Experimental and Molecular Pathology 85 (2008) 174–188

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Microenvironment of the murine mammary carcinoma 4T1: Endogenous IFN-γ affects tumor phenotype, growth, and metastasis

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ARTICLE INFO

Article history:
Received 12 May 2008
Available online 28 May 2008

Keywords:
Tumor
Microenvironment
Mammary
Carcinoma
4T1
IFN-γ
Metastasis
Murine

ABSTRACT

IFN-γ has a profound influence on growth and metastasis of solid tumors. This is true for the murine mammary carcinoma 4T1 which grows faster and metastasizes much more readily when transplanted into the mammary fatpads of IFN-$\gamma^{-/-}$ mice. We were interested in determining which infiltrating hematopoietic cells produce IFN-γ within the 4T1 tumor microenvironment. 4T1 tumors were infiltrated with progressively increasing numbers of F4/80$^+$/CD11c$^+$ myeloid cells, many of which were also Gr-1$^+$, and Gr-1$^+$/CD11b$^+$ granulocytes. Only small numbers of CD4 T cells, CD8 T cells, NK cells, and γδ T cells, the most likely IFN-γ-producing cells, were seen at any time point. Sensitive intracellular cytokine staining and flow cytometry revealed no tumor-infiltrating hematopoietic cells with detectable levels of intracellular IFN-γ, although IFN-γ mRNA transcripts were detected in tumor tissue. However, a progressive increase in the expression of three IFN-γ-inducible surface membrane proteins (B7-H1, I-A^d, and ICAM-1) on growing 4T1 tumor cells indicated the presence of biologically active IFN-γ in the tumor microenvironment. Moreover, 4T1 tumor cells from *in vitro* culture expressed these surface molecules 48 h after intratumoral injection into mature tumors. These data suggest that very low amounts of endogenous IFN-γ elaborated by infiltrating hematopoietic cells within the microenvironment of a solid tumor can achieve biologically active concentrations and affect tumor phenotype, growth, and metastasis.

Introduction

Solid tumors are composed of tumor cells and supporting stroma, endothelial cells, and infiltrating hematopoietic cells all embedded in a complex extracellular matrix (van Kempen et al., 2003). A variety of biologically active molecules are elaborated into the tumor microenvironment by tumor cells themselves or tumor-infiltrating hematopoietic cells (Freitas et al., 1997), including factors that affect angiogenesis, tumor proliferation, immune suppression, and metastasis (Leek et al., 1994; Mocellin et al., 2001; Ben-Baruch, 2003). Interferon γ (IFN-γ) is a pleiotropic cytokine secreted by a variety of tumor-infiltrating hematopoietic cells (Boehm et al., 1997; Farrar and Schreiber, 1993), and there is considerable experimental evidence suggesting that endogenous IFN-γ plays a role in inhibiting tumor development, growth, and metastasis (Barth et al., 1991; Dighe et al., 1994; Kaplan et al., 1998; Coughlin et al., 1998; Shankaran et al., 2001; Street et al., 2001; Pulaski et al., 2002; Ostrand-Rosenberg et al., 2002).

The mouse mammary carcinoma 4T1 was originally isolated from a spontaneously arising mammary tumor in BALB/cfC3H mice (Dexter et al., 1978; Miller and Heppner, 1979). The 6-thioguanine-resistant 4T1 tumor metastasizes via the hematogenous route to liver, lungs, bone, and brain, making it a good model of human metastatic breast cancer (Heppner et al., 2000). 4T1 grows progressively and causes a uniformly lethal disease even after excision of the primary tumor (Morecki et al., 1998; Pulaski et al., 2000). Recently, it has been shown that 4T1 grows more rapidly and metastasizes more readily when transplanted into the mammary fatpads of IFN-γ knockout (IFN-$\gamma^{-/-}$) mice (Pulaski et al., 2002; Shi et al., 2004), suggesting an important role for IFN-γ in immunity to this tumor. We were interested in characterizing the 4T1 tumor-infiltrating hematopoietic cells, particularly with respect to the cellular source or sources of IFN-γ in the tumor microenvironment.

Materials and methods

Mice

Eight week-old female BALB/c mice were obtained from Charles River/NIH (Waltham, MA). Eight week-old female C.129S7(B6)-*Ifng*^{tm1Ts}/J (IFN-$\gamma^{-/-}$) mice and their wild-type BALB/cJ (IFN-$\gamma^{+/+}$) controls were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in a ventilated barrier rack (Lab Products, Inc., Seaford, DE) in a temperature-controlled room with a 12 h photoperiod. Mice were given food and water *ad libitum*. This research was done under a protocol approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

4T1 tumor cell culture

The 6-thioguanine-resistant 4T1 mouse mammary carcinoma was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640

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0014-4800/$ – see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.yexmp.2008.05.002

Table 1
Antibody reagents used for flow cytometry experiments

Reagent	Supplier	Cat. no/lot no.	Isotype	Clone
Anti-mouse CD16/32 (Fc Block)	eBioscience	14-0161-85	R-IgG2b k	93
FITC-anti-mouse CD45	eBioscience	11-0451/E000250	R-IgG2b k	30-F11
FITC-anti-mouse CD11c	eBioscience	11-0114-82	H-IgG	N418
FITC-anti-mouse F4/80	eBioscience	11-4801	R IgG2a k	BM8
FITC-anti-mouse gamma delta TCR	eBioscience	11-5811-81/ E000867	H-IgG	UC7-13D5
FITC-anti-mouse Gr-1 (Ly-6G)	eBioscience	11-5931/E003725	R IgG2b	RB6-8C5
FITC-R-IgG1 k	eBioscience	11-4301/E003775	R-IgG1 k	eBRG1
FITC-anti-mouse IFN-γ	eBioscience	11-4311/E3119	R-IgG1 k	XMG1.2
FITC-Strepavidin	eBioscience	11-4311-87/ E005326	n/a	n/a
PE-Rat-IgG2a k	eBioscience	12-4321/E00513	R IgG2a k	eBR2a
PE-Rat IgG2b K	eBioscience	12-4331	R IgG2b k	KLH/G2b-1-2
PE-anti-mouse CD11b	eBioscience	12-0112/E000173	R IgG2b k	M1/70
PE-anti-mouse B7-H1 (PD-L1)	eBioscience	12-5982/E006192	R IgG2a L	MIH5
PE-anti-mouse MHC Class II (d, b not s) ($I\text{-}A^d$)	eBioscience	12-5321-82/ E008130	R IgG2b k	M5/114.15.2
PE-anti-mouse CD54 (ICAM-1)	eBioscience	12-0541/E000042	R IgG2b k	YN1/1.7.4
PE-anti-mouse CD3e	eBioscience	12-0031/E003659	H-IgG	145-2C11
PE-anti-mouse CD4 (L3T4)	PharMingen	553730/M075833	R IgG2b k	GK1.5
PE-anti-mouse CD45	eBioscience	12-0451-82/ E011938	R IgG2a k	30-F11
PE-anti-mouse CD49b (DX5)	eBioscience	12-5971-81/ E010636	R IgM k	DX5
PE-anti-mouse CD11b	PharMingen	557397/M041985	R IgG2b k	M1/70
PE-anti-mouse F4/80	eBioscience	12-4801-80/ E010365	R IgG2a k	BM8
PE-Strepavidin	eBioscience	12-4312/E004394	n/a	n/a
PE-Cy5-anti-mouse CD45	eBioscience	15-0451/E004642	R IgG2b k	30-F11
PE-Cy5-anti-mouse F4/80	Serotec	MCA497C/010604	R IgG2b	CI;A3-1 (F4/80)
PE-Cy5-anti-mouse CD3e	eBioscience	15-0031/E004767	H-IgG	145-2C11
PE-Cy5-anti-mouse Gr-1 (Ly-6G)	eBioscience	15-5931/E004050	R IgG2b	RB6-8C5
PE-Cy7-anti-mouse CD8a (Ly-2)	PharMingen	552877/ 0000061910	R IgG2a k	53-6.7
PE-Cy7 Strepavidin	eBioscience	25-4325/E12003	n/a	n/a
Biotin-anti-mouse CD119 (IFN-γRα)	PharMingen	558771/ 0000064387	R IgG2a k	GR20
Biotin-anti-mouse F4/80	Serotec	MCA497C/010604	R IgG2b	CI;A3-1 (F4/80)

medium supplemented with 10% fetal bovine serum (both from Hyclone, Logan, UT), plus 1.0 mM sodium pyruvate, and 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Cambrex, Walkersville, MD). The strongly adherent tumor cells were routinely cultured in standard Falcon plastic tissue culture plates (Fisher Scientific, Fair Lawn, NJ), and cells were removed from the substrate by treatment with 0.25% trypsin/1 mM EDTA (Sigma Chemicals, St. Louis, MO). To avoid loss of membrane proteins caused by trypsin treatment, tumor cells for flow cytometric studies were grown on Falcon non-tissue culture plastic plates (Fisher Scientific, Fair Lawn, NJ) and harvested by treatment with ice-cold 0.5% ethylenediaminetetraacetic acid (EDTA) in Hank's balanced salt solution (HBSS) followed by repetitive pipetting. Viability of cells was determined by 0.25% trypan blue dye exclusion.

4T1 tumors in vivo

Early passage 4T1 tumor cells were harvested from culture by trypsin treatment, then 1.0×10^5 viable cells in 50 μl of HBSS were injected into the mid-abdominal mammary fatpads of recipient mice. Tumor growth was assessed morphometrically using electronic calipers, and tumor volumes were calculated according to the formula $V(\text{mm}^3)=L$ (major axis)$\times W^2$ (minor axis)/2 (Carlsson et al., 1983). All experiments were terminated when tumors reached an average diameter of 16 mm.

Fluorophore-labelling of tumor cells

4T1 tumor cells harvested from culture using trypsin were incubated with 3 μM carboxyfluorescein diacetate succiniimidyl ester (CFSE, Sigma Chemical Co., St. Louis, MO) for 10 min at 37 °C. The CFSE-labeled cells were washed three times by centrifugation (250 ×g for 6 min), then some were replated in tissue culture and some tested for viability and used for intratumoral injections or injections into mammary fatpads.

Enzymatic digestion of primary 4T1 tumors

Primary tumors were carefully excised and surface blood was removed by rinsing in HBSS. The tumors were minced with scissors in 2.5 ml HBSS, then the cells and fragments were added to 2.5 ml filter-sterilized collagenase type I (Sigma Chemicals, St. Louis, MO) at 10 mg/ml and incubated for 1 h at 37 °C on a platform rocker. Cells were washed twice by centrifugation (250×g for 10 min at 4 °C), tested for viability, then resuspended in HBSS with 0.1% sodium azide (Sigma) and 1% bovine serum albumin (Fisher Scientific, Fair Lawn, NJ) (staining buffer).

Liver and lung metastasis assays

Lungs and livers were carefully excised and surface blood was removed by rinsing in HBSS. The organs were minced with scissors in 2.5 ml HBSS, then cells and fragments were

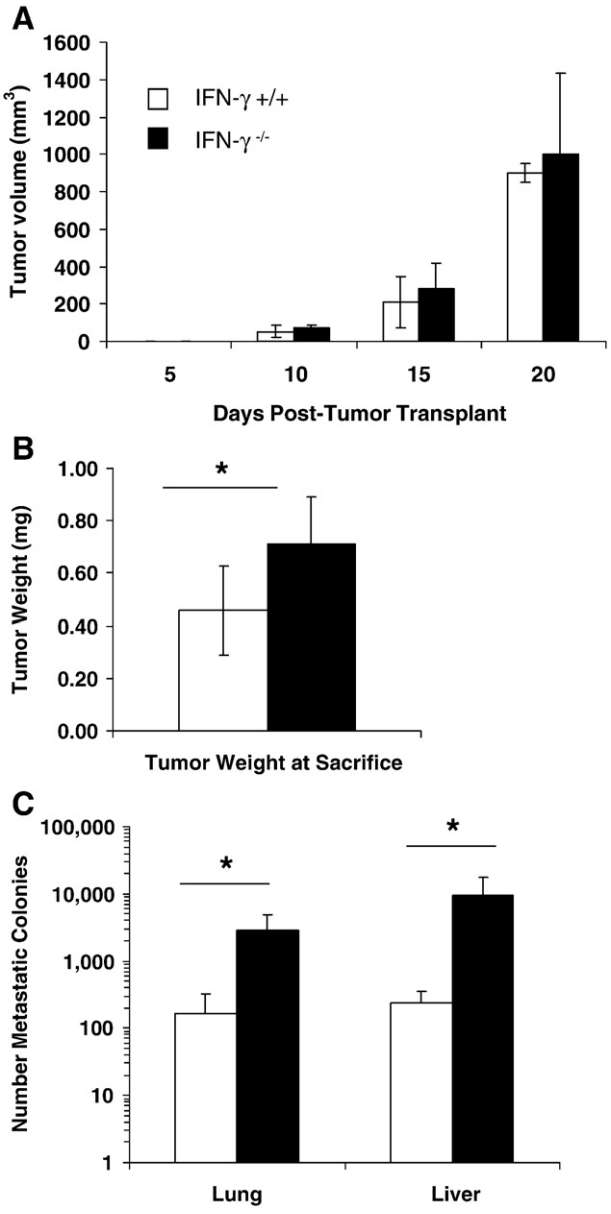

Fig. 1. Differences in primary tumor growth and metastasis of 4T1 tumors transplanted into IFN-$\gamma^{+/+}$ and IFN-$\gamma^{-/-}$ mice. 1×10^4 4T1 tumor cells were injected into the abdominal mammary fatpads of five wild-type and five knockout mice. (A) On days 5, 10, 15, and 20 after transplantation, tumor volumes were determined. (B) At 20 days post-tumor transplant, tumor weights were measured and (C) metastatic colonies in lungs and liver were determined by clonogenic assay. Data represent the means±one standard deviation.