Onset of Abnormal Blood and Lymphatic Vessel Function and Interstitial Hypertension in Early Stages of Carcinogenesis

Jeroen Hagendoorn,1 Ricky Tong,1 Dai Fukumura,1 Qingcong Lin,2 Jennifer Lobo,1 Timothy P. Padera,1 Lei Xu,2 Raju Kucherlapati,2 and Rakesh K. Jain2

1Edwin L. Steele Laboratory for Tumor Biology, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School and 2Harvard Medical School-Partners Healthcare Center for Genetics and Genomics, Boston, Massachusetts

Abstract

Recent improvements in diagnostic methods have opened avenues for detection and treatment of (pre)malignant lesions at early stages. However, due to the lack of spontaneous tumor models that both mimic human carcinogenesis and allow direct optical imaging of the vasculature, little is known about the function of blood and lymphatic vessels during the early stages of cancer development. Here, we used a spontaneous carcinogenesis model in the skin of DNA polymerase η–deficient mice and found that interstitial fluid pressure was already elevated in the hyperplastic/dysplastic stage. This was accompanied by angiogenic blood vasculature that exhibited altered permeability, vessel compression, and decreased smooth muscle actin–positive perivascular cell coverage. In addition, the lymphatic vessels in hyperplastic/dysplastic lesions were partly compressed and nonfunctional. These novel insights may aid early detection and treatment strategies for cancer. (Cancer Res 2006; 66(7): 3360-4)

Introduction

Although it is well established that angiogenesis precedes neoplastic transformation (1, 2), it is unclear how the newly formed blood vessels function and affect the local tissue microenvironment. Moreover, the status of lymphatic vessels during early stages of carcinogenesis is unknown (3). Because optimal scheduling of antiangiogenic agents can increase the effectiveness of conventional cytotoxic therapy by reversing abnormalities in tumor blood vasculature (4), understanding the functional changes in blood and lymphatic vasculature may have broad implications for treatment of early cancer stages.

Tumor blood and lymphatic vessels are structurally and functionally abnormal. This abnormality can result from an imbalance in molecular factors regulating vessel growth and maturation as well as from compressive mechanical forces generated by proliferating neoplastic cells (5, 6). Abnormal growth and maturation can lead to hyperpermeable, tortuous blood vessels whereas compressive forces can cause vessel lumen collapse and thus restrict flow. Although changes in vascular structure during the transition from hyperplasia/dysplasia to neoplasia have been investigated in several murine spontaneous tumor models (7, 8), to date, corresponding changes in blood and lymphatic vessel function and the interstitial fluid pressure have not been reported. We have recently developed mice genetically deficient for DNA polymerase η (pol η) that exhibit a reproducible pattern of skin squamous carcinomas induced by UVB irradiation (described in ref. 9). Here, we use pol η−/− mice to measure interstitial fluid pressure and the structural and functional status of blood and lymphatic vessels in the early stages of carcinogenesis.

Materials and Methods

Animals and UVB irradiation. Mice made deficient in pol η by targeted deletion of exon 4 of the pol η gene were used in these experiments (9). These mice exhibit a well-defined and reproducible pattern of UVB light-induced carcinogenesis similar to human xeroderma pigmentosum variant (shown in detail in Figs. 4 and 5.4 of ref. 9). Pol η−/− and wild-type (WT) mice were UVB irradiated thrice a week at a dose of 3.75 kJ/m2 each time with a bank of two UVB lamps. UVB flux was measured by a UVX-31 digital radiometer (Blak-Ray lamp Model XX-15M and Model UVX-31, UV Products, Inc., Upland, CA). For all the experiments, Pol η−/− mice were used that had received ~10 weeks of irradiation (and had developed hyperplastic/dysplastic lesions) or at least 5 months of irradiation (tumor stage) and compared with WT control mice that had received 3 or 5 months of irradiation.

Interstitial fluid pressure measurements. Interstitial fluid pressure was measured using the wick-in-needle technique (10). One or two interstitial fluid pressure measurements were obtained per ear in WT mice (n = 5) and in pol η−/− mice during the hyperplastic/dysplastic stage (n = 8) and the tumor stage (n = 6). Briefly, 26-gauge needles with a 1-mm side hole 2 mm from the tip were filled with two surgical sutures (6-0 ethilon). The needle was connected to a pressure transducer by polyethylene tubing filled with sterile heparinized saline. The pressure transducer was connected to an amplifier and the signal was sent to a chart recorder. For all measurements, good fluid communication was confirmed by compressing and decompressing the tubing with a screw clamp and verifying a proper response. The mean pressure was taken before and after compression-decompression.

Blood and lymphatic vessel function. To assess vascular function intravitaly, angiography and lymphangiography were done in WT, hyperplastic/dysplastic, and tumor-bearing animals (n = 3-5 per group per experiment). Angiography was done using i.v. injection of FITC-dextran (Mw, 2,000,000; Sigma, St. Louis, MO; ref. 6). Lymphangiography was done after slow, intradermal injection of 1 to 2 μL of FITC-dextran (Mw, 2,000,000; Sigma) in the peripheral ear or the surface of tumors. The vessels were imaged by single-photon fluorescence microscopy as previously described (11).

Vascular permeability measurements. The effective microvascular permeability coefficient was measured as described (12) using tetramethylrhodamine–labeled or cyanine-5-labeled bovine serum albumin.

Immunohistochemistry and image analysis. For immunohistochemistry, WT, hyperplastic/dysplastic, and tumor-bearing animals (n = 3-5 per group) were injected i.v. with biotinylated Lycopersicon esculentum lectin (10 μg/g body weight; Vector Laboratories, Inc., Burlingame, CA).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

J. Hagendoorn, R. Tong, and D. Fukumura contributed equally to this work.

Requests for reprints: Rakesh K. Jain, Edwin L. Steele Laboratory for Tumor Biology, Department of Radiation Oncology, Massachusetts General Hospital, 100 Blossom Street, Cox 7, Boston, MA 02114. Phone: 617-726-4083; Fax: 617-724-1819; E-mail: jain@steele.mgh.harvard.edu.

©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-05-2655
After 5 minutes, animals were sacrificed by pericardial perfusion with 4% formaldehyde. Five-micron-thick paraffin sections were prepared from excised, fixed ears. Lectin was detected by horseradish peroxidase–conjugated streptavidin (Vector Elite ABC kit, Vector Laboratories). Immunohistochemistry was done for MEGA32 (1:200; BD Biosciences, San Jose, CA), α-smooth muscle actin (α-SMA; 1:1,000; Sigma), and LYVE-1 (1:2,000; Upstate, Charlottesville, VA) using microwave antigen retrieval (DakoCytomation, Glostrup, Denmark) and Envision Plus Kit (DakoCytomation). To quantify blood and lymphatic vessel morphology and pericyte coverage, digital images were taken from immunohistochemistry sections. Vessels (lectin/MECA32 or LYVE-1 positive structures) were hand-traced using ImageJ image analysis software as described (6) to calculate the perimeter and aspect ratio—the ratio of maximum to minimum diameter—of stained vessels. Perivascular cell coverage was quantified by double staining of perfused lectin and α-SMA. Total number of vessels and vessels covered by pericytes were counted in all groups.

**Gene array and quantitative PCR.** To determine expression of genes involved in angiogenesis, total RNA was extracted from WT and hyperplastic/dysplastic pol ε−/− ears (n = 2–3 per group) using TRIzol reagent (Invitrogen, Carlsbad, CA). To screen for relative expression of multiple genes, cDNA arrays containing 96 genes involved in angiogenesis were used according to the instructions of the manufacturer (GEArray Q Series). Chemiluminescent spots were quantified by densitometry and normalized with β-actin (FluoroChem 8000 system). A change of >3-fold in pol ε−/− versus WT ears was considered significant differential expression. The mRNA levels of several molecules associated with angiogenesis [Ang1, Ang2, EDG1, IFNγ, SPHK1, Tie1, transforming growth factor (TGF)-α, TGF-β receptor 3, vascular cell adhesion molecule, vascular endothelial growth factor (VEGF), VEGF receptor 1, VEGF receptor 2, platelet-derived growth factor (PDGF)-B, PDGF-C, PDGF-D, and PDGFR-β] were further determined using real-time quantitative PCR. Quantitative real-time PCRs were done on the ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). All experiments were done in duplicate and relative quantification done by comparing to β-actin. A serial dilution of template was used to verify primer efficiency. Primer sequences can be found in Supplementary Fig. S1.

**Statistics.** Results are presented as mean ± SE. Student t test was used to evaluate statistical significance (defined as P < 0.05). F test was used to calculate equality of variances. All experiments were done under the guidelines of the Massachusetts General Hospital Animal Care and Use Committee.

**Results.** Elevated interstitial fluid pressure in hyperplastic/dysplastic lesions and tumors. We measured interstitial fluid pressure in the ears of WT and pol ε−/− mice in the hyperplastic/dysplastic stage or with established tumors. In the hyperplastic/dysplastic stage, interstitial fluid pressure was increased compared with WT mice (4.5 ± 0.8 versus −0.2 ± 0.7 mm Hg, respectively; P < 0.05). In tumors, interstitial fluid pressure was even further increased (9.7 ± 1.1 mm Hg; P < 0.05; Fig. 1A). Thus, these data show that interstitial fluid pressure is already increased in the hyperplastic/dysplastic stage of carcinogenesis.

**Abnormal vasculature in hyperplastic/dysplastic lesions and tumors.** Elevated interstitial fluid pressure can result from abnormal blood vessels as well as lymphatic vessels. Thus, we first did intravitral microscopy to analyze vascular function. Angiography revealed that hyperplastic/dysplastic lesions in pol ε−/− mice had abnormal, dilated vessels with multiple leaky spots (Fig. 1B and C). The microvascular permeability in hyperplastic/dysplastic lesions displayed a wide variability compared with WT ears (9.37 ×
Immunohistochemistry also revealed that vascular density was increased in hyperplastic/dysplastic regions had an increased permeability. In addition, αSMA-positive perivascular cell coverage was decreased in hyperplastic/dysplastic lesions and even further decreased in tumors (Fig. 1G-I and K). Taken together, these data show that angiogenic vasculature becomes chaotic, hyperpermeable, and functionally abnormal in the hyperplastic/dysplastic stage of carcinogenesis.

Abnormal lymphatics in hyperplastic/dysplastic lesions and tumors. We next assessed whether structural and functional abnormalities of the lymphatics occurred in hyperplastic/dysplastic lesions. Lymphangiography in WT mice showed normal lymphatic networks (Fig. 2A). In contrast, pol η−/− ears in the hyperplastic/dysplastic stage showed irregular lymphatic networks that were locally compressed or dilated (Fig. 2B) whereas pol η−/− tumors contained no functional lymphatics (data not shown). Immunohistochemistry confirmed that WT ears had circular, open lymphatic vessels (Fig. 2C). Lymphatic vessels in hyperplastic/dysplastic lesions were more compressed compared with WT controls (Figs. 2D and 3A) and only 82% of vessels had an open lumen versus 100% in controls (Fig. 3B). Perilesional lymphatics, on the other hand, were dilated as shown by their increased perimeter (128.9 ± 6.9 versus 89.2 ± 2.1 μm; P < 0.05; Figs. 2D and 3C). Tumor lymphatics had a decreased perimeter compared with lymphatics in premalignant lesions (78.3 ± 3.2 versus 90.1 ± 3.1 μm; P < 0.05), were even more compressed, and only 10% had an open lumen (Figs. 2E and 3A-C).

Gene array analysis of hyperplastic/dysplastic lesions. To determine if molecules that have a role in blood and lymphatic vessel formation and remodeling were induced or blocked, we did gene array analysis for 96 genes associated with angiogenesis (Table 1). Gene array densitometry and subsequent quantitative PCR showed no different expression of major determinants of blood and lymphatic vessel formation and remodeling in the hyperplastic/dysplastic pol η−/− ears versus WT, except for a 3.9-fold up-regulation of IFNγ as confirmed by quantitative PCR (Fig. 3D). Possibly, accumulation or activation of immune cells in hyperplastic/dysplastic lesions contributed to this up-regulation of IFNγ (13).

Discussion

Although the importance of angiogenesis during early neoplastic transformation is well recognized, there are no reports that assess the function of blood and lymphatic vasculature during the early stages of cancer development, presumably due to a lack of suitable animal models. In contrast to other transgenic mice (14), pol η−/− mice exhibit UV light-induced multistep carcinogenesis in the ears that can be easily accessed with intravital microscopy. Using this novel approach, we found that blood vessels begin to show abnormalities such as increased vascular density, dilation, and hyperpermeability. Blood vessels are also collapsed and have incomplete perivascular cell coverage in the hyperplastic/dysplastic stage. Gene array analysis did not detect different expression of major determinants of blood and lymphatic vessel formation and remodeling in the hyperplastic/dysplastic stage, except for up-regulation of IFNγ. These findings are consistent with earlier work that showed no different expression of well-known angiogenic molecules in the hyperplasia stage (7). IFNγ can be expressed by lymphocytes involved in an immune reaction against tumor cells and stroma (15). At the same time, lymphocytes may play a role in early tumor progression by expression of matrix metalloproteinase 9 (16). Further investigation will need to address the recruitment and role of lymphocytes and other immune cells in early stages of cancer.

In concert with our previous findings (6, 11, 17), we found no functional lymphatics in established pol η−/− tumors. Surprisingly, even in the hyperplastic/dysplastic stage, lesional lymphatics were more compressed than normal vessels and a significant fraction had no open lumen. At the same time, perilesional vessels were increased in size, possibly providing partially compensatory drainage of the excess interstitial fluid (3). Nearly all lymphatic structures inside tumors were compressed and their size was decreased, suggesting that these are remnants of lymphatic vessels. Thus, lymphatic vessel compression with resultant functional abnormalities in lymph drainage already occurs during early stages of carcinogenesis.

Both abnormal, hyperpermeable blood vasculature and impaired lymphatic function lead to increased interstitial fluid pressure, a
major transport barrier for the delivery of therapeutic agents in tumors (18). The earliest increase in interstitial fluid pressure reported before the current study was within days of implanting cancer cells in the dorsal skinfold chamber (19). Using our spontaneous tumor model, we observed increased interstitial fluid pressure in the hyperplastic/dysplastic stage. Therefore, similar to tumors, delivery of chemopreventive agents to hyperplastic/dysplastic lesions may be hindered by elevated interstitial fluid pressure.

Table 1. Angiogenic gene array analysis of the ears of pol η−/− mice in the hyperplastic/dysplastic stage and WT mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>KO</th>
<th>Gene</th>
<th>WT</th>
<th>KO</th>
<th>Gene</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adamts1</td>
<td>0.141</td>
<td>0.329</td>
<td>Fn1</td>
<td>0.388</td>
<td>0.319</td>
<td>Serpinb2</td>
<td>0.970</td>
<td>1.156</td>
</tr>
<tr>
<td>Bnase4</td>
<td>0.750</td>
<td>0.799</td>
<td>Hgf</td>
<td>0.371</td>
<td>0.372</td>
<td>Serpinf1</td>
<td>1.016</td>
<td>0.355</td>
</tr>
<tr>
<td>Cd36</td>
<td>0.421</td>
<td>0.684</td>
<td>Hif1a</td>
<td>0.835</td>
<td>0.408</td>
<td>Sparc</td>
<td>1.147</td>
<td>0.595</td>
</tr>
<tr>
<td>Cdhh5</td>
<td>0.918</td>
<td>0.827</td>
<td>Ifna1</td>
<td>0.183</td>
<td>0.295</td>
<td>Tek</td>
<td>0.615</td>
<td>0.568</td>
</tr>
<tr>
<td>Chga</td>
<td>0.447</td>
<td>0.880</td>
<td>Ifng</td>
<td>0.082</td>
<td>0.519</td>
<td>Tgfα</td>
<td>0.011</td>
<td>0.185</td>
</tr>
<tr>
<td>Coll18a1</td>
<td>0.107</td>
<td>0.149</td>
<td>Ifgam</td>
<td>0.270</td>
<td>0.396</td>
<td>Tgfb1</td>
<td>0.117</td>
<td>0.115</td>
</tr>
<tr>
<td>Csf3</td>
<td>0.101</td>
<td>0.205</td>
<td>Mmp2</td>
<td>0.182</td>
<td>0.085</td>
<td>Tgfb2</td>
<td>0.453</td>
<td>0.451</td>
</tr>
<tr>
<td>Ctgf</td>
<td>1.205</td>
<td>0.755</td>
<td>Msr1</td>
<td>0.706</td>
<td>0.642</td>
<td>Tgfb3</td>
<td>0.970</td>
<td>0.685</td>
</tr>
<tr>
<td>Edg1</td>
<td>0.285</td>
<td>0.253</td>
<td>Nos3</td>
<td>0.482</td>
<td>0.678</td>
<td>Tgfr1</td>
<td>0.292</td>
<td>0.433</td>
</tr>
<tr>
<td>Efnα5</td>
<td>0.250</td>
<td>0.286</td>
<td>Pdgfra</td>
<td>0.232</td>
<td>0.376</td>
<td>Tgfr3</td>
<td>0.033</td>
<td>0.282</td>
</tr>
<tr>
<td>Efnβ2</td>
<td>0.905</td>
<td>0.568</td>
<td>Pecam1</td>
<td>0.128</td>
<td>0.270</td>
<td>Thbs1</td>
<td>0.948</td>
<td>0.372</td>
</tr>
<tr>
<td>Eng</td>
<td>0.102</td>
<td>0.097</td>
<td>Plau</td>
<td>0.158</td>
<td>0.210</td>
<td>Thbs2</td>
<td>0.635</td>
<td>0.549</td>
</tr>
<tr>
<td>Ephb4</td>
<td>0.706</td>
<td>0.572</td>
<td>Ptgα1</td>
<td>0.256</td>
<td>0.459</td>
<td>Thbs3</td>
<td>0.180</td>
<td>0.181</td>
</tr>
<tr>
<td>Fgf1</td>
<td>0.112</td>
<td>0.157</td>
<td>Ptgα2</td>
<td>0.596</td>
<td>0.643</td>
<td>Thb4</td>
<td>0.150</td>
<td>0.127</td>
</tr>
<tr>
<td>Fgf16</td>
<td>0.883</td>
<td>0.783</td>
<td>Ptn</td>
<td>0.286</td>
<td>0.253</td>
<td>Timp2</td>
<td>0.962</td>
<td>0.568</td>
</tr>
<tr>
<td>Fgf7</td>
<td>1.009</td>
<td>0.510</td>
<td>Rsn</td>
<td>0.837</td>
<td>0.741</td>
<td>Vcam1</td>
<td>0.153</td>
<td>0.399</td>
</tr>
<tr>
<td>Fgf11</td>
<td>1.078</td>
<td>0.536</td>
<td>Serpinb5</td>
<td>1.002</td>
<td>0.885</td>
<td>Vegfb</td>
<td>0.087</td>
<td>0.172</td>
</tr>
<tr>
<td>Fk5</td>
<td>1.152</td>
<td>0.850</td>
<td>Serp1</td>
<td>0.177</td>
<td>0.338</td>
<td>Actb</td>
<td>1.264</td>
<td>0.945</td>
</tr>
</tbody>
</table>

NOTE: Values represent relative gene expression normalized to GAPDH. The genes included in this array that showed a spot intensity below 10% of GAPDH in both WT and KO were Adamts18, Angpt1, Angpt2, Ctgf, Efnα2, Efg, Efgc, Ehrb2, Ets1, F2, Fg2, Fg4, Fg6, Fgf3, Fgfr4, Fgfr, Kdr, Cxcl1, Idbx1, Idbx3, Ifnb1, Ifg1, Il10, Il12a, Iga5, Itgα3, Mdk, Mmp9, Nrp1, Pdgfa, Pdgfb, Pdgfrb, Cxcl4, Pgf, Plau, Ccl2, Smad1, Spp1, Tgfr2, Tie1, Timp1, Tnc, Tnf, Vegfa, and Vegfc.

Abbreviation: KO, pol η−/− mice in the hyperplastic/dysplastic stage.
pressure. Moreover, these findings suggest that optimal scheduling of antiangiogenic agents to normalize the tumor vasculature and enhance the efficacy of chemoradiation therapy, as shown in previous studies (20–22), may also be useful to treat early stages of cancer. Furthermore, elevated interstitial fluid pressure could be used to detect early stages of cancer when the disease is more amenable to treatment (19). Thus, these novel insights may be useful in early cancer detection and treatment.

Acknowledgments

Received 7/27/2005; revised 1/13/2006; accepted 2/2/2006.

Grant support: NIH grants CA-84301 and EH-11040 (R. Kucherlapati), CA-80124 and CA-85140 (R.K. Jain), and CA-96915 (D. Fukumura).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Sylvie Roberge, Carolyn Smith, and Melanie Fortier for excellent technical assistance; Drs. Yves Boucher and Satoshi Kashiwagi for helpful suggestions; and Tara Belezos for assistance.

References

Onset of Abnormal Blood and Lymphatic Vessel Function and Interstitial Hypertension in Early Stages of Carcinogenesis

Jeroen Hagendoorn, Ricky Tong, Dai Fukumura, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/7/3360

Cited articles
This article cites 22 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/7/3360.full#ref-list-1

Citing articles
This article has been cited by 19 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/66/7/3360.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.