Increased Blood Clotting, Microvascular Density, and Inflammation in Eotaxin-Secreting Tumors Implanted into Mice

Michael Samoszuk,* Tom Deng,[†]
Mark J. Hamamura,[‡] Min-Ying Su,[‡]
Nicholas Asbrock,* and Orhan Nalcioglu[‡]

From the Pathology Department* and the Center for Functional Onco-Imaging,[‡] University of California, Irvine; and the Department of Pathology and Laboratory Medicine,[†] University of California, Los Angeles, California

An important theme that is emerging in cancer research is the interaction between tumor cells and the host stroma. Because many types of human cancer are infiltrated by eosinophils that are believed to mediate an anti-tumor cytotoxic effect, we developed and studied a transfected B16 murine melanoma cell line that secretes high levels (510 pg/ml/100,000 cells/day) of eotaxin, a chemokine that recruits and activates primarily eosinophils. Here we report that there was increased inflammation (eosinophils, mast cells, mononuclear cells), blood clotting, and microvascular density within the tumors produced by subcutaneous implants of eotaxin-secreting tumor cells in 10 C57BL/6 compared to tumors produced by wild-type tumor cells. The extensive blood clotting in the eotaxin-transfected tumors was associated with significantly decreased blood flow to the tumors as measured by magnetic resonance imaging [(mean maximum signal enhancement of eotaxin-secreting tumors, 147 ± 57 ($n = 7$) compared to 202 ± 36 signal enhancement units ($n = 8$) for the wild-type melanoma cells; $P = 0.04$ by two-tailed, unpaired t -test]. Surprisingly, there was no significant difference between the growth rates or mean masses of the eotaxin-secreting tumors (750 ± 280 mg, $n = 10$) and the wild-type tumors (780 ± 290 , $n = 10$) after 20 days of growth *in vivo*, despite the significantly slower growth rate *in vitro* of the eotaxin-secreting tumor cells. We conclude that eotaxin and the resultant tumor-infiltrating inflammatory cells are not likely to mediate a significant anti-tumor effect *in vivo*. Instead, elevated eotaxin is associated with increased inflammation, microvascular density, and blood clotting. Thus, eotaxin and eosinophils may play a more complex role in modulating the growth of tumors than the simple, anti-tumor cytotoxic effect that has been previously proposed. (*Am J Pathol* 2004, 165:449–456)

An important theme that is emerging in cancer research is the interaction that occurs between cancer cells and the tumor microenvironment that consists primarily of fibroblasts, endothelial cells, and inflammatory cells.^{1–4} In particular, there is now increasing evidence that inflammatory cells may play a critical role in promoting rather than retarding tumor progression.^{5,6} Indeed, such evidence has prompted Hanahan and Weinberg⁷ to propose that “important new inroads [in cancer research] will come from regarding tumors as complex tissues in which mutant cancer cells have conscripted and subverted normal cell types to serve as active collaborators in their neoplastic agenda. The interactions between the . . . malignant cells and these supporting co-conspirators will prove critical to understanding cancer pathogenesis.”

One of the types of inflammatory cells that is commonly seen in the stroma of many types of human cancer is the eosinophil.^{8–13} Long considered to be primarily a mediator of allergic responses in diseases such as asthma and parasitic infections, the eosinophil is a rare type of granulocyte that also appears to play a critical role in tissue remodeling associated with wound healing.¹⁴ Because eosinophil granules also contain a number of powerful cytotoxic compounds, some investigators have proposed that eosinophils may suppress tumor growth by mediating a cytotoxic response to tumor cells.^{15–17} At this time, however, the precise role of eosinophils in promoting or retarding the growth of cancers remains uncertain.^{18,19}

To explore the role of eosinophils in modulating tumor growth in more detail, we developed an experimental model consisting of murine B16 melanoma cells that were transfected to secrete high levels of eotaxin, a potent chemoattractant and activator of eosinophils^{17,20} and, to a lesser extent, mast cells and mononuclear cells.^{21,22} Here we describe the functional and histological properties of tumors produced by this eotaxin-secreting cell line. Our results suggest that eotaxin produces significant functional effects within tumors but is unlikely to elicit an effective cytotoxic response by eosinophils against the tumor cells.

Supported by a College of Medicine Committee on Research Award.

Accepted for publication April 6, 2004.

Address reprint requests to Michael Samoszuk, M.D., Pathology Department, University of California, Irvine, Building 10, Route 40, 101 The City Drive, Orange, CA 92868. E-mail: msamoszu@uci.edu.

Materials and Methods

Creation of Eotaxin-Secreting Transfected Cell Line

Total RNA was first isolated from fresh frozen mouse muscle using a Trizol kit (Invitrogen, Carlsbad, CA). The first-strand cDNA was then synthesized from total RNA using a cDNA synthesis kit (Invitrogen). The cDNA was amplified by polymerase chain reaction (PCR) in 20 μ l of TaqDNA polymerase mixture with forward and reverse primers derived, respectively, from upstream and downstream of the coding regions of the mouse eotaxin precursor.^{17,20} The forward primer was 5' CCGGGCAGTA-CTTCCATCTGT CT 3' and the reverse primer was 5' TTGCATATGCAAGAAATTACAC 3'. The PCR amplification was performed under the following conditions: 94°C for 1 minute, 57°C 1 minute, and 72°C 1 minute for 27 cycles. The PCR products were purified by the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). A 526-bp PCR fragment was then ligated to the TA cloning vector using the TOPO cloning kit (Invitrogen). The 526-bp insert fragment was excised by *Eco*RI (Promega, Madison, WI) and was gel-purified and subcloned to the *Eco*RI site of PCDNA3 0.1 (+) vector (Invitrogen).

The DNA sequence and orientation for the eotaxin insert in the PCNA 3.1(+) vector were verified on double-stranded DNA using T 7 primer (Davis Sequencing, Davis, CA). A sense-oriented and sequence-verified eotaxin clone was chosen for further transfection. The plasmids were grown in *Escherichia coli*. On verification of the proper sequence, the B16 F1 melanoma cell line (American Type Culture Collection, Manassas, VA) was transfected using a Superfect Transfection Kit (Qiagen). Selection of transfected tumor cells was performed with G 418, a neomycin eukaryotic homologue. We then confirmed production of eotaxin by the transfectants by using an enzyme-linked immunosorbent assay (Quantikine; R&D Systems, Minneapolis, MN) to measure murine eotaxin in the cell culture supernatants. Transfected cell lines that secreted high (>500 pg/10⁶ cells/48 hours) levels of eotaxin were then subcloned, recharacterized, and expanded for further studies.

Characterization of Eotaxin-Secreting Cell Line in Vitro

A transfected cell line that continued after subcloning and expansion to secrete high levels of eotaxin (510 pg/ml/100,000 cells/day as measured by the Quantikine enzyme-linked immunosorbent assay) was selected for further study. Total RNA was isolated from 3 $\times$ 10⁶ eotaxin-transfected cells and from wild-type B16 cells using the Qiagen RNeasy minikit (Qiagen). Reverse transcriptase (RT)-PCR was then performed on the isolated RNA using a Titanium One-Step RT-PCR kit (Clontech/BD Biosciences, Palo Alto, CA). A forward oligo DNA primer with sequence (5' ccgggcagt aactccatc tgtctc 3') and a reverse oligo DNA primer with sequence (5' ttgcatatg-caagaaattacac 3') were used in the RT-PCR reaction at a

concentration of 45 μ mol/L (primers synthesized by Invitrogen). A mouse β -actin primer (Clontech) served as the positive control. The RT-PCR reaction and amplification were performed under the following conditions: 50°C 1 hour, 94°C 5 minutes, (92°C 1 minute, 55°C 1 minute, 72°C 1 minute) $\times$ 30, 68°C 2 minutes. The amplified RT-PCR product was then subjected to electrophoresis on a 1.5% agarose gel (0.5 μ g/ml ethidium bromide) for 1.5 hours at 80 V. The gel was visualized using a BioDoc-It system (UVP, Upland, CA).

To confirm the production of eotaxin by the transfected cell line, we also performed immunoprecipitation and Western blotting studies on cell lysates. The eotaxin-transfected cells and the B16 wild-type control cells were first lysed using the Mammalian Cell Protein Extraction reagent (Pierce Biotechnology, Rockford, IL). The lysates were then immunoprecipitated using the Seize X Mammalian Immunoprecipitation kit (Pierce Biotechnology) according to the manufacturer's instructions. For the immunoprecipitation study, we used a rat monoclonal anti-mouse eotaxin antibody (catalog number MAB420, R&D Systems).

Proteins bound to the immunoprecipitation beads were eluted and boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer for 5 minutes. These samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 35 minutes at 200 V using the Mini-Protean 3 Cell (Bio-Rad Laboratories, Hercules, CA) in two identical 15% polyacrylamide gels. One gel was directly visualized using a silver-stain kit (Bio-Rad Laboratories), while the other gel was transferred for subsequent Western blotting onto a polyvinylidene difluoride membrane (Pall Corp., Port Washington, NY) for 1 hour at 100 V. The eotaxin bound to this membrane was then visualized using the rat monoclonal antibody to eotaxin (5 μ g/ml), rabbit anti-rat antibody conjugated to horseradish peroxidase (Rockland Immunochemical Inc., Gilbertsville, PA), and the Opti-4CN detection kit (Bio-Rad Laboratories). The positive control used for the immunoprecipitation and Western blotting studies was recombinant murine eotaxin (R&D Systems).

To compare the *in vitro* growth rates of the B16 wild-type cells and the eotaxin-transfected cells, we performed a kinetic analysis of cell viability and proliferation. Both cell lines were separately seeded at a concentration of 10,000 cells per 100 μ l of medium (Dulbecco's minimal essential medium and 10% fetal bovine serum) into multiple wells of 96-well plates and then cultured in an incubator at 37°C and 5% CO₂. Each day, three wells of each cell type were then assayed for numbers of viable cells using the MTT cell proliferation assay in accordance with the manufacturer's directions (American Type Culture Collection). In brief, cells were incubated with the MTT reagent for 2.5 hours and visually checked under the microscope to ensure complete staining. Detergent reagent was then added and left overnight to extract all of the chromogen. The absorbance of each well at 570 nm was then measured on a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories). The optical density at each time point was directly proportional to the numbers of viable cells.

Animal Studies

C57BL/6 mice (10 female, 10 male), 6 weeks old, were purchased from Jackson Laboratories, Bar Harbor, ME. Using tuberculin syringes, we injected 5×10^5 wild-type or eotaxin-secreting B16 tumor cells into the shaved and epilated dorsal skin of groups of 10 mice (five male and five female). The mice were then examined daily for any evidence of toxicity, and the bidimensional diameters of the tumors were measured using a digital electronic caliper. The cross-sectional areas of the tumors on each day were then approximated by calculating one-half of the product of the two diameters. On day 20 after tumor injection, we performed contrast-enhanced, magnetic resonance imaging of the eotaxin-secreting and wild-type tumors, using procedures that we have previously described in detail.²³ The maximum signal enhancement for each tumor was recorded, and we then compared the mean maximum signal enhancements of the eotaxin-secreting tumors and the wild-type tumors using a two-tailed, unpaired *t*-test. We also examined T2-weighted images of the tumors to identify regions of water (shown with bright signal intensity) and hemorrhage/clotting (dark signal intensity).

At the conclusion of the magnetic resonance imaging study, the surviving mice were sacrificed and the tumors and normal organs (liver, lung, spleen, kidney) removed for histological studies. After carefully dissecting away normal connective tissue and fat from the tumors, we measured the mass of each tumor and then calculated the mean masses of the eotaxin-secreting and wild-type tumors. All tissues were then fixed in neutral-buffered formalin and processed for routine histological studies using hematoxylin and eosin (H&E)-stained sections of the tissues or other special stains as described below. To determine the effects of eotaxin secretion on tumor metastasis, we also microscopically examined the normal organs from each of the mice for the presence of micro-metastases.

Quantification of Inflammatory Cells

To quantify the numbers of eosinophils in the tumors, the tissue sections were stained using Luna's method for eosinophil granules. We counted the bright red eosinophils in 10, nonoverlapping, $\times 40$ microscopic fields of the interface between each tumor and its adjacent connective tissue. A standard Giemsa stain was used in a similar manner to identify and count mast cells in the tumors. Mononuclear inflammatory cells and neutrophils were identified and counted on the sections that were routinely stained with H&E.

Assessment of Microvascular Density and Blood Clotting

The microvascular density in the stroma adjacent to the tumors was measured using the digital dissection procedure that we have previously described in detail.²⁴ In brief, the percentage of the total surface area of each

Figure 1. RT-PCR, immunoprecipitation, and Western blot of eotaxin gene-transfected cell line. mRNA extracted from the eotaxin gene-transfected cell line and parental wild-type cells were subjected to RT-PCR with amplification primers specific for murine eotaxin. **A:** A strong band migrating at the expected size for eotaxin amplification product (526 bp) was detected in the eotaxin-transfected cell line but not in the wild-type parental cell line. Cell lysates were then immunoprecipitated with a polyclonal antibody directed against murine eotaxin, and the immunoprecipitate was visualized with a silver stain. **B:** The eotaxin gene-transfected cell line yielded an 8.4-kd protein band that co-migrated with murine eotaxin-positive control material. **C:** A Western blot of cell lysates confirmed the presence of immunoreactive eotaxin in the transfected cell line but not in the wild-type cell line.

digital image occupied by blood vessels was recorded, and we then calculated the mean MVD for each type of tumor by averaging the percentages from 10 images from each of 10 tumors of each type.

For assessment of blood clotting, we examined tissue sections that were routinely stained with H&E for the presence of intraluminal fibrin clots with coalescent red blood cells and/or lines of Zahn. Ten nonoverlapping, $\times 40$ fields from each tumor were examined, and we recorded the percentage of vessels in each microscopic field with microscopic evidence of thrombosis. From these data, we then calculated the mean percentages of blood vessels in the eotaxin and wild-type tumors with histological evidence of thrombosis.

Collagen Stain

To compare fibrosis in the eotaxin-secreting and wild-type tumors, we stained sections of the tumors with Masson's trichrome stain to produce a sky blue color in regions of collagen fibrosis. The connective tissue adjacent to the tumors was then graded on a semiquantitative scale (0 = no collagen; 1 = occasional collagen fibrosis in $<25\%$ of microscopic fields; 2 = collagen fibrosis in $>25\%$ of microscopic fields). All morphometric studies were performed by a pathologist with no previous knowledge of the type of tumor being examined.

Results

Characterization of Eotaxin-Secreting Cell Line

The results of the RT-PCR, immunoprecipitation, and Western blotting studies are illustrated in Figure 1; A to C. A strong band at 526 bp was evident in the PCR amplification product derived from mRNA extracted from the

Figure 2. *In vitro* growth rates of eotaxin gene-transfected cell line and wild-type cell line as measured by MTT cell proliferation assay. Transfected and wild-type cells were initially seeded at the same density into wells of a 96-well plate. At varying intervals, the numbers of metabolically active cells were compared using the dye reduction assay. Wild-type cells initially grew much faster than the transfected cells.

eotaxin-transfected cell line. This band corresponded to the expected size of the eotaxin amplification product and was not detected in the amplification product derived from the wild-type (parental) cell line. In the immunoprecipitation study, a protein band migrating at 8.4 kd was detected in the lysate of the eotaxin-transfected cell line but not in the lysate from the wild-type cells; this protein band corresponded to the known molecular mass of murine eotaxin and also co-migrated with an eotaxin-positive control. Immunoblotting of the lysate confirmed that the immunoprecipitated material was reactive with antibody directed against murine eotaxin. On the basis of these studies, therefore, we concluded that the transfected cell line secreted eotaxin, and the wild-type parental cells did not.

The results of the *in vitro* cell proliferation assay are shown in Figure 2. Although the wild-type and eotaxin-secreting cell lines were seeded at the same density, the wild-type cells appeared to grow initially at a much higher rate than the eotaxin-secreting cell line, as evidenced by the spectrophotometric measurement of numbers of metabolically active tumor cells at different time points. By 100 hours, however, the numbers of viable cells were nearly equal, suggesting that the growth of the wild-type cells had reached a plateau. On the basis of this study, we concluded that the eotaxin-secreting tumor cells grew more slowly than the wild-type cells *in vitro*.

Animal Studies

The growth rates of the eotaxin-secreting tumors and wild-type tumors were nearly identical *in vivo* (Figure 3). At the end-point of the experiment, the average mass of the 10 tumors produced by the eotaxin-secreting cell line was statistically identical to the average mass of the 10 wild-type tumors (Figure 4A). Metastases were not detected in any of the normal organs in animals with either type of implanted tumor. There were no microscopically evident abnormalities in the normal organs from the mice bearing the eotaxin-secreting tumors.

The magnetic resonance imaging studies were successfully completed on eight mice with eotaxin-secreting tumors and seven mice with wild-type tumors. Complete

Figure 3. *In vivo* growth rates of eotaxin-secreting and wild-type tumor cells implanted into skin of B16 mice. The tumor area was measured on each day with digital electronic calipers. Although there was substantial variation in the tumor size at each day of the experiment, there was no significant difference in the mean tumor areas of the eotaxin-secreting and wild-type tumors.

data from the remaining mice could not be obtained because of technical problems such as inability to establish and maintain tail vein access or death during the imaging procedure. The results of the magnetic resonance studies of signal intensity are shown in Figure 4B. Although there was substantial mouse-to-mouse variation in the maximum magnetic resonance image signal intensity, the mean maximum signal enhancement of the wild-type tumors was statistically greater than the mean maximum signal enhancement of the eotaxin-secreting tumors. This result is consistent with a greater degree of blood perfusion in the wild-type tumors.

Gross examination of the tumors revealed that 8 of 10 eotaxin-secreting tumors had evidence of extensive surface ulceration, compared to only 2 of 10 wild-type tumors. Representative T2-weighted images of eight different tumors are presented in Figure 5. The eotaxin-secreting tumors in these images generally had missing tops, consistent with the ulceration that was evident on gross examination. By contrast, the wild-type tumors appeared to have generally intact surface epithelium.

Quantification of Inflammatory Cells

Microscopic evaluation of the eotaxin-secreting tumors revealed substantially greater eosinophilia at the interface between tumor and connective tissue than in wild-

Figure 4. Comparison of masses (A) and blood perfusion (B, magnetic resonance signal enhancement) in wild-type and eotaxin gene-transfected tumors. At the end of 20 days of growth, the average mass of the eotaxin-transfected tumors was statistically identical to the average mass of the wild-type tumors. There was significantly lower blood perfusion (signal enhancement), however, in the eotaxin-transfected tumors. This result is consistent with impaired blood flow caused by thrombosis.

Figure 5. Representative T2-weighted magnetic resonance images of eotaxin-secreting and wild-type tumors. Most of the eotaxin-secreting tumors had missing tops, consistent with necrotic ulceration. By contrast, the wild-type tumors generally had smooth intact surfaces.

type tumors (Figure 6A). Interestingly, we were not able to detect intact eosinophils within the deeper portions of any of the eotaxin-secreting tumors, perhaps because the internal eosinophils had undergone complete degranulation that rendered them unrecognizable amid the debris of necrotic tumor cells. Mast cells were readily evident at the interface between tumor and connective tissue in both types of tumor implants (Figure 6B). Greater than 50% of the mast cells adjacent to eotaxin-secreting tumors appeared to be undergoing degranulation (Figure 6C), however, compared to less than 10% of the mast cells in the wild-type tumors. Overall, there were significantly more inflammatory cells (eosinophils, mast cells, mononuclear cells) in the eotaxin-secreting tumors than in the wild-type tumors (Figure 7). Infiltration by neutrophils, however (less than 3 per $\times 40$ field) was similar in both types of tumors (data not graphed).

Assessment of Microvascular Density and Blood Clotting

Numerous clotted blood vessels were visible in the eotaxin-secreting tumors (Figure 6D), whereas the blood vessels in the wild-type tumors were generally free of clots (Figure 6E). We also noted an apparent increase in the number of small blood vessels in the connective tissue adjacent to the eotaxin-secreting tumors (Figure 6F), and many of these small blood vessels appeared to be occluded by coalescent masses of red blood cells. Morphometric analysis confirmed that the microvascular density and proportion of clotted blood vessels were both significantly greater in the eotaxin-secreting tumors compared to the wild-type tumors (Figure 8).

Collagen Stain

There was no significant difference in collagen fibrosis (Figure 6G) between the eotaxin-secreting tumors (average score, 0.7) and wild-type tumors (average score, 0.9).

Discussion

We have developed and characterized a transfected, murine melanoma cell line that secretes functionally active eotaxin that is not normally produced by the parental,

Figure 6. Microscopic appearances of representative eotaxin-secreting and wild-type tumors. **A:** The interface between tumor and adjacent connective tissue contained numerous bright red eosinophils with characteristic bilobed nuclei (Luna's stain). **B** and **C:** Mast cells with deep purple granules were also readily identified at the tumor-stroma interface in the wild-type tumors (Giemsa stain, **B**) and eotaxin-secreting tumors (Giemsa stain, **C**). In the latter case, many of the mast cells showed histological evidence of structural disintegration and degranulation as manifested by aggregates of free purple granules. **D:** The blood vessels in the eotaxin-secreting tumors (H&E stain) often were partially or totally occluded by fibrin clots with lines of Zahn or aggregated red blood cells. **E:** By contrast, the blood vessels in the wild-type tumors generally contained red blood cells without evidence of significant clotting (H&E stain). **F:** In addition, the interface between eotaxin-secreting tumor and adjacent connective tissue often had multiple small blood vessels that were occluded by coalescent masses of red blood cells (H&E stain), suggestive of granulation tissue with extensive thrombosis. **G:** Well-organized collagen fibers (blue stain, Masson trichrome) were relatively infrequent in the eotaxin-secreting as well as the wild-type tumors.

wild-type cell line. Although the eotaxin-secreting cells grew more slowly than the wild-type cells *in vitro*, the eotaxin-secreting tumors grew at the same rate as the wild-type tumor cells when implanted into the skin of mice. Moreover, we have demonstrated that the eotaxin-

Figure 7. Quantification of inflammatory cells in eotaxin-secreting and wild-type tumors. Eosinophils were identified as bright red cells in tumors stained by Luna's method and counted in multiple nonoverlapping microscopic fields as described in the text. Similarly, mast cells were identified by their dark purple cytoplasmic granules in tissue sections stained with Giemsa stain. Mononuclear cells (lymphocytes, macrophages) and neutrophils were presumptively identified by their characteristic microscopic appearance in sections stained with H&E. Eosinophils, mast cells, and mononuclear cells were all significantly ($P < 0.05$) increased in the eotaxin-secreting tumors compared to wild-type tumors by two-tailed t -test. Neutrophils were relatively infrequent ($<3/40 \times$ field) in both types of tumors, and there was no significant difference between the two types of tumors (data not graphed).

secreting tumors had more extensive inflammation (eosinophilia, mast cells, mononuclear cells), widespread thrombosis, ulceration, and blood vessel formation than the wild-type tumors. The histological evidence of thrombosis was accompanied by functional evidence of impairment in blood flow as measured by magnetic resonance imaging studies. We conclude, therefore, that eotaxin produces significant histological and functional changes in tumors but is unlikely to elicit an effective

Figure 8. Morphometric quantification of microvascular density and proportion of clotted blood vessels in eotaxin-secreting and wild-type tumors. The percentage of surface area of each tumor image containing blood vessels was determined by the digital dissection procedure described in the text. Clotted blood vessels were identified by the presence of intraluminal fibrin with coalescent red blood cells and/or lines of Zahn. There were significantly (two-tailed t -test) more blood vessels in the eotaxin-secreting tumors, and significantly more of these blood vessels had histological evidence of clotting, compared to the wild-type tumors.

cytotoxic response by eosinophils or other inflammatory cells against the tumor cells.

Because eotaxin is a powerful chemoattractant for mast cells, macrophages, and T cells in addition to eosinophils,^{21,22} the effects of eotaxin that we observed on tumor histology and blood flow could potentially be attributed to multiple cell types in addition to eosinophils. Indeed, our morphometric analysis confirmed that the effects of eotaxin were not limited to eosinophils but included recruitment to the tumors of mast cells and mononuclear cells as well.

There are a number of well-established mechanisms by which these cells could modulate the connective tissue around tumors. For example, eosinophils are a potent source of transforming growth factor- α in healing cutaneous wounds,¹⁴ and eotaxin by itself can also induce an *in vivo* angiogenic response, even in the absence of eosinophils.²⁵ Inflammatory mast cells have also been shown to up-regulate angiogenesis and connective tissue remodeling in tumors,²⁶ and tissue macrophages play many important roles in wound healing. Thus, our finding of increased microvascular density within the eotaxin-secreting tumors could be attributed to multiple factors, including eosinophils mediating a wound-healing response by transforming growth factors, eotaxin itself promoting a direct angiogenic effect, mast cells up-regulating tumor angiogenesis and tissue remodeling, and the effects of macrophages on wound healing.

In view of the well-known effects of eosinophils on tissue fibrosis and the increased microvascular density that we observed in the eotaxin-secreting tumors, we were somewhat surprised to discover that there was no apparent change in collagen fibrosis in the eotaxin-secreting tumors. Although the explanation for this discrepancy between increased blood vessels and unaltered fibrosis was not investigated in this study, it is possible that the relatively brief time course of our experiment (20 days) was insufficient for the collagen fibrosis to develop fully and become detectable by the Masson trichrome stain that we used. An alternate explanation is that the rapidly proliferating tumor cells simply obliterated any nascent fibrosis before it could become organized and visible.

The presence of extensive thrombosis within the blood vessels of the eotaxin-secreting tumors was also somewhat unexpected. In retrospect, we believe that it was probably attributable mostly to the infiltrating eosinophils because eosinophil granule proteins such as eosinophil peroxidase have been shown to have potent procoagulant activity.²⁷ In addition, eosinophil cationic protein enhances factor XII-dependent reactions, thereby shortening the coagulation time of normal plasma,²⁸ and eosinophil cationic granule proteins potentially inhibit the anticoagulant activity of the glycosylated form of thrombomodulin.²⁹ Thus, a number of eosinophil-related mechanisms could account for the increased thrombosis and associated impaired blood flow that we observed in the eotaxin-secreting tumors. We acknowledge, however, that other inflammatory cells such as mast cells and mononuclear cells may also have contributed significantly to modulating hemostasis within the tumors. Fur-

thermore, we have not excluded the possibility that eotaxin itself may have some procoagulant activity by mechanisms that as yet remain undefined. Regardless of the mechanism of the increased blood clotting, we believe that the increased blood clotting almost certainly explains the apparent inconsistency between the increased microvascular density and concurrent decreased blood flow that we observed in the eotaxin-secreting tumors.

The normal growth rate of the eotaxin-secreting tumors *in vivo* initially seems to be inconsistent with previous reports that eosinophils were responsible for interleukin-4-induced tumor suppression^{15,17} and the widespread perception that eosinophils are responsible for killing parasites. A more recent study, however, implicated neutrophils, rather than eosinophils, in the growth suppression that was reported in the interleukin-4-secreting tumors.³⁰ Moreover, eosinophils infiltrating interleukin-5, gene-transfected tumors do not suppress tumor growth,³¹ and infiltration by eosinophils of natural, interleukin-5-secreting human tumors such as lymphoma is not associated with tumor regression.¹¹ Finally, we note that the *in vitro* studies that demonstrated an anti-tumor cytotoxic effect mediated by eosinophils used exceptionally high ratios of eosinophils to tumor cells (10:1 to 100:1) that probably do not accurately reflect the natural conditions in tumors.¹⁶ Consequently, on the basis of our studies, we conclude that eotaxin is unlikely to elicit an effective anti-tumor response by eosinophils or other inflammatory cells *in vivo*.

The presence of extensive ulceration and diminished blood flow within the eotaxin-secreting tumors appears somewhat paradoxical in view of the role of inflammatory cells in mediating a wound healing response. There is evidence, however, that eosinophils negatively affect wound re-epithelialization and that depletion of eosinophils actually accelerates open skin wound epithelial closure.³² In addition, the enhanced blood clotting associated with eosinophilia could have accelerated superficial tissue necrosis and infarction. It is not clear, however, whether the increased microvascular density was secondary to the ulceration and necrosis or primarily triggered by some other mechanism in the eotaxin-secreting tumors.

In summary, our results are significant because they demonstrate the complex effects of eotaxin on tumors and the apparent absence of an effective anti-tumor response mediated by eosinophils and other inflammatory cells elicited by eotaxin. Our findings, therefore, are consistent with the accumulating evidence that inflammation does not effectively retard the growth and progression of cancers.^{5,6} Further work is needed, therefore, to determine what role eosinophils and other inflammatory cells may play in regulating the growth and progression of tumors.

Acknowledgments

We thank Dr. Huali Wang, Vivian Fong, and Thu Huynh for their excellent technical assistance in the imaging experiments and characterization of the cell line.

References

1. Bissell MJ, Radisky D: Putting tumours in context. *Nat Rev Cancer* 2001, 1:46–54
2. Park CC, Bissell MJ, Barcellos-Hoff MH: The influence of the micro-environment on the malignant phenotype. *Mol Med Today* 2000, 6:324–329
3. Tlsty TD: Stromal cells can contribute oncogenic signals. *Semin Cancer Biol* 2001, 11:97–104
4. Erickson AC, Barcellos-Hoff MH: The not-so innocent bystander: microenvironment as a therapeutic target in cancer. *Exp Opin Ther Targets* 2003, 7:71–88
5. Coussens LM, Werb Z: Inflammation and cancer. *Nature* 2002, 420:860–867
6. Hanahan D, Lanavecchia A, Mihich E: Fourteenth annual Pezcoller symposium: the novel dichotomy of immune interaction with tumors. *Cancer Res* 2003, 63:3005–3008
7. Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 2000, 100:57–70
8. Samoszuk M: Eosinophils and human cancer. *Histol Histopathol* 1997, 12:807–812
9. Samoszuk MK, Nathwani BN, Lukes RJ: Extensive deposition of eosinophil peroxidase in Hodgkin's and non-Hodgkin's lymphomas. *Am J Pathol* 1986, 125:426–429
10. Samoszuk M, Ravel J, Ramzi E: Epstein-Barr virus and interleukin-5 mRNA in AIDS-related lymphomas with eosinophilia. *Hum Pathol* 1992, 23:1355–1359
11. Samoszuk M, Ramzi E, Cooper DL: Interleukin-5 mRNA in three T-cell lymphomas with eosinophilia. *Am J Hematol* 1993, 42:402–404
12. Samoszuk MK, Nguyen V, Gluzman I, Pham JH: Occult deposition of eosinophil peroxidase in a subset of human breast carcinomas. *Am J Pathol* 1996, 56:87.90
13. Samoszuk M, Lin F, Rim P, Strathearn G: New marker for blood vessels in human ovarian and endometrial cancers. *Clin Cancer Res* 1996, 2:1867–1871
14. Todd R, Donoff BR, Chiang T, Chou MY, Elovic A, Gallagher GT, Wong DT: The eosinophil as a cellular source of transforming growth factor alpha in healing cutaneous wounds. *Am J Pathol* 1991, 138:1307–1313
15. Tepper RI, Coffman RL, Leder P: An eosinophil-dependent mechanism for the anti-tumor effect of interleukin-4. *Science* 1992, 257:548–551
16. Mattes J, Hulett M, Xie W, Hogan S, Rothenberg ME, Foster P, Parish C: Immunotherapy of cytotoxic T-cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. *J Exp Med* 2003, 197:387–393
17. Rothenberg ME, Luster AD, Leder P: Murine eotaxin, and eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proc Natl Acad Sci USA* 1995, 92:8960–8964
18. Wong DT, Bowen SM, Elovic A, Gallagher GT, Weller PF: Eosinophil ablation and tumor development. *Oral Oncol* 1999, 35:496–501
19. Fernandez-Acenero MJ, Galindo-Gallego M, Sanz J, Aljama A: Prognostic influence of tumor-associated eosinophilic infiltrate in colorectal carcinoma. *Cancer* 2000, 88:1544–1548
20. Jose PJ, Griffiths-Johnson DA, Collins PD, Walsh DT, Moqbel R, Totty NF, Truong O, Hsuan JJ, Williams TJ: Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J Exp Med* 1994, 179:881–887
21. Jundt F, Anagnostopoulos I, Bommer K, Emmerich R, Muller G, Foss HD, Royer HD, Stein H, Dorken B: Hodgkin/Reed-Sternberg cell induce fibroblasts to secrete eotaxin, a potent chemoattractant for T cells and eosinophils. *Blood* 1999, 94:2065–2071
22. Menzies-Gow A, Ying S, Sabroe I, Stubbs VL, Soler D, Williams TJ, Kay AB: Eotaxin (CCL11) and eotaxin-2 (CCL24) induce recruitment of eosinophils, basophils, neutrophils, and macrophages as well as features of early- and late-phase allergic reactions following cutaneous injection in human atopic and nonatopic volunteers. *J Immunol* 2002, 169:2712–2718
23. Samoszuk M, Corwin M, Yu H, Wang J, Nalcioğlu O, Su, MY: Inhibition of thrombosis in melanoma allografts in mice by endogenous mast cell heparin. *Thromb Haemost* 2003, 90:351–360

24. Samoszuk M, Leonor L, Espinoza F, Carpenter PM, Nalcioglu O, Su MT: Measuring microvascular density in tumors by digital dissection. *Analyt Quant Cytol Histol* 2002, 24:15–22
25. Salcedo R, Youn HA, Ponce ML, Ward JM, Kleinman H, Murphy WJ, Oppenheim JJ: Eotaxin (CCL11) induces in vivo angiogenic responses by human CCR3+ endothelial cells. *J Immunol* 2001, 166: 7571–7578
26. Coussens LM, Raymond WW, Bergers G, Laig-Webster M, Behrendt- sen O, Werb Z, Caughey GH, Hanahan D: Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogene- sis. *Genes Dev* 1999, 13:1382–1397
27. Samoszuk M, Corwin M, Hazen SL: Effects of human mast cell tryptase and eosinophil granule proteins on the kinetics of blood clotting. *Am J Hematol* 2003, 73:18–25
28. Venge P, Dahl R, Hallgren R: Enhancement of factor-XII dependent reactions by eosinophil cationic protein. *Thromb Res* 1979, 14: 641–649
29. Slungaard A, Vercollotti GM, Tran T, Gleich GR, Key NS: Eosinophil granule proteins impair thrombomodulin function. *J Clin Invest* 1993, 91:1721–1730
30. Noffz G, Qin Z, Kopf M, Blankenstein T: Neutrophils but not eosino- phils are involved in growth suppression of IL-4 secreting tumors. *J Immunol* 1998, 160:345–350
31. Kruger-Krasagakes S, Li W, Richter G, Diamanstein T, Blankenstein T: Eosinophils infiltrating interleukin-5-gene-transfected tumors do not suppress tumor growth. *Eur J Immunol* 1993, 23:992–995
32. Yang J, Torio A, Donoff RB, Gallagher GT, Egan R, Weller PF, Wong DT: Depletion of eosinophil infiltration by anti-IL-5 monoclonal anti- body (TRFK-5) accelerates open skin wound epithelial closure. *Am J Pathol* 1997, 151:813–819