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Research Article

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Epithelial–neutrophil Activating Peptide (ENA-78) Is an Important Angiogenic Factor in Non-small Cell Lung Cancer

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Abstract

We report here the role of the CXC chemokine, epithelial neutrophil activating peptide (ENA-78), as an angiogenic factor in human non-small cell lung cancer (NSCLC). In freshly isolated human specimens of NSCLC, elevated levels of ENA-78 were found that strongly correlated with the vascularity of the tumors. In a SCID mouse model of human NSCLC tumorigenesis, expression of ENA-78 in developing tumors correlated with tumor growth in two different NSCLC cell lines. Furthermore, passive immunization of NSCLC tumor-bearing mice with neutralizing anti-ENA-78 antibodies reduced tumor growth, tumor vascularity, and spontaneous metastases, while having no effect on the proliferation of NSCLC cells either in vitro or in vivo. These findings suggest that ENA-78 is an important angiogenic factor in human NSCLC. (*J. Clin. Invest.* 1998. 102:465–472.) Key words: chemokines • angiogenesis • metastasis • malignancy • apoptosis

Introduction

Angiogenesis is a key step in the progression to and maintenance of a malignant phenotype (1). Net angiogenic activity is dictated by the balance of expression of factors that either promote or inhibit angiogenesis (2). The number of such angiogenic and angiostatic factors is extensive, and likely reflects the redundancy that is inherent in a biologic process as vital as angiogenesis. The CXC chemokine family is unique with respect to the process of angiogenesis. This family consists of related chemokines that are either angiogenic or angiostatic depend-

ing upon the presence of a three amino acid motif, the glutamic acid-leucine-arginine (ELR)¹ motif, which immediately precedes the first cysteine in the primary structure (3). The presence of the ELR motif in members of the CXC chemokine family confers potent angiogenic activity, whereas members that lack the ELR motif inhibit angiogenesis (3).

Previously, we have studied the role of CXC chemokines in regulating net angiogenic activity in human non-small cell lung cancer (NSCLC), and hypothesized that the relative expression of angiogenic, as compared with angiostatic, members of the CXC chemokine family is an important determinant of net angiogenic activity in NSCLC. Previous studies have shown that IL-8 (an ELR-CXC chemokine) is a potent angiogenic factor (4, 5), whereas interferon- γ -inducible protein 10 (IP-10; a non-ELR-CXC chemokine) is an endogenous angiostatic factor in human NSCLC (6). In this study, we hypothesize that epithelial neutrophil activating peptide (ENA-78), a related ELR-CXC chemokine, is an important regulator of NSCLC angiogenic activity.

We find in this report that ENA-78 is significantly expressed in freshly isolated human NSCLC. Furthermore, as assessed by vessel density, the expression of ENA-78 in these tumors directly correlates with tumor vascularity. This correlation is markedly stronger than that found for IL-8. This finding was paralleled in a model of human NSCLC tumorigenesis in severe combined immunodeficient (SCID) mice, in which we found a direct correlation between tumor size and expression of ENA-78. Subsequent neutralization of ENA-78 in vivo demonstrated significant inhibition of angiogenic activity (assessed by vessel density) associated with a reduction in tumor size and metastatic potential. Additionally, tumors from mice treated with anti-ENA-78 antibodies demonstrated an increased ratio of apoptotic to proliferating cells, whereas neutralization of ENA-78 did not induce apoptosis or alter proliferation of NSCLC cells in vitro. These data support the notion that expression of angiogenic CXC chemokines is a significant source of angiogenic activity in human NSCLC tumors, and suggests a need to exploit this property in a therapeutic setting.

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1. Abbreviations used in this paper: ELR, glutamic acid-leucine-arginine; ENA-78, epithelial neutrophil activating peptide-78; GRO, growth-related oncogene; IP-10, interferon- γ -inducible protein 10; NSCLC, non-small cell lung cancer; PCNA, proliferating cell nuclear antigen; SCID, severe combined immunodeficiency; TUNEL, TdT-mediated dUTP nick end labeling.

Methods

Reagents. Polyclonal rabbit anti-human ENA-78 sera was produced by immunization of rabbits with ENA-78, (R & D Systems, Inc., Minneapolis, MN) in multiple intradermal sites with CFA. This antibody has been well characterized previously for its neutralizing capacity (7, 8). The ENA-78 antiserum specificity has been confirmed by Western blot analysis against recombinant human ENA-78 and was found to neutralize 30 ng of ENA-78 at a dilution of 1:1000 (7). Furthermore, in a sandwich ELISA, this antibody is specific for ENA-78 without cross-reactivity to a panel of 12 other recombinant human cytokines or the murine chemokines KC and MIP-2 (8). The antiprotease buffer for tissue homogenization consisted of 1× PBS with 2 mM phenylmethylsulfonyl fluoride, and 1 µg/ml each of antipain, aprotinin, leupeptin, and pepstatin A. Anti-proliferating cell nuclear antigen (PCNA) conjugated with horseradish peroxidase, antifactor VIII-related antigen coupled with horseradish peroxidase, and appropriate control antibodies were purchased from DAKO Corp. (Carpinteria, CA). The apoptosis detection assay (in situ cell death detection kit) was purchased from Boehringer Mannheim (Mannheim, Germany).

Normal human lung and NSCLC tumor tissue. Tissue specimens were obtained from consented individuals undergoing thoracotomy ($n = 126$) for suspected NSCLC in accordance with the University of Michigan I.R.B. approval. Samples of tumor, and normal lung distal to tumor, were homogenized and sonicated in antiprotease buffer upon recovery from the operating room. Specimens were centrifuged at 900 g for 15 min, filtered through 0.45 µm Sterile Acrodiscs (Gelman Sciences, Inc., Ann Arbor, MI), and frozen at -70°C until thawed for assay by a specific ENA-78 ELISA. A portion of this specimen was lyophilized (SpeedVac; Savant Instruments, Inc., Farmingdale, NY), normalized to an equivalent amount of total protein, and used in the corneal micropocket model of neovascularization for analysis of angiogenic activity. Additionally, a portion of tumor was also fixed in 4% paraformaldehyde and imbedded in paraffin for histologic analysis. University of Michigan Hospital pathologists determined the cell type. Only cases of NSCLC were included in our analysis (squamous cell carcinoma, $n = 53$; adenocarcinoma, $n = 73$). Normal lung tissue was examined and used as a control only if the histology appeared normal without evidence for inflammation or malignant cell invasion ($n = 40$).

Human NSCLC cell lines. The A549 human adenocarcinoma cell line (American Type Culture Collection, Rockville, MD) was maintained in RPMI 1640 (Whitaker Biomedical Products, Whitaker, CA), 1 mM glutamine, 25 mM Hepes buffer, 100 U/ml penicillin, 100 ng/ml streptomycin, and 10% FCS. Cells were cultured and passaged at 37°C in room air/5% CO_2 . Calu-1 (squamous cell carcinoma) cell culture media consisted of Eagle's MEM, supplemented with 1 mM glutamine, 25 mM Hepes buffer, 100 U/ml penicillin, 100 ng/ml streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, and 10% FCS. For proliferation assays, 5×10^4 cells were plated in 35-mm wells and allowed to grow to 50% confluence, serum starved for 24 h, then treated with either control or neutralizing anti-ENA-78 antibodies or recombinant ENA-78, in the presence of complete media with 1% serum. Cells were then trypsinized, and counted on a hemocytometer. For inoculation into mice, the cells were trypsinized, harvested, washed, and resuspended in serum-free media.

Human NSCLC-SCID mouse chimeras. 4–6-wk-old female CB17-SCID mice (Taconic Farms, Inc., Germantown, NY) with serum Ig $< 1 \mu\text{g/ml}$ were injected subcutaneously with human A549 or Calu-1 cells (10^6 cells in 100 µl) into each flank. The animals were maintained in sterile laminar flow rooms and killed in groups of six. At time of death, anticoagulated (heparin 50 U/500 µl of blood) blood was collected and centrifuged. Tumors were dissected from the mice and measured with a Thorpe caliper (Biomedical Research Instruments, Rockville, MD). Tumor volume was calculated using the formula [volume = $(d_1 \times d_2 \times d_3) \times 0.5236$], where d_n represents the three orthogonal diameter measurements. A portion of the tumor

was fixed in 4% paraformaldehyde for histologic analysis and immunohistochemistry. The other portion of the tumor was snap frozen for subsequent homogenization and sonication in antiprotease buffer followed by filtration through 0.45-µm filters (Acrodiscs; Gelman Sciences, Inc.). The filtrate was stored at -70°C for later analysis of total protein and ENA-78 by ELISA. The lungs were inflated with 4% paraformaldehyde, and prepared for histopathology. In the ENA-78 depletion studies, A549 tumor-bearing SCID mice received intraperitoneal (i.p.) injections of 500 µl of either neutralizing rabbit anti-human ENA-78, control (pre-immune) serum, or no treatment, every 48 h for 8 wk, starting at the time of cell inoculation. Tumor specimens from these mice were processed as described above.

Quantitation of lung metastases by light microscopy. Two H & E-stained lung sections from each of sixteen A549 tumor-bearing mice treated with either anti-ENA-78 or control antibodies were examined under low magnification ($\times 40$) to count spontaneous metastases. In addition, using an Olympus BH-2 microscope (Olympus, Tokyo, Japan) coupled to a Sony 3CCD camera (Sony Corp., Park Ridge, NJ) and a Macintosh IIfx computer (Apple Computer, Inc., Cupertino, CA), the total area of metastatic tumor burden per lung section was quantitated (square pixels) using NIH Image 1.55 software. Data were expressed as either the number of metastases per lung section or the area of metastatic tumor per section (square pixels at $\times 40$).

Corneal micropocket model of angiogenesis. In vivo angiogenic activity of human tumors was assayed in the avascular cornea of Long Evans rat eyes, as previously described (9–11). In brief, equal volumes of lyophilized tumor specimens normalized to total protein, were combined with sterile Hydron (Interferon Sciences Inc.) casting solution. 5-µl aliquots were pipetted onto an inverted sterile polypropylene specimen container, and polymerized overnight in a laminar flow hood under UV light. Before implantation, pellets were rehydrated with normal saline. Animals were given i.p. ketamine (150 mg/kg) and atropine (250 µg/kg) for anesthesia. Rat corneas were anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution followed by implantation of the Hydron pellet into an intracorneal pocket (1–2 mm from the limbus). 6 d after implantation, animals received heparin (1,000 U) and ketamine (150 mg/kg) i.p., followed by a 10-ml perfusion of colloidal carbon via the left ventricle. Corneas were harvested and photographed. Positive neovascularization responses were defined as sustained directional ingrowth of capillary sprouts and hairpin loops towards the implant. Negative responses were defined as either no growth or only an occasional sprout or hairpin loop displaying no evidence of sustained growth.

Immunohistochemistry for ENA 78. Tumor specimens were fixed in 4% paraformaldehyde for 24 h. Paraffin-embedded tissue sections were dewaxed with xylene and rehydrated through graded concentrations of ethanol. Samples were then stained for ENA-78 using a modification of our previously described technique (12). In brief, nonspecific binding sites were blocked with normal goat serum (BioGenex Labs, San Ramon, CA), washed, and overlaid with a 1:1000 dilution of either control (rabbit) or rabbit anti-human ENA-78 serum. Slides were then rinsed and overlaid with secondary biotinylated goat anti-rabbit IgG (1:35) and incubated for 60 min. After washing twice with Tris-buffered saline, slides were overlaid with a 1:35 dilution of alkaline phosphatase conjugated to streptavidin (BioGenex Labs), and incubated for 60 min. Fast Red (BioGenex Labs) reagent was used for chromogenic localization of ENA-78 antigen. After optimal color development, sections were immersed in sterile water, counterstained with Mayer's hematoxylin, and cover-slipped with an aqueous mounting solution.

Quantitation of vessel density. Quantitation of vessel density was performed using a modification of the previously described method (13). In brief, tissue sections (both human tumors and A549-SCID mouse tumors) were dewaxed with xylene and rehydrated through graded concentrations of ethanol. Slides were stained for endothelial cells using antibody to Factor VIII-related antigen conjugated to horseradish peroxidase (DAKO Corp.). DAB (Vector Laboratories, Inc., Burlingame, CA) reagent was used for chromogenic

localization of Factor VIII-related antigen. After optimal color development, sections were immersed in water and cover-slipped. Tumor specimens were scanned at low magnification ($\times 40$) to identify vascular hot spots. Areas of greatest vessel density were then examined under higher magnification ($\times 200$) and counted. Any distinct area of positive staining for Factor VIII-related antigen was counted as a single vessel. Results were expressed as the number of vessels per high power field ($\times 200$).

Measurement of apoptosis and proliferation. Sections of A549 tumors from SCID mice treated with either control or anti-ENA-78 antibodies were stained using either the TdT-mediated dUTP nick end labeling (TUNEL) method to detect apoptosis or PCNA antibody conjugated to horseradish peroxidase (DAKO Corp.). Cells were counted in three high power fields per tumor ($\times 400$) after scanning at low power ($\times 40$) to select areas devoid of frank necrosis. Results were expressed as the number of apoptotic nuclei per $\times 400$ field, or the number of proliferating (PCNA positive) cells/ $\times 400$ field.

ENA-78 ELISA. Antigenic ENA-78 was quantitated in aqueous homogenates of human and mouse NSCLC tumors using a modification of a double ligand method as previously described (8). This method consistently detected ENA-78 concentrations greater than 50 pg/ml in a linear fashion. Tumor samples were run in parallel for total protein content (Pierce Chemical Co., Rockford, IL), and results were expressed as nanogram of ENA-78 per milligram total protein.

Statistical analysis. The studies involved a minimum of six human NSCLC/SCID mouse chimeras at each time point or for each manipulation. Groups of data were evaluated by analysis of variance to indicate groups with significant differences. Data that appeared statistically significant were compared by Student's *t* test for comparing the means of multiple groups, and were considered significant if *P* values were < 0.05 . Results were presented as means \pm SEM. Data were analyzed on a Macintosh computer using the Statview 4.5 statistical software package (Abacus Concepts, Inc., Berkeley, CA).

Results

ENA-78 levels are elevated in freshly isolated human NSCLC. We first hypothesized that levels of ENA-78 would be elevated in freshly resected specimens of human NSCLC relative

Table I. Composite of Data Demonstrating Inhibition of NSCLC-induced Corneal Neovascularization by ENA-78 Neutralizing Antibody

Test sample	No. Positive/total	
	Control	Anti-ENA-78
Squamous cell carcinoma	8/8	0/8
Adenocarcinoma	7/7	1/8

Control antibodies were normal rabbit serum.

to normal lung tissue. Tumor specimens were processed immediately after resection and homogenized in an aqueous anti-protease buffer. Levels of ENA-78 were determined by ELISA, and standardized to total protein (nanogram ENA-78 per milligram of protein). ENA-78 levels were elevated sixfold in tumor tissue compared with normal lung homogenates (19.2 ± 4.7 versus 3.1 ± 1.0 ng/mg, *P* = 0.05; Fig. 1 A). Immunolocalization of ENA-78 in paraffin embedded NSCLC tumor sections revealed that the major cellular source of ENA-78 was the tumor cells (Fig. 1 B).

ENA-78 is an angiogenic factor in NSCLC. To determine whether ENA-78 was a significant angiogenic factor present in the tumor specimens, we assessed in vivo angiogenic activity of freshly isolated human NSCLC homogenates, normalized to equal amounts of total protein, in the rat corneal micropocket assay. Tumor specimens were pretreated with either control or anti-ENA-78 antibodies, and incorporated into hydon pellets. 15 of 15 tumor-control pellets induced a strongly positive angiogenic response in the cornea (Fig. 2, A and C). In contrast, only 1 of 16 anti-ENA-78-treated tumor pellets induced angiogenic activity (Fig. 2, B and D; Table I).

To confirm the role of ENA-78 as a NSCLC tumor-derived

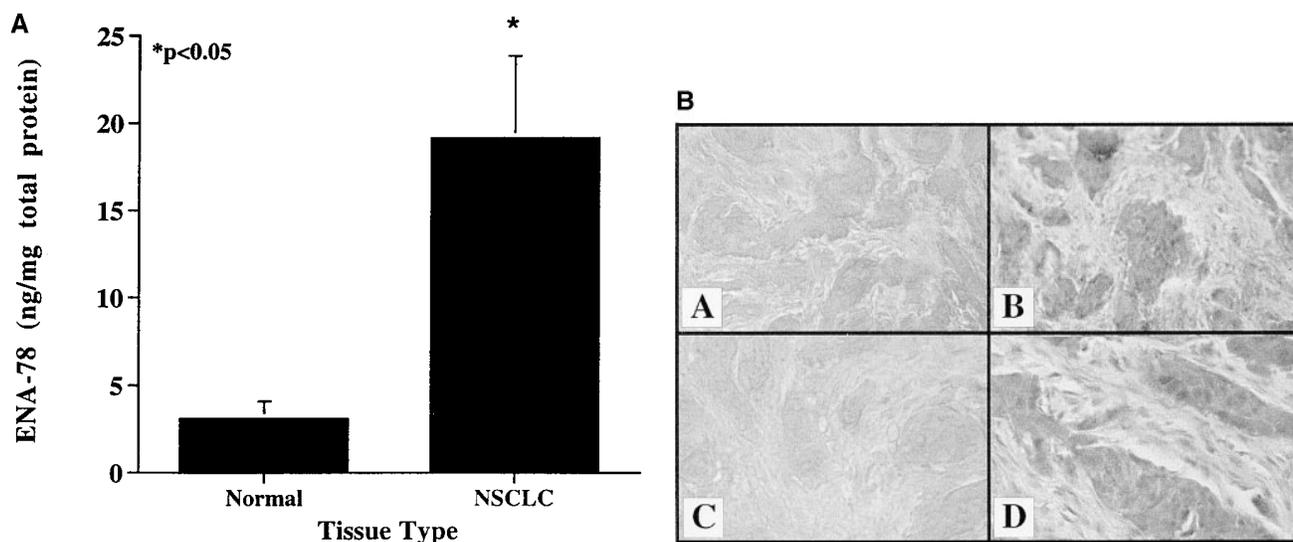


Figure 1. Levels of ENA-78 are elevated in human NSCLC. (A) ENA-78 levels in freshly isolated human NSCLC (19.2 ± 4.7 ng/ng, *n* = 126), as compared with normal lung tissue (3.1 ± 1.0 ng/mg, *P* = 0.05). Only histologically normal lung tissue, free of inflammation and tumor cells were included for comparison. (B) Immunolocalization of ENA-78 in a representative specimen of NSCLC. Panels A and C, 1:500 normal rabbit serum primary antibody ($\times 200$ and $\times 400$, respectively). Panels B and D, 1:500 anti-ENA-78 primary antibody; ($\times 200$ and $\times 400$, respectively).

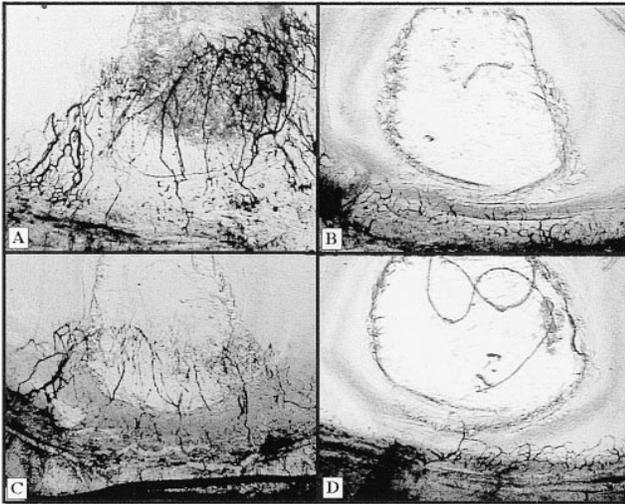


Figure 2. In vivo angiogenic activity of human NSCLC homogenates. Corneal neovascularization in response to squamous cell carcinoma pretreated with control (A) or neutralizing anti-ENA-78 antibodies (B). Adenocarcinoma pretreated with control (C) or anti-ENA-78 antibodies (D). All photographs $\times 20$.

angiogenic factor, vessel density was assessed in paraffin-embedded NSCLC tumor sections. Factor VIII-related antigen was used as a marker of microvessels, and vessel density was compared with the level of ENA-78 from the corresponding tumor homogenates. Vessel density correlated significantly with levels of ENA-78 (Fig. 3; $r = 0.83$, $P = 0.003$, $n = 12$). In addition, vessel density was compared with levels of IL-8, a CXC chemokine that we have shown is an important angiogenic factor in NSCLC (5). Levels of ENA-78 demonstrated a much greater correlation with vessel density than levels of IL-8 ($r = 0.83$ versus 0.55, data not shown).

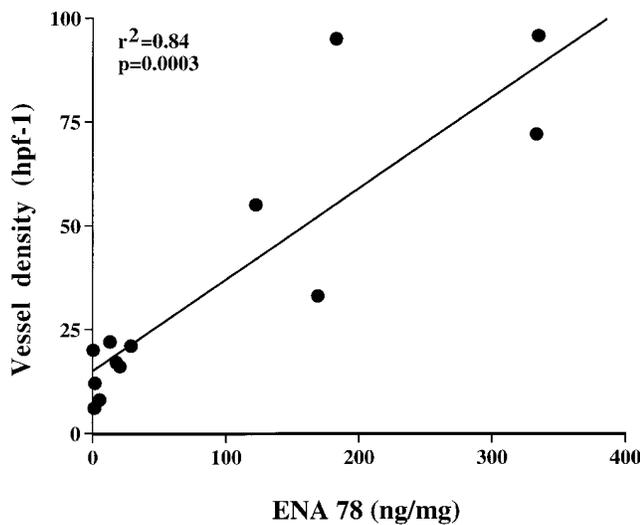


Figure 3. Human NSCLC vessel density correlates with levels of ENA-78. Vessel density was assessed by Factor VIII-related antigen immunolocalization in histologic sections of NSCLC and compared with the level of ENA-78 (nanogram per milligram of total protein) detected by ELISA in the corresponding tumor homogenate.

Expression of ENA-78 correlates with NSCLC tumor growth in SCID mice. The above findings were obtained from human specimens of NSCLC tumors, and were important in that they established that ENA-78 accounts for a significant portion of the angiogenic activity in NSCLC. However, we were interested in determining the role of ENA-78 in the biology of NSCLC tumor growth, which is a dynamic process evolving over time. To study the role of ENA-78 in a longitudinal fashion, we employed an animal model of tumorigenesis in which SCID mice were injected with human NSCLC cells (A549 and Calu-1 cell lines) and tumors were characterized temporally for the expression of ENA-78. Tumor size increased over a period of 8–12 wk. Interestingly, A549 (adenocarcinoma) cells were associated with a more aggressive phenotype, and expressed much higher levels of ENA-78 than Calu-1 (squamous cell) tumors. However, regardless of cell type, expression of ENA-78 directly correlated with the growth of the tumors ($r = 0.78$, $P = 0.04$ for A549; $r = 0.83$, $P = 0.003$ for Calu-1; Fig. 4).

Neutralization of ENA-78 inhibits tumorigenesis and metastasis of NSCLC in SCID mice. To assess the role of ENA-78 in the growth of NSCLC, mice were inoculated with the more aggressive cell type, A549 (adenocarcinoma), followed by passive immunization with either neutralizing ENA-78 or control antibodies. Antibodies were given every other day for a period of 8 wk (0.5 cc intraperitoneal injection). Depletion of ENA-78 via passive immunization resulted in a right and

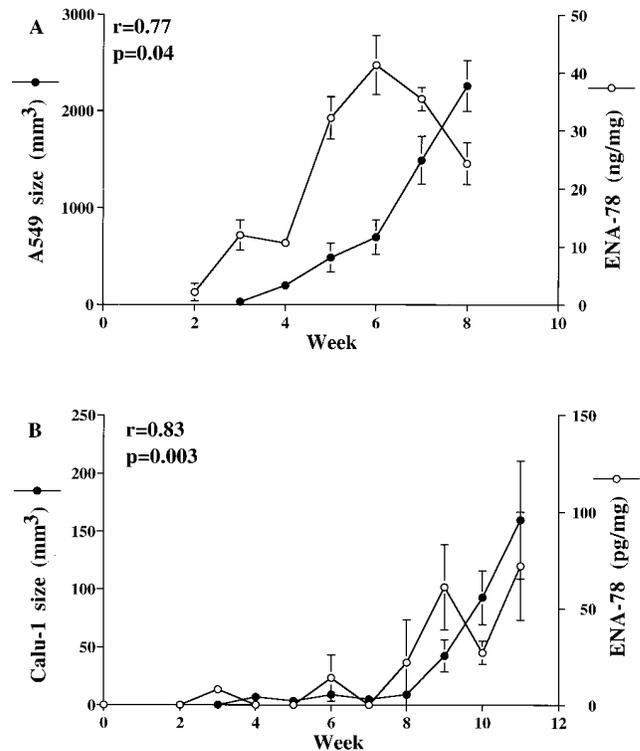


Figure 4. Expression of ENA-78 correlates with tumor size. (A) Tumor growth and expression of ENA-78 (nanogram per milligram of total protein) by A549 cells in SCID mice. (B) Tumor growth and expression of ENA-78 (picogram per milligram of total protein) by Calu-1 cells in SCID mice.

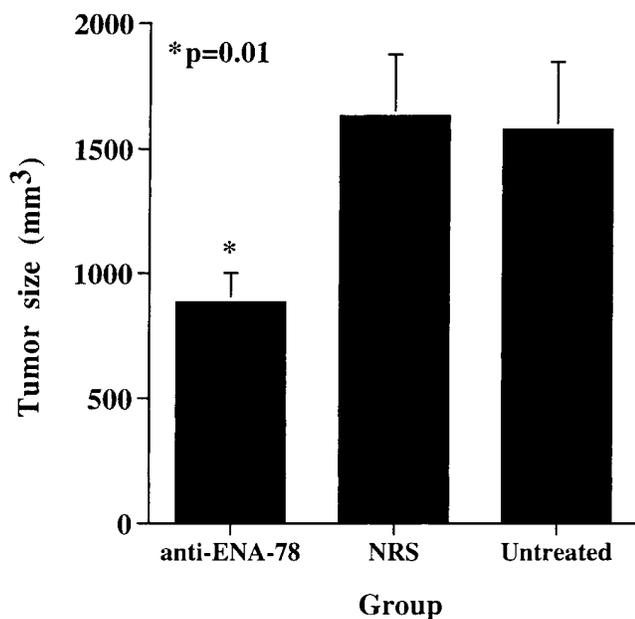


Figure 5. Inhibition of A549 tumor growth in SCID mice treated with anti-ENA-78 antibodies. Mice were inoculated with tumor cells on day 0, and treatment groups were passively immunized with either rabbit antihuman-ENA-78, or normal rabbit serum every other day for 7 wk.

downward shift of the growth curve, such that animals treated with anti-ENA-78 had significantly smaller tumors than those receiving control serum (883.8 ± 117.9 versus 1632.5 ± 245.5 mm³; $P = 0.01$; Fig. 5). A549 tumors in an untreated group were not significantly different from the control antibody-treated group (Fig. 5).

We next assessed the occurrence of spontaneous lung metastases in A549 tumor-bearing SCID mice treated with either anti-ENA-78 or control antibodies. Treatment with anti-ENA-78 significantly reduced the number of metastases in histologic sections of the lungs of SCID mice (1.1 ± 0.2 versus 6.7 ± 2.5 metastases per section, $P = 0.03$; Fig. 6 A). Additionally the size of the metastases, as assessed by digital image analysis, were also markedly smaller in the anti-ENA-78-treated group (6.0 ± 3.2 versus $47.9 \pm 18.5 \times 10^3$ pixels per lung section, $\times 40$; $P = 0.04$; Fig. 6 B). Thus, both the primary and metastatic tumor growth were inhibited in the presence of neutralizing anti-ENA-78 antibodies.

Neutralization of ENA-78 reduces tumor vascularity and increases apoptosis of tumor cells in vivo. To demonstrate the mechanism by which inhibition of ENA-78 reduces tumor growth, neovascularization was assessed using morphometric analysis of Factor VIII-related antigen as a marker for vessels. Tumors from mice treated with anti-ENA-78 had significantly less vessel density than tumors from animals treated with control serum (14.7 ± 1.9 versus 26.2 ± 4.4 vessels/hpf; $\times 200$; $P = 0.03$; Fig. 7). Since prior studies have determined that inhibition of angiogenesis is accompanied by an increased rate of apoptosis in tumor cells (14, 15), we assessed apoptosis in situ using the TUNEL method and counted the number of TUNEL-positive cells per $\times 400$ field. A549 tumors from anti-ENA-78-treated mice had significantly more apoptotic cells

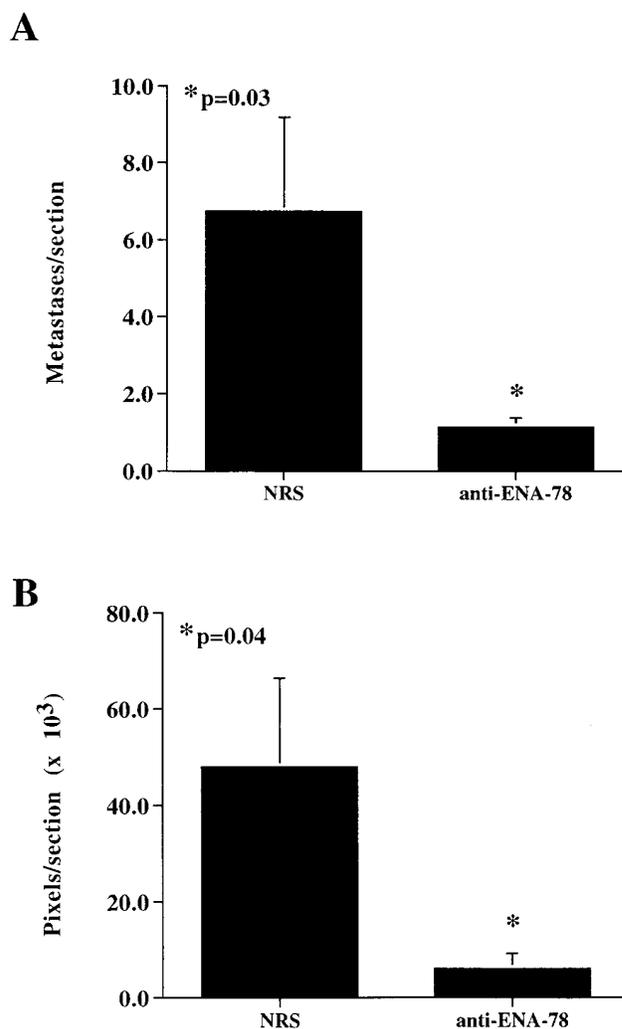


Figure 6. Reduction in both the number (A), and size (B) of metastases in A549 tumor-bearing SCID mice treated with anti-ENA-78 as compared with control antibodies. Metastases were counted at a magnification of 40 on histologic sections of lungs from SCID mice, ($n = 2$ sections per mouse, eight mice per group). Size of metastases were assessed by digital image analysis ($\times 40$).

per high power field than tumors from control antibody-treated mice (42.8 ± 7.6 versus 13.1 ± 1.8 cells/hpf, $P = 0.003$; Table II). In contrast, there was no difference in the rate of proliferation of A549 cells in SCID mice under these same conditions as assessed by PCNA immunolocalization (Table II).

Anti-ENA-78 antibodies have no effect on proliferation or apoptosis of A549 cells in vitro. To exclude the possibility that anti-ENA-78 directly inhibited proliferation of A549 cells, we performed in vitro proliferation assays in the presence of anti-ENA-78, control antibodies, or in the presence of exogenous ENA-78. Neither the addition of exogenous ENA-78, nor the inhibition of endogenous ENA-78 with neutralizing antibodies had any direct effect on the rate of in vitro proliferation of A549 cells (Table III). Likewise, the inhibition of ENA-78 in vitro did not induce apoptosis of A549 cells grown in culture (Table IV). These data support the contention that the effect

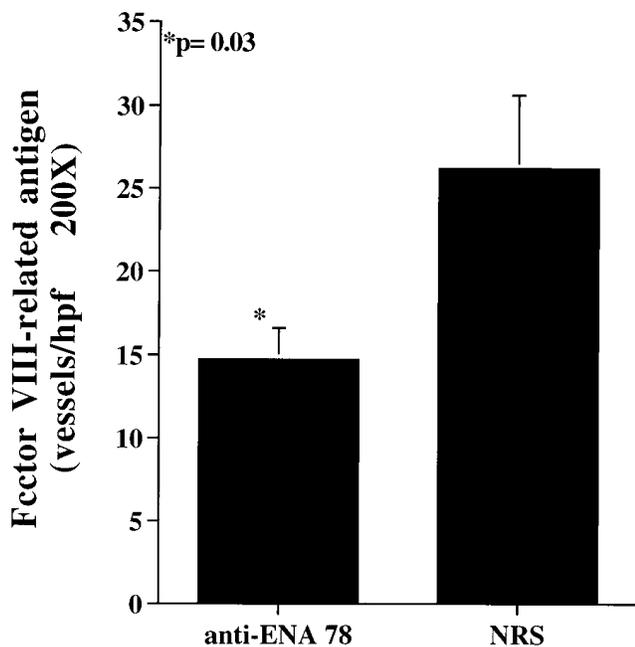


Figure 7. Vessel density in sections of A549 tumors from anti-ENA-78, or control antibody-treated SCID mice. Vessels were defined as any area of distinct positive staining for Factor VIII-related antigen ($n = 10$ sections per treatment group).

of ENA-78 on tumor growth of NSCLC is related to its angiogenic activity.

Discussion

The dependence of solid tumor growth on angiogenesis presents an opportunity to develop new therapies to treat malignant tumors. Lung cancer remains the leading cause of cancer related deaths because of its high incidence and poor response to chemotherapy and radiation. The key to new therapeutic advances relies on a greater understanding of the biology of this disease. In this study, we have found that ENA-78 is an important factor that regulates angiogenesis in NSCLC.

ENA-78 is a member of the CXC chemokine family. Although originally defined as a family of leukocyte chemoattractants (16), CXC chemokines are also important in the regulation of angiogenesis (3, 17–21). Specifically, the presence of the ELR motif dictates the angiogenic activity for these cyto-

Table II. A549 Cell Proliferation (PCNA) and Apoptosis (TUNEL) in SCID Mice Treated with Either Normal Rabbit Serum (NRS) or Anti-ENA-78

Group	PCNA+/hpf	TUNEL+/hpf
NRS	211.5±17.8	13.6±1.8
Anti-ENA-78	214.1±7.9	42.8±7.6
	$P = 0.9$	$P = 0.003$

Cells were counted in five high power fields (hpf; ×400) in six sections from each group.

Table III. 5×10^4 A549 Cells were Plated in 35-mm Plates and Adhered for 24 h

	Antibody		ENA-78		
	NRS	Anti-ENA-78	0 ng/ml	1 ng/ml	10 ng/ml
Cells ($\times 10^4$)					
24 h	41.3±4.1	40.2±1.9	43.1±6.2	42.5±2.0	41.8±3.4
72 h	102.7±14	95.5±3.7	105.7±8.6	98.3±5.0	112.5±4.9

Cells were serum starved for 24 h and 1% serum added. Cells were exposed to either control (NRS) or Anti-ENA-78 antibodies (1:500). Alternatively, cells were exposed to recombinant human ENA-78 in the doses indicated. At 24 and 72 h, cells were trypsinized and counted on a hemacytometer. Conditions were run in triplicate, and the experiment was repeated twice ($P > 0.5$ for all comparisons).

kines (3). IL-8, ENA-78, granulocyte chemotactic peptide-2, growth-related oncogene alpha (GRO α), GRO β , and GRO γ are all ELR-CXC chemokines with potent angiogenic activity (3, 19). In contrast, IP-10, monokine induced by gamma-interferon (MIG), and platelet factor-4 (PF-4) lack the ELR motif and are all angiostatic factors (3, 17, 20–22).

Net angiogenic activity is determined by a balance of positive and negative regulators of angiogenesis. Our laboratory has hypothesized that NSCLC tumor-derived angiogenic activity is due, in part, to an imbalance in the expression of angiogenic (ELR) versus angiostatic (non-ELR) CXC chemokines. This hypothesis predicts that the ELR-CXC chemokines are overexpressed in malignant tumors, and that non-ELR-CXC chemokines are relatively underexpressed. Previously, we have found that expression of IP-10 (a non-ELR-CXC chemokine) correlates inversely with tumor size, and that reconstitution of tumor-associated IP-10 reduced tumor growth in a SCID mouse model of human NSCLC. Coupled with the findings in this report, our data support the notion that NSCLC-derived angiogenic activity is regulated by the balance of expression of members of the CXC chemokine family.

In this study we examined both freshly isolated human tumors, and a SCID mouse model of human NSCLC tumorigenesis. We found ENA-78-dependent angiogenic activity in human specimens of NSCLC. Importantly, we also found that histologic tumor vascularity directly correlated with the levels of ENA-78. We confirmed the biological relevance of this finding in a SCID mouse model of tumorigenesis using the A549

Table IV. In Vitro Apoptosis of A549 Cells in the Presence of Neutralizing ENA-78 or Control (Normal Rabbit Serum) Antibodies

Condition	TUNEL+/hpf
Control	50.0±4.3
Anti-ENA-78	46.0±3.9
	$P = 0.5$

Cells were plated on slide wells with 1:500 dilutions of the indicated antibody. Cells were stained using the TUNEL assay and counted in three high power fields (hpf; ×200) in eight sections from each group.

and Calu-1 human NSCLC cell lines in SCID mice. ENA-78 expression by these tumors was found to directly correlate with tumor growth. Moreover, when A549 tumor-bearing animals were passively immunized with anti-ENA-78, both tumor growth and spontaneous metastases were markedly attenuated. This reduction in tumor growth was accompanied by a decrease in vascularity, and an increase in apoptosis of the A549 tumor cells from anti-ENA-78-treated mice. The apoptosis of A549 cells in vivo was not due to a direct effect of depletion of ENA-78, as there was no in vitro evidence that ENA-78 had any effect on apoptosis of NSCLC cells. This is consistent with the work of O'Reilly and co-workers (14, 15) who have found previously that angiostatic therapy of murine tumors is associated with increased apoptosis. Similarly, in vivo and in vitro proliferation of A549 cells was unaffected by the presence of anti-ENA-78.

The findings of this study paralleled our previous studies of IL-8 in regulating angiogenesis of NSCLC. We originally found that IL-8 was an angiogenic factor in surgical specimens of human NSCLC (5). However, in this study we found that ENA-78 had a much stronger correlation with tumor vascularity than IL-8. We have reported previously that expression of IL-8, comparable to ENA-78, correlated strongly with tumor size in the SCID mouse-human NSCLC chimera. However, the levels of ENA-78 expressed in freshly isolated human tumors, and human NSCLC in SCID mice were three- and tenfold greater than IL-8, respectively (4).

Many studies have now confirmed that ELR-CXC chemokines are important angiogenic factors in solid tumors. For example, expression of IL-8 has been correlated with angiogenesis in lung, gastric, and CNS cancers (23–25). Additionally, both IL-8 as well as GRO α , GRO β , and GRO γ have been implicated in the control of angiogenesis in malignant melanoma (26–29). This is the first report of the role of ENA-78 as a tumor-derived angiogenic factor.

While we found a significant correlation of ENA-78 expression with tumor vascularity and tumor growth in this study, neutralization of ENA-78 did not completely inhibit tumor growth. This finding reflects the fact that the angiogenic activity induced by NSCLC tumors is probably due to many overlapping or redundant factors acting in parallel. In addition to ENA-78 and IL-8, there are other angiogenic factors that we have not accounted for, and that are beyond the scope of this study, including other ELR-CXC chemokines. A potential strategy for future study would be to target the putative receptor for CXC chemokine-mediated angiogenesis. While this receptor remains unknown, evidence suggests that the receptor which mediates the angiogenic activity of CXC chemokines is CXC chemokine receptor 2 (28, 30). Finally, other non-chemokine angiogenic factors may likely play a role in NSCLC angiogenesis as well, such as basic fibroblast growth factor, or vascular endothelial cell growth factor.

An interesting feature of NSCLC is the wide range of levels of ENA-78 found in freshly isolated human tumors. A question raised by this observation is whether this disparity has prognostic value. The correlation of tumor vascularity with the risk of recurrence of NSCLC suggests that tumor vessel density may be an independent prognostic factor for patients with NSCLC (31). Indeed this finding has been confirmed by Harpole et al. (32). The strong correlation of ENA-78 levels with tumor vascularity suggests that expression of ENA-78 may also be a prognostic factor for patients with early stage

NSCLC. Preliminary studies from our laboratory suggest that elevated tumor levels of ENA-78 are a significant risk factor for recurrence after resection of stage I adenocarcinoma of the lung (our unpublished observations).

In summary, we have found that ENA-78 is an important determinant of angiogenic activity in human NSCLC. The biology of ENA-78 in human NSCLC, and in a SCID mouse model of human NSCLC tumorigenesis suggest that strategies targeting CXC chemokine mediated angiogenesis may be beneficial for the treatment of lung cancer.

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