The Secretory Leukocyte Protease Inhibitor Is a Type 1 Insulin-Like Growth Factor Receptor–Regulated Protein that Protects against Liver Metastasis by Attenuating the Host Proinflammatory Response

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Abstract

The secretory leukocyte protease inhibitor (SLPI) can attenuate the host proinflammatory response by blocking nuclear factor κB (NF-κB)–mediated tumor necrosis factor α (TNF-α) production in macrophages. We have previously shown that highly metastatic human and mouse carcinoma cells, on their entry into the hepatic microcirculation, trigger a rapid host proinflammatory response by inducing TNF-α production in resident Kupffer cells. Using GeneChip microarray analysis, we found that in mouse Lewis lung carcinoma subclones, SLPI expression was inversely correlated with tumor cell ability to induce a proinflammatory response and metastasize to the liver and with type 1 insulin-like growth factor receptor expression levels. To establish a causal relationship between SLPI expression and the metastatic phenotype, we generated, by transfection, multiple clones of the highly metastatic H6.25, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1. Phone: 514-934-1934, ext. 36692; Fax: 514-843-1411; E-mail: pnina.brodt@mhbc.mcgill.ca.
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Introduction

The secretory leukocyte protease inhibitor (SLPI) is an 11.7-kDa, nonglycosylated, single-chain protein that is produced by different cell types including lung epithelial cells, secretory cells of the salivary glands, and various host inflammatory and immune cells such as macrophages, neutrophils, and B lymphocytes (1–3). It was initially identified as a serine proteinase inhibitor with activity against neutrophil elastase, cathepsin G, trypsin, and chymotrypsin (4) and its major function was thought to be tissue protection from inflammation-induced damage caused by these enzymes. More recently, it has been recognized as an anti-inflammatory modulator with a broad range of activities that may or may not be related to its antiprotease functions. These include increasing host resistance to bacterial and viral infection (5, 6), down-regulation of prostaglandin E2 and matrix metalloproteinase-1 and -9 production (7), and attenuation of the macrophage response to lipopolysaccharide (LPS; ref. 8).

Whereas the precise mechanisms mediating these various activities have not been fully elucidated, the evidence suggests that they are related to the ability of SLPI to inhibit NF-κB activation and, as a result, reduce tumor necrosis factor α (TNF-α) production and attenuate TNF-α-induced inflammation in response to various stimuli (3, 9–11). Collectively, these activities have identified SLPI as an endogenous regulator of innate host immunity and inflammation of which the main function is to provide tissue protection from the extreme effects and damage caused by an excessive inflammatory response.

TNF-α is a key cytokine in the acute phase of inflammation (12). It also contributes to malignant progression (12–14). Direct evidence for the involvement of TNF-α in malignancy comes from the observation that mice lacking the TNF-α gene are resistant to skin carcinogenesis (15). In other studies, it has been shown that the i.p. administration of recombinant TNF-α in mice led to a significant increase in the number of liver metastases, in several models including metastatic lymphoma (16) and solid tumors (17–19). This effect may be mediated through several mechanisms including TNF-α-induced tumor cell motility and increased production of metalloproteinases, resulting in enhanced invasion and angiogenesis (13, 20). In addition, TNF-α can induce the expression of several adhesion receptors on vascular endothelial cells. These include E- and P-selectin that can mediate tumor-endothelial cell adhesion and trigger a cascade of cellular interactions that are required for tumor cell transmigration (19–24).

In a series of studies, we have previously shown that tumor cell entry into the hepatic microvasculature can trigger a rapid proinflammatory cascade that begins with increased local TNF-α and interleukin 1 (IL-1) production by activated Kupffer cells and leads to up-regulated expression of E-selectin and other vascular adhesion receptors, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on the hepatic sinusoidal vessels (22, 25). Furthermore, a blockade of TNF-α mediated E-selectin induction by mouse-specific C-raf antisense oligonucleotides or the inhibition of E-selectin function by specific neutralizing antibodies resulted in a marked reduction in the number of experimental liver metastases following the

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injection of human colorectal carcinoma CX-1 and mouse Lewis lung carcinoma derived H-59 cells, respectively (21, 23). These results showed that E-selectin plays an important role in the establishment of hepatic metastases by these tumor cells. Taken together, these results suggested that metastatic tumor cells can trigger an endogenous, local host proinflammatory response that can promote liver metastasis through the up-regulation of vascular adhesion receptors (22, 23, 25). This response was tumor specific and could not be observed with a Lewis lung carcinoma subline (M-27) that is poorly metastatic to the liver (22, 25).

Using RNA chip microarray analysis to identify genes that are differentially expressed in the two carcinoma cell lines of common origin, H-59 and M-27, with divergent potentials to induce a proinflammatory response and metastasize to the liver, we identified SLPI as a potentially protective gene against liver metastasis. The objective of this study was to evaluate the antimetastatic function of SLPI and explore its role in the regulation of the host proinflammatory response to liver-invading tumor cells.

Materials and Methods

Cell lines. The origin and metastatic properties of in vivo derived Lewis lung carcinoma subclones H-59 and M-27 were previously described in detail (26). H-59 cells are highly metastatic, primarily to the liver. M-27 cells are moderately metastatic to the lung and do not metastasize to the liver. To produce green fluorescent protein (GFP)–expressing H-59 tumor cells, the cells were transduced with the VLTR-GFP retrovirus as we previously described (25, 27). The control macrophage cell line ANA-1 was derived from the bone marrow of C57BL/6 mice and previously characterized in detail (28). It was used here as a reference cell line in assays characterizing the secretory properties of the carcinoma lines. All cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT) in a humidified incubator containing 5% CO2.

Plasmids and transfection. Metastatic H-59 carcinoma cells were transfected with the pCDNA3.1 vector (Invitrogen, Carlsbad, CA) encoding a full-length mouse SLPI cDNA. Clones were selected in culture medium containing 400 μg/mL G418 (Life Technologies, Inc., Grand Island, NY). Mock-transfected cells were generated by transfection with the pcDNA3.1/neo plasmid under identical conditions. M-27\(^{\text{M-27g}}\) cells and M-27\(^{\text{IGF\text{-}IR}}\) cells were generated as we previously described in detail (29). All transfected clones were maintained in culture medium containing 200 μg/mL G418 and analyzed by reverse transcription-PCR (RT-PCR) and Western blotting to ascertain SLPI mRNA and protein expression and secretion, respectively.

RT-PCR. Total cellular RNA was extracted from snap-frozen liver fragments using Trizol (Invitrogen) according to the instructions of the manufacturer. Two micrograms of total RNA were reverse transcribed using the Thermoscript RT-PCR System (Invitrogen) with Random Hexamers as primer. The mixture was incubated for 10 minutes at 25°C, then for 45 minutes at 55°C, and finally for 5 minutes at 85°C. The upstream and downstream primers used were as follows: for TNF-α, 5′-GAGATGC-TACTTGAGTCATCGTGC-3′ and 5′-CCCTTCAGAGAACTCAGGAATGGG-3′ (expected product, 331 bp); for E-selectin, 5′-GTACATACATGCTCCAGT-GAG-3′ and 5′-CACACGGTTCTGACGATGTG-3′ (expected product, 469 bp); for SLPI, 5′-TCACTGGTGCTCTGCTGCTG-3′ and 5′-GCTCCCTTGGCAGAC- CATTG-3′ (expected product, 300 bp); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GGAAGTGGTGGTTGACGATTG-3′ and 5′-AAAGTCAAGTGGTGGATGACC-3′ (expected product, 520 bp). cDNA amplification was done with the Platinum Taq DNA polymerase kit (Invitrogen) using 30 cycles of amplification for SLPI, TNF-α, and E-selectin and 26 cycles for GAPDH, each for 30 seconds at 94°C, 58°C (TNF-α, GAPDH), or 56°C (SLPI), and 30 seconds at 72°C, followed by a 7-minute incubation at 72°C. The amplified DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel without further purification.

Real-time PCR. RNA extraction and reverse transcription were done as described above. The cDNA amplification was done in a LightCycler (Roche Applied Science, Indianapolis, IN) using the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Applied Science) and a standard PCR reaction mixture containing 3 mmol/L MgCl2, 0.5 mmol/L each of the TNF-α, GAPDH, and IGF-IR primers, and 2 μL of cDNA. Twenty microliters of this reaction mixture were initially incubated for 10 minutes at 95°C to denature the DNA and activate the FastStart Taq DNA Amplification was then done

![Figure 1. SLPI expression levels are inversely correlated to IGF-IR expression and the liver-metastasizing potential. A, representative data of RT-PCR analyses done on RNA extracted from the tumor cells and results of laser densitometry based on three experiments; columns, mean ratio of SLPI/GAPDH relative to H-59 cells that were assigned a value of 1 (left) or mean ratio of IGF-IR/GAPDH relative to M-27 cells that were assigned a value of 1 (right); bars, SD. B, to assess the SLPI protein expression level, Western blot analysis was done on 48-hour serum-free conditioned media obtained from the tumor cells and concentrated 40-fold before analysis.](https://www.aacrjournals.org/doi/10.1158/0008-5472.CAN-05-3043)
using 46 cycles of denaturation (95°C, 10 seconds, ramp rate 20°C/s), annealing (58°C, 5 seconds, ramp rate 20°C/s), and extension (72°C, 26 seconds, ramp rate 20°C/s). A single fluorescence reading was taken at each extension step. The crossing points, marking the cycle when the fluorescence of a given sample significantly exceeded the baseline signal, were recorded and expressed as a function of cycle number. The data were analyzed using the LightCycler software.

Western blot assay. Subconfluent monolayers (~8 × 10⁶ cells) were washed extensively to remove serum and the cells were cultured in serum-free medium for 48 hours at 37°C. To assess the levels of secreted SLPI, the conditioned media were first concentrated 40 or 100-fold and then analyzed by Western blotting. Fifty micrograms of protein were loaded per lane of a 15% SDS-polyacrylamide gel and the separated proteins transferred onto nitrocellulose membranes and probed with a rabbit polyclonal antibody to mouse SLPI as previously described (3). A peroxidase-conjugated affinity-purified donkey anti-rabbit immunoglobulin G (Cedarlane, Hornby, Ontario, Canada) was used as secondary antibody at a dilution of 1:10,000 and the enhanced chemiluminescence system used to visualize the protein bands.

Analysis of the activity of SLPI secreted by gene-transfected H-59 cells. To confirm that H-59 cells transfected with the SLPI gene secreted an active protein that is able to modify TNF-α production by macrophages, an in vitro assay was established. Serum-free conditioned media were harvested from 24-hour cultures of control, mock-transfected, and SLPI-transfected H-59 cells. The media were filtered to ensure that they were cell-free and sterile and added (or not) to monolayers of ANA-1 cells that were preseeded 24 hours earlier in 24-well plates at a density of 5 × 10⁵ per well for a 1-hour incubation at 37°C. To each well, 100 ng/mL LPS were then added for a 6 hours incubation, the ANA-1 cell supernatants harvested, and TNF-α levels analyzed by ELISA using the protocol described in detail elsewhere (25, 30).

Cell invasion assay. Tumor cell invasion was assessed in vitro using Matrigel essentially as we previously described (31). Briefly, the assay was done using 8-μm pore size Nuclepore filters (Transwell, Corning, Acton, MA) that were precoated with 0.23 mg/mL Matrigel (Collaborative Research, Bedford, MA). To each filter, 5 × 10⁵ cells were added and insulin-like growth factor (IGF-I; 10 ng/mL) was used to induce cell motility. Triplicate filters were used for each cell type and assay condition and 20 random fields were counted per filter.

Intraspinal/portal injections. C57BL/6 female mice (6-10 weeks old) were anesthetized by an i.m. injection of 0.5 mg/kg Acepromazine followed by an i.m. injection of ketamine/xylazine at final concentrations of 100 and 10 mg/kg, respectively. The spleens were exposed through a small abdominal incision, 10⁶ or 10⁷ tumor cells in 0.1 mL saline or saline alone were injected into the spleen, and the animals were splenectomized 1 minute later. Fourteen days post inoculation of 10⁶ tumor cells, the animals were euthanized, the livers removed, and the hepatic metastases enumerated immediately without prior fixation of the livers. For measurement of TNF-α and E-selectin mRNA, livers were harvested at different time intervals, ranging from 20 minutes to 24 hours post inoculation of 2 × 10⁶ tumor cells. Liver fragments used for mRNA extraction were snap-frozen in liquid N₂ and then stored at −80°C until they were used. All the procedures were carried out in accordance with the policies and regulations of the McGill Animal Care facility.

Immunohistochemistry and fluorescence microscopy. Following tumor cell injection, the livers were perfused with 50 mL of a 4% paraformaldehyde solution, fixed for 48 hours in the same fixative, transferred into a 30% sucrose solution for 4 days, and then placed in optimum cutting temperature medium before freezing at −80°C. Immunohistochemistry was done on 7-μm cryostat sections. The sections were incubated first in a blocking solution (1% Donkey serum, 1% goat serum, 0.1% bovine serum albumin, 0.1% Triton X-100 in PBS) for 30 minutes and then overnight at 4°C with a rat monoclonal antibody to murine E-selectin (PharMingen, Mississauga, Ontario, Canada), used at a dilution of 1:50. After several washes with PBS, the sections were incubated for 1 hour with an Alexa 568 goat anti-rat antibody (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:200 and mounted in Prolong Gold antifade reagent (Molecular probes). Representative digitized images were acquired using a Zeiss microscope. To monitor tumor cell retention in the liver during the first 6 hours following injection, mice were injected with 10⁵ GFP+ tumor cells and the livers removed at different time intervals thereafter, ranging from 20 minutes to 6 hours, and processed for fluorescence microscopy as described above. Representative digitized images were acquired using the Zeiss microscope and the fluorescent tumor cells were enumerated.

Results

Identification of SLPI as a potential regulatory gene in liver metastasis. Using Affymetrix 74A DNA microarray chips, we did a
comparative gene expression analysis of carcinoma cells with divergent potentials to metastasize to the liver. RNA was extracted from liver-metastatic H-59 and nonmetastatic M-27 carcinoma cells. Among several interesting candidate genes, we found that SLPI mRNA was expressed at dramatically higher levels (>2 log difference in gene expression) in M-27 as compared with H-59 cells. Using this analysis, we were also able to confirm our previously published findings that the IGF-I receptor (IGF-IR) was differentially expressed in these two cell lines (32, 33).

Previously we have shown that in this carcinoma model, the metastatic potential correlated with IGF-IR expression levels (29, 31). It was therefore of interest to determine whether SLPI expression was regulated by IGF-IR. To this end, SLPI expression levels were analyzed in M-27 cells overexpressing IGF-IR (M-27IGF-IR) and compared with those in wild-type cells. Results of mRNA and protein analyses (Fig. 1) showed an inverse correlation between the expression levels of SLPI and IGF-IR in these cells. We previously reported that IGF-IR overexpression in M-27 cells resulted in the acquisition of a liver-metastasizing potential (29, 31). The present results show that SLPI gene expression was in fact lost in these cells, confirming the inverse correlation between SLPI expression and the metastatic phenotype and identifying SLPI as an IGF-IR–regulated gene. Together, these data suggested that SLPI may be directly involved in the regulation of liver metastasis.

Production and characterization of H-59 cells overexpressing SLPI. To determine whether SLPI expression was causally related to the protection against liver metastasis, we transfected H-59 cells with the pCDNA3.1-SLPI expression vector and several stable clones were selected in G418-containing medium. Using
RT-PCR and Western blot analysis, we identified three clones (SLPI-1, SLPI-2, and SLPI-3) expressing SLPI mRNA (Fig. 2A) and secreting high levels of the SLPI protein (Fig. 2B). As expected, mock-transfected H-59 clones (Mock-1 and Mock-2) had no detectable SLPI mRNA or protein expression (Fig. 2A and B).

Assessment of the biological activity of SLPI produced by gene-transfected H-59 carcinoma cells. Previously, it has been reported that SLPI suppressed LPS-induced NF-κB activation and TNF-α and nitric oxide production in macrophages, suggesting that SLPI can inhibit the LPS induced responses via an autocrine mechanism (3). To determine whether SLPI-transfected cells produced functionally active SLPI protein, which is able to inhibit TNF-α production by macrophages, we used an in vitro assay of macrophage activation. Serum-free conditioned media harvested from SLPI-transfected and control H-59 cells were added to monolayers of a murine macrophage cell line (ANA-1) 1 hour before the addition of LPS and the cultures were incubated for an additional 6 hours. TNF-α production was assessed in the macrophage supernatants using an ELISA. Nontreated LPS-activated macrophages were used as positive controls. Results in Fig. 3 show that whereas medium conditioned by H-59 cells had no effect, or even increased TNF-α production by the macrophages, medium conditioned by SLPI-expressing cells blocked TNF-α production, reducing it to background levels. This suggested that the cells produced a fully active SLPI, which is able to inhibit TNF-α production by macrophages. A similar effect was observed when medium conditioned by the SLPI-producing nonmetastatic M-27 cells was tested. TNF-α levels detectable in H-59 and M-27 conditioned medium were low (0.0205 and 0.0214 ng/mL, respectively) and comparable to those detected in control, nonstimulated ANA-1 cells (0.0205 ng/mL).

SLPI expression inhibits TNF-α induction in the liver in response to infiltrating tumor cells. In a series of reports, we previously showed that the influx of metastatic tumor cells into the liver triggers a rapid proinflammatory response. Specifically, we found that Kupffer cell–associated TNF-α and IL-1 production were increased in response to the tumor cells and this led to upregulated expression of E- and P-selectin, VCAM-1, and ICAM-1 on hepatic sinusoidal endothelial cells. This response was restricted to highly metastatic H-59 carcinoma cells and was not triggered by poorly metastatic M-27 cells (22, 25). To determine whether SLPI expression played a regulatory role in these divergent abilities to induce host reactivity, we compared the course of host inflammation induced by wild-type (nontransfected) H-59 cells to the

Figure 5. SLPI expression reduces E-selectin gene induction in the liver in response to infiltrating tumor cells. Mice were inoculated with 2 × 10^6 tumor cells by the intrasplenic/portal route and the livers were removed at the time intervals indicated. Total RNA was extracted and analyzed by RT-PCR. For each of the cell types, three livers were analyzed per each time point and each analysis was done thrice. A, representative data. B, results of laser densitometry. Columns, means of the data obtained with H-59 (n = 3), Mock-1 and Mock-2 (Mock, n = 6), and SLPI-1, SLPI-2, and SLPI-3 (SLPI, n = 9) cells; bars, SD. They are expressed as the ratios of E-selectin/GAPDH relative to the control group (livers of saline injected mice), which were assigned a value of 1 (**, P < 0.01, Student’s t test). C, results of immunohistochemistry done on cryostat sections of livers derived from the animals 8 hours following the injection of GFP-expressing nontransfected (left) or SLPI-transfected (right) H-59 cells. The sections were stained with rat anti-murine E-selectin antibodies and an Alexa 568 goat anti-rat antibody (red staining). Representative of 60 fields analyzed using the 40× objective. D, results of tumor cell quantification based on fluorescence microscopy analysis done on liver cryostat sections that were obtained at different intervals post injection of 10^6 GFP⁺ tumor cells. Columns, mean number of tumor cells observed using the 40× objective in a total of 80 fields, representing a total of four liver sections per time point (P > 0.05 at all time points); bars, SD.
inflammatory response induced by carcinoma cells stably expressing the SLPI gene. Using real-time PCR, we monitored hepatic TNF-α mRNA levels at different time intervals post intrasplenic/portal tumor inoculation. As shown in Fig. 4, the inoculation of untransfected or mock-transfected H-59 cells triggered a time-dependent induction of TNF-α mRNA that was evident at 1 hour, peaked at 6 hours, and persisted for up to 10 hours post tumor cell injection. Saline-injected animals did not express TNF-α mRNA. Interestingly, H-59 carcinoma cells stably expressing SLPI also initially induced TNF-α mRNA synthesis in the inoculated mice but the expression was short-lived and was significantly reduced relative to mice injected with control cells by 6 hours post tumor cell inoculation. SLPI expression reduces E-selectin gene induction in the liver in response to infiltrating tumor cells. In agreement with our previous reports (22), vascular endothelial E-selectin mRNA expression in mice inoculated with H-59 cells began to increase within 2 hours, reaching maximal levels at 8 hours, and remaining high for up to 10 hours following tumor inoculation. A similar pattern was observed in mice inoculated with mock-transfected H-59 cells. In contrast, we found a weak and short-lived increase in E-selectin expression in mice injected with H-59 carcinoma cells expressing SLPI, with levels declining to the baseline as early as 8 hours following tumor inoculation (Fig. 5). Immunohistochemistry done on liver cryostat sections obtained from tumor-injected animals confirmed these differences in E-selectin induction by the tumor cells. Figure 5C illustrates representative images of vascular E-selectin staining obtained in livers from mice injected with GFP-expressing H-59 tumor cells (8 hours following injection). No immunostaining was observed in livers from mice injected with SLPI-1-transfected tumor cells. This rapid decline in TNF-α production and the subsequent E-selectin induction were not due to a difference in the total number of cells retained in the liver following tumor inoculation, as determined by fluorescence microscopy analysis and shown in Fig. 5D.

Expression of IGF-IR increases TNF-α production in response to tumor cell injection. We previously reported that M-27 carcinoma cells transfected with the IGF-IR gene acquired a liver-metastasizing potential (29). We have shown here (Fig. 1) that these cells lose the expression of SLPI mRNA and do not produce detectable levels of the protein (Fig. 1). It was therefore of interest to test whether these cells could induce TNF-α synthesis in vivo on entry into the liver. To this end, the tumor cells were injected via the intrasplenic/portal route and at different intervals thereafter; hepatic TNF-α production was measured by RT-PCR. The results in Fig. 6 show that IGF-IR gene expression in these cells and the resulting loss of SLPI production restored the ability of the cells to induce TNF-α production in the liver to levels generally observed following the injection of highly metastatic H-59 cells.

Expression of SLPI prevents experimental liver metastasis. Finally, we tested the effect of SLPI secretion and the resulting loss of TNF-α and E-selectin induction on the ability of H-59 cells to colonize the liver. Tumor cells were inoculated by the intrasplenic/portal route and hepatic metastases were enumerated 14 to 21 days later. Results in Fig. 7 show that SLPI expression in H-59 cells altered their metastatic potential. In mice inoculated with H-59 cells that secreted SLPI, the number of hepatic metastases was reduced by 66% to 80% (P < 0.001) relative to wild-type cells and by 54% to 70% (P < 0.005) relative to mock-transfected cells. Results of a Matrigel invasion assay, shown in Fig. 7C, suggest that this reduction in the metastatic potential was not due to reduced cell invasion (29, 34).

Discussion
Our results identify SLPI as an inhibitor of liver metastasis and link this function to its ability to suppress the tumor cell induced host proinflammatory response during the early stages of liver colonization. The host inflammatory response and its major mediator, TNF-α, play a dual role in tumor progression and metastasis. On one hand, TNF-α can inhibit tumor growth through its cytocidal and proapoptotic activities (35), but on the other hand, it can promote tumor progression through several alternate mechanisms that include promotion of tumor cell growth (36), induction of tumor cell motility and invasion (20, 37, 38), up-regulation of vascular endothelial cell adhesion receptors as shown by us and others (22, 23, 39), and the induction of angiogenesis (13, 40, 41). The ultimate effect of TNF-α may depend on its concentrations, on tumor cell susceptibility to its proapoptotic effects, and on the stage of the disease at which the tumor and its microenvironment are subject to its actions (12–14, 42).

Our results suggest that the response of host inflammatory cells in the liver, Kupffer cells in particular, may be modulated in a
paracrine fashion by tumor-derived soluble factors that can either induce or attenuate cytokine production. Indeed, both autocrine and paracrine effects of SLPI in inflammation have been described. SLPI was initially identified as a macrophage-secreted protein that attenuates LPS-induced nitric oxide and TNF-α production in an autocrine fashion (8). Transfection of macrophages with a eukaryotic expression vector encoding SLPI cDNA (3) or using adenoviral gene delivery (9) resulted in the inhibition of LPS-induced nitric oxide and TNF-α production.

Figure 7. Expression of SLPI prevents experimental liver metastasis. Liver metastases were enumerated 14 days after the intrasplenic/portal injection of 10^5 tumor cells. A, representative livers. B, results obtained with each individual cell type. Cumulative data from three independent experiments are shown. The median numbers of metastases/liver are indicated [P < 0.001 for mice injected with each of the SLPI clones and P < 0.005 for mice injected with the uncloned population of SLPI-transfected H-59 cells (H-59SLPI) as compared with mice injected with H-59 or mock-transfected cells (Mann-Whitney test)]. The two latter groups did not significantly differ from each other. C, results of the Matrigel invasion assay. Columns, mean number of cells per field in three filters counted per tumor type and based on analysis of 20 random fields per filter; bars, SD.
TNF-α production by the transfected cells. In vivo production of both local and systemic SLPI in response to bacterial infection has been well documented. Moreover, in a model of hepatic ischemia/reperfusion induced in mice by partial hepatectomy, the exogenous administration of SLPI was shown to attenuate liver and lung inflammatory injury, diminish neutrophil accumulation in both organs, and reduce serum levels of TNF-α and macrophage inflammatory protein 2, suggesting that SLPI can act in a paracrine fashion to attenuate inflammation-induced damage in sites remote from the site of the inflammatory insult (43, 44). The present results show that high endogenous SLPI production by poorly metastatic M-27 carcinoma cells or SLPI-transfected H-59 cells led to reduced TNF-α production in the liver as compared with control, highly metastatic H-59 cells. These findings, taken together with our previous results, suggest that in the present model, tumor-derived SLPI was acting in a paracrine fashion to block cytokine production by Kupffer cells localized in sinusoidal vessels around the incoming tumor cells.

The liver is the major endocrine source of IGF-I production. Previously, we have shown that hepatic IGF-I acts as a paracrine, growth-promoting factor to enhance the growth of metastatic H-59 cells in the liver (32, 33). The present data show that in addition to its growth promoting effect, IGF-I may also regulate the tumor-induced inflammatory response by suppressing SLPI production, thereby allowing sufficient local TNF-α levels to be produced and initiate the molecular cascade required for metastases formation. IGF-I may also act as a survival factor, protecting the tumor cells, as well as resident hepatic cells, from TNF-α-induced apoptosis (45–47).

The mechanism underlying the negative regulatory effect of IGF-I on SLPI transcription remains to be elucidated. The transcriptional activators of SLPI synthesis are yet to be defined. The identification of LPS as an inducer of both TNF-α and SLPI implies that SLPI synthesis may be regulated by NF-κB. In gill cells, IGF-I was identified as an inhibitor of NF-κB activation and this was mediated through serine phosphorylation of IκB (48). The inhibition of NF-κB-mediated signaling may be one mechanism by which a negative regulatory effect on SLPI production is transmitted.

To date, few studies have examined the role of SLPI in tumorigenesis and metastasis. Interestingly, however, it was recently reported that elevated levels of SLPI increased both the s.c. growth and the lung-colonizing potential of 3LL cells, and this was attributed to the protease inhibitory function of SLPI (49). Whereas the reason for the discrepancy between these and our own results is not presently clear, it may be related to the different anatomic sites used to evaluate tumor growth in the two studies (i.e., s.c. and lung metastases in one and liver metastases in the other). It is conceivable that SLPI and, consequently, TNF-α and inflammation play different and even opposing regulatory roles in tumor growth in these sites. IGF-IR expression levels in the tumor cells used in the different studies may also have contributed to the divergent results by affecting their susceptibility to TNF-α-induced apoptosis. This interpretation seems to be in agreement with our findings that a reduction in IGF-IR expression in H-59 cells by antisense RNA reduced their liver-metastasizing potential but not their s.c. growth (33). It is of interest to note in this respect that M-27 cells can metastasize spontaneously to the lung but not to the liver (26).

These site-specific growth patterns indicate that SLPI and IGF-IR expression may differently affect tumor growth in different anatomic sites, possibly because the host inflammatory responses generated in these sites are distinct or they could affect tumor progression in diverse ways.

Our results, taken together with previous studies, provide further evidence that the host inflammatory response, through its molecular mediators, such as TNF-α and SLPI, plays a crucial role in regulating the tumor microenvironment and thereby may control the fate of disseminating tumor cells.

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