intraperitoneally with Beetle luciferin (Promega Corp.) in PBS at 126 mg/kg body weight. Animals were placed in a dark box supplemented with a controlled light to take pictures of the background gray-scale image and then exposed to the CCD to generate a pseudo-color image that represents light intensity. For colocalization of the bioluminescent emission on the animal body, gray-scale and pseudo-color images were merged and quantification of bioluminescence was performed applying Meta Imaging Series software (Molecular Devices).

Tumor irradiation

Mice were anesthetized and in vivo bioluminescent imaging was used to delineate the luciferase-expressing pancreatic tumors. The tumor margins were marked on the skin and the animals were restrained by adhesive tape during irradiation. Tumors were irradiated applying brachytherapy afterloader (192 Nucletron microSelectron HDR) through bronchial sleeve applicator. The dose (6 Gy) was calculated to 1.0 cm isodose line and 0.5-cm width silicon bolus was placed above and below the sleeve to achieve dose homogeneity. The treatment field was designed to cover the tumors and protect the rest of the body. The prescribed dose was confirmed by film dosimetry. Variation of dose inside the tumors was estimated to be within ±10% of the prescribed dose. Tumor growth was monitored at the indicated times immediately prior to irradiation and 7 days after irradiation by whole-body bioluminescent imaging and quantification of bioluminescence, applying Meta Imaging Series software.
Statistics
The results are presented as the mean ± SD. Differences between groups were assessed by the unpaired Student’s t test. Chi test was performed to examine the association between IR and PANC1-Luc tumor dissemination in vivo.

Results
IR increases heparanase expression and invasiveness of pancreatic carcinoma cells in vitro and in vivo
As radiation-induced tumor aggressiveness was previously studied applying primarily in vitro model systems (8, 9, 13), we initially investigated whether similar mechanism operates in vivo. For this purpose, human pancreatic carcinoma PANC1 cells, genetically engineered to express the luciferase gene (PANC1-Luc), were implanted orthotopically into the pancreas of SCID mice, and whole-body bioluminescent imaging was applied to monitor tumor progression. Once the primary orthotopic tumors became detectable by bioluminescent imaging (2 weeks postinjection, Supplementary Fig. S1A), the mice were divided into 2 groups (n > 10 in each group); tumors growing in mice from the experimental group were treated with IR (6 Gy), as shown in Supplementary Fig. S1B and described in Materials and Methods, whereas mice of the control group remained untreated. Whole-body bioluminescent imaging was repeated 7 days post–IR treatment to assess tumor progression. As expected, the increase in total bioluminescent signal emitted by the IR-treated primary tumors was approximately 2-fold lower than that emitted by the untreated tumors, indicating inhibitory effect of IR on tumor cell proliferation rate. This finding is in agreement with the inhibitory effect exerted by IR on Panc1-LUC cell proliferation in vitro (Fig. 1A). However, whole-body imaging analysis revealed Panc1-LUC tumor dissemination, evidenced by the appearance of newly formed bioluminescent foci on day 7 post-IR, in 73% of IR-treated mice versus only 23% of untreated mice (chi-square test P = 0.008). Notably, the increase in overall tumor area (which includes both primary and secondary tumor sites and reflects tumor spread rather than growth) did not differ statistically in IR-treated and untreated mice on day 7 post–IR treatment (not shown). Collectively these results suggest that along with its antiproliferative action on PANC1 cells, IR may promote dissemination of orthotopic pancreatic tumor in vivo. These findings are in full agreement with the ability of IR to augment invasiveness of PANC1 cells in vitro (Fig. 2B and ref. 9).

Critical involvement of heparanase in tumor cell invasiveness (and particularly in aggressive behavior of pancreatic cancer (33–36) prompted us to examine the effect of IR on heparanase expression in this experimental setting. For this purpose, PANC1-Luc orthotopic tumors growing in SCID mice were either untreated or irradiated with a dose of 6 Gy, as described previously. Twenty-four hours postirradiation, the tumor-bearing mice were sacrificed, their tumors excised, and heparanase mRNA expression levels in IR-treated versus untreated tumor tissue were compared by quantitative real-time PCR (qRT-PCR). Because our experimental model consists of PANC1 human pancreatic carcinoma cells growing in a mouse host, utilization of primers specific to either human or mouse heparanase mRNA sequences enabled us to distinguish between tumor- and host-derived heparanase. As shown in Figure 2A, qRT-PCR analysis with human heparanase specific primers revealed a 4-fold increase in heparanase mRNA levels in the orthotopic tumor following in vivo irradiation. When mouse heparanase specific primers were utilized, no significant difference in host-derived heparanase expression was detected between irradiated and nonirradiated tumors (not shown). To further validate a stimulatory effect of IR on heparanase expression, 2 pancreatic cancer cell lines, PANC1 and AsPc-1 (characterized by relatively low basal level of heparanase expression) were untreated or treated with clinically relevant doses of IR, and heparanase mRNA levels were assessed by RT-PCR. As shown in Figure 2B, RT-PCR analysis demonstrated a dose-dependent increase in heparanase mRNA levels following cell exposure to IR. Consistent with the above findings, increased levels of heparanase protein in IR-treated versus untreated pancreatic cancer cell lines were detected by immunofluorescent staining (Fig. 2C), and confirmed by increased enzymatic activity (Fig. 2D), further corroborating a stimulatory effect of IR on heparanase expression. Noteworthy, treatment with gemcitabine, the backbone of chemotherapy in pancreatic cancer (5), often given concurrently with radiation therapy, did not abolish IR-induced heparanase expression in pancreatic carcinoma cells (not shown).

Effect of IR on heparanase expression is mediated by Egr1 transcription factor
We next investigated the molecular mechanism underlying IR-driven increase of heparanase expression in pancreatic cancer. The Egr1 transcription factor is an important regulator of heparanase promoter activity. Egr1 was shown to
control heparanase expression acting as either activator or repressor of heparanase transcription, depending on the cell and tissue type (41, 42). Notably, IR was reported to affect Egr1 expression in several cell types (43–45). Applying immunoblot analysis to investigate IR-induced changes in Egr1 protein levels, we found that in PANC1 cells transient increase (3-fold) in Egr1 during the first 15 minutes post-IR was followed by a profound and continuous downregulation of Egr1, as compared with its basal levels in the untreated cells (Fig. 3A and B). These results are in accordance with the RT-PCR data showing decreased levels of Egr1 mRNA in IR-treated versus untreated PANC1 and AsPc-1 cells (Fig. 2B, middle). Of note, in pancreatic carcinoma cells the decrease in Egr1 levels occurred in parallel with IR-induced overexpression of heparanase (Fig. 2B).

These observations led us to hypothesize that, unlike in the majority of cancer cells, in pancreatic carcinoma Egr1 acts as a repressor rather than activator of heparanase transcription. To test this assumption, PANC1 cells were cotransfected with plasmids encoding for luciferase (LUC) driven by the heparanase promoter (46) together with either Egr1 expressing vector (pEgr1) or empty pcDNA3 vector. Luciferase activity was measured in cell lysates 48 hours posttransfection and normalized with β-galactosidase. Four-fold decrease in heparanase promoter activity was detected in PANC1 cells cotransfected with Egr1 expressing vector, as compared with cells cotransfected with empty vector ($P = 0.0001$; Fig. 3C), validating the inhibitory effect of Egr1 on heparanase promoter in pancreatic carcinoma. Moreover, ChIP analysis demonstrated decreased occupancy of the heparanase promoter by Egr1 in IR-treated versus untreated cells 4 hours post-IR (Fig. 3D), in further support of the role of Egr1 in IR-induced heparanase expression.

**Specific heparanase inhibitor abolishes IR-induced invasiveness of Panc-1 cells in vitro**

We next utilized the Matrigel invasion assay (47) to test whether the stimulatory effect of IR on pancreatic carcinoma cell invasiveness is due (at least in part) to the IR-induced increase in heparanase expression. For this purpose, PANC1 cells added to the upper compartment of Boyden chambers were untreated or irradiated with 10 Gy and allowed to migrate through Matrigel in the presence or absence of a
specific inhibitor of heparanase enzymatic activity SST0001 (\(100\text{NA}, \text{RO-H}, 100\% \text{ N-acetylated, 25}\% \text{ glycol-split heparin}; \text{ref. 37}). SST0001 inhibits the heparanase enzyme at 10 nano-molar concentrations \textit{in vitro} (37), as well as melanoma metastasis (48) and myeloma tumor growth (29) \textit{in vivo}. As shown in Figure 4, IR markedly (4-fold) enhanced invasion of PANC1 cells through Matrigel-coated filters and this enhancement was abolished in the presence of SST0001.

Enhanced antitumor effect of combined SST0001 and IR treatment

To examine the effects of combining SST0001 and radiation \textit{in vivo}, SCID mice were orthotopically injected with PANC1-LUC cells. Two weeks after cell injection, when the bioluminescent pancreatic tumors were detected (as described in Materials and Methods), the mice were divided into 4 groups \((n = 7)\) and treated with either radiation alone (6 Gy), SST0001 alone (600 \(\mu\text{g/mouse}, \text{administered daily i.p.}), \text{radiation (6 Gy)} \text{ followed by treatment with SST0001}, \text{or vehicle alone (saline, administered daily i.p.)}. Progression of the orthotopic PANC1-Luc tumors was monitored by measurements of the total tumor size (including primary and secondary sites) on day 1 and day 7 of the experiment, applying Meta Imaging Series software and the effect of the different treatments on PANC1-Luc tumor spread was estimated as the change in total tumor size between experimental day 1 and 7. Administration of both SST0001 and IR, but not each treatment alone, attenuated PANC1-Luc tumor spread, as evidenced by an unchanged total tumor size (tumor area on day 7 divided by tumor area on day 1 = 1) measured in mice under combined treatment,
versus approximately 3-fold increased tumor size observed in mice that were untreated or treated with either SST0001 or IR alone (Fig. 5). In addition, newly-formed bioluminescent tumor foci were detected on day 7 post-IR in only 21% of mice that received combined treatment with SST0001 and radiation, as compared with more than 70% of mice that received IR treatment alone (chi-square test $P = 0.009$).

Discussion

Although improved surveillance and therapy have decreased the mortality of most cancer types during the last decades, the impact of these advances on pancreatic carcinoma patients remains rather modest. Pancreatic cancer is the fourth leading cause of cancer death in the United States and the 5-year survival rate is less than 5% (2). This poor outcome is often explained by a high intrinsic resistance of pancreatic tumors to conventional treatment, primarily due to overexpression of different effector molecules that prevent the normal response to drugs and radiotherapy (3). Thus, more data about the identity of downstream effectors responsible for pancreatic cancer cell resistance to therapy are needed to properly address the disease treatment. Targeting these effector molecules may represent an attractive therapeutic approach in pancreatic carcinoma. Considerable progress has been made in deciphering the role of multiple mutations that render pancreatic cancer resistance to treatment by triggering protooncogene expression or inactivating tumor suppressor genes (3). Much less is known about the involvement of ECM-degrading enzymes in pancreatic cancer resistance. Here we demonstrate for the first time the functional importance of heparanase in modulating response of pancreatic cancer to radiotherapy and describe a new molecular mechanism underlying radioresistance of pancreatic tumors.

It should be noted that like nearly any known treatment modality, radiotherapy may exert an adverse effect upon metastasis under some conditions (49–51). Several researchers have observed that local irradiation increases invasiveness/aggressiveness of different tumor cell types, including pancreatic cancer (8–14). In light of these findings it is conceivable that radiation-induced aggressiveness may hamper effectiveness of radiotherapy in pancreatic and probably other types of cancer. Our present study indicates that stimulation of heparanase expression by IR may be an integral part of this phenomenon.

Heparanase plays a pivotal role in creating a supportive microenvironment for tumor development and dissemination (16, 32, 52). Potential contributions of heparanase activity to pancreatic cancer progression (via enhanced tumor growth, invasiveness, angiogenesis, metastasis) are well documented (33–35), and further supported by a significant independent association of heparanase positivity with pancreatic adenocarcinoma dedifferentiation and lymph node metastasis (36). Here we have shown that IR augments heparanase expression...
levels in untreated PANC1 cells (Fig. 3A). At the same experimental setting, ChIP analysis revealed a marked decrease in occupancy of the heparanase promoter by Egr1 following IR treatment (Fig. 3D). In this respect, it should be noted that applying quantitative real-time PCR we observed that pancreatic carcinoma cell lines (i.e., PANC1, AsPc-1) are characterized by relatively higher basal levels of Egr1, as compared with cancer cells of nonpancreatic origin (i.e., breast and colon carcinoma, melanoma, glioma; not shown). In addition, we found that pancreatic carcinoma cells express high basal levels of NGFI-A/Egr1-binding protein NAB2 (3- to 6-fold higher than in breast and colon carcinoma; not shown). NAB2 is a transcriptional corepressor that directly interacts with Egr1 and represses activation of its target promoters (54). It is therefore plausible that in the presence of both Egr1 and NAB2 the heparanase promoter is repressed in pancreatic cells. However, following IR-associated temporal decrease in Egr1, this repression is released, allowing for activation of the heparanase promoter. Clearly, as heparanase is not the only target gene of Egr1, additional Egr1-controlled genes may contribute to the observed effect of IR on tumor aggressiveness in vivo as well.

Combination of radiotherapy with drugs that inhibit IR-induced tumor aggressiveness may be an attractive strategy to diminish adverse prometastatic action while retaining the therapeutic benefit of radiation, thus reducing resistance of pancreatic cancer to treatment. Our study presents evidence that SST0001, a specific inhibitor of heparanase enzymatic activity (37) attenuates radiation-induced invasiveness in an orthotopic model of pancreatic cancer (Fig. 5). Spread of orthotopically growing pancreatic tumors was significantly reduced in mice treated with a combination of SST0001 and IR, as compared with either modality alone (Fig. 5). The ability of SST0001 to inhibit IR-induced tumor invasiveness (Fig. 4) is a most likely explanation for the enhanced antitumor effect of combined SST0001 and IR treatment in this system. It should be noted that SST0001 alone did not exert a significant inhibitory effect on PANC1-Luc orthotopic tumor growth (Fig. 5). A likely explanation for the lack of effectiveness of SST0001 monotherapy in this system is that PANC1 cells are characterized by low endogenous levels of heparanase. Interestingly, in a model of orthotopic Panc02 mouse pancreatic carcinoma (55, 56), expressing high levels of endogenous heparanase, administration of SST0001 alone resulted in 2-fold inhibition of orthotopic Panc02 tumor growth (P = 0.044, Supplementary Fig. S2).

Attenuation of IR-mediated pancreatic tumor spread by SST0001 represents a proof of concept for heparanase inhibition as a relevant approach to diminish radio-resistance in IR treated pancreatic cancer patients. Our results warrant further studies aimed at identifying the most effective dose and schedule administration schemes for combined SST0001 and IR treatment, toward future translation to clinical setting. In parallel, elucidation of the molecular mechanism underlyng heparanase upregulation in pancreatic cancer in response to IR will help to better define target patient populations in which future antiheparanase therapies could be particularly beneficial.

![Figure 5. Enhanced antitumor effect of combined SST0001 and IR treatment. SCID mice were orthotopically injected with PANC1-LUC cells and either remained untreated (white bar), treated with conformal IR (6Gy) alone (grey bar), SST0001 alone (daily i.p injection of 600 µg/mouse; white dotted bar) or IR in combination with SST0001 (administered daily i.p. for 7 days; grey dotted bar). A, tumor progression was monitored by whole-body bioluminescence imaging of the orthotopic PANC1-LUC tumor, prior (day 1) and 7 days after (day 7) administration of IR. Representative images are shown. B, the effect of different treatments on tumor spread presented as the change of total tumor size (primary + secondary tumors), analyzed applying Meta Imaging Series software. Tumor spread was attenuated by a combined treatment with IR and SST0001 (*, P = 0.037), but not IR or SST0001 alone.](cancerres.aacrjournals.org)
Disclosure of Potential Conflicts of Interest

C. Pisano is employed by Sigma-Tau Industrie Farmaceutiche Riunite S.p.A, and is listed as inventor on a patent for SST0001. A. Meirovitz, E. Hermano, I. Lerner, E. Zcharia, T. Peretz, and M. Elkin declare no conflict of interest.

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