Responsiveness of Stromal Fibroblasts to IFN-\(\gamma\) Blocks Tumor Growth via Angiostasis

Yu Lu, Wei Yang, Chuan Qin, Lianfeng Zhang, Jingjing Deng, Shubai Liu and Zhihai Qin

*J. Immunol.* 2009;183;6413-6421; originally published online Oct 19, 2009; doi:10.4049/jimmunol.0901073

http://www.jimmunol.org/cgi/content/full/183/10/6413

**Supplementary Data**

http://www.jimmunol.org/cgi/content/full/jimmunol.0901073/D1

**References**

This article cites 49 articles, 20 of which can be accessed free at:

http://www.jimmunol.org/cgi/content/full/183/10/6413#BIBL

**Subscriptions**

Information about subscribing to *The Journal of Immunology* is online at http://www.jimmunol.org/subscriptions/

**Permissions**

Submit copyright permission requests at http://www.aai.org/ji/copyright.html

**Email Alerts**

Receive free email alerts when new articles cite this article. Sign up at http://www.jimmunol.org/subscriptions/etoc.shtml
The importance of stromal cells for tumor is akin to soil for seed. However, the interaction among these cells is far from understood. In this study, we show that stromal fibroblasts exist not only during tumor progression but also during regression stage, together with immune effector cells. Coinjection of stromal fibroblasts with tumor cells often promotes tumor growth. However, the presence of IFN-γ significantly impairs the ability of these cells to promote tumor growth due to a reduced angiogenesis. The mechanism relies mainly on the IFN-γ-mediated down-regulation of vascular endothelial growth factor production by fibroblasts. The results reveal a novel link between immune cells and nonbone marrow-derived stromal cells, and define stromal fibroblasts as the main targets of IFN-γ in tumor immunity. The Journal of Immunology, 2009, 183: 6413–6421.

The stroma of solid tumors is a complex microenvironment for malignant cells and supplies growth factors and blood (1–4). It is composed of a variety of normal cell types, including bone marrow-derived immune cells such as macrophages, granulocytes, and T cells, as well as nonhematopoietic cells (4). Fibroblasts are the main cellular component of the tumor stroma and are one of the groups of nonhematopoietic cells whose importance in the progression, growth, and spread of cancer cells has been recognized for a long time (4–6). During tumor-induced angiogenesis, stromal fibroblasts contribute actively to neovascularization through various factors (6). Fibroblasts have been shown to be the principal source of host-derived vascular endothelial growth factor (VEGF) in both spontaneous and implanted tumor models (7). Activated fibroblasts produce extracellular matrix (ECM) that is rich in fibronectin and type I collagen, both of which are conducive to initiating tumor angiogenesis (8). Additionally, carcinoma-associated fibroblast (CAF)-derived stromal cell-derived factor 1 (SDF-1) mediates the recruitment of endothelial cells (9).

Little is known about the interaction between stromal components during tumor growth or rejection. Aside from direct cell-cell contact, cytokines may play an essential role as mediators of tumor stromal cell cross-talk. A series of studies, including ours, demonstrate a critical requirement for IFN-γ in tumor growth inhibition (10–13). In addition, high levels of exogenous TNF-α or IL-4 also mediate the suppression of tumor growth through the IFN-γ pathway (14, 15), highlighting the key role of IFN-γ in tumor immunity (10–16). Consistent with the pleiotropic activities of this cytokine, the IFN-γ receptor (IFN-γR) is expressed on almost all cell types (17–19). Although direct killing of tumor cells by IFN-γ is proposed (20), there is also evidence showing that IFN-γ contributes to tumor rejection through its effect on nonhematopoietic stromal cells (11). Both endothelial cells and fibroblasts exhibit high IFN-γR expression levels (18). In unstimulated fibroblasts, the IFN-γR is expressed on their cell surface (21). In tumor-associated fibroblasts, IFN-γ is found to up-regulate the correlative molecules B7-H1 (PD-L1) and B7-DC (PD-L2), indicating that activated fibroblasts express IFN-γR (22). It has been suggested that endothelial cells may participate in the IFN-γ-mediated disruption of the tumor vasculature (23). However, little is known about how IFN-γ acts on stromal fibroblasts during antitumor immune responses.

In the present study, we observed that stromal fibroblasts exist locally at the tumor site during both tumor progression and regression phase. Coinjection of stromal fibroblasts with tumor cells promotes tumor growth. However, the IFN-γ responsiveness of these cells is sufficient to impair tumor growth, indicating that IFN-γ plays a key role in altering stromal fibroblast function. Our results clearly define stromal fibroblasts as the main cellular targets of IFN-γ in anti-tumor immunity. Moreover, we elucidated the effect of IFN-γ on stromal fibroblasts, which leads to down-regulated VEGF production and angiostasis in the tumor.

Materials and Methods

Mice

Wild-type C57BL/6 and BALB/c mice were purchased from Experimental Animal Center of Weitonglihua (Beijing, China). IFN-γR−/− mice on C57BL/6 background were originally obtained from The Jackson Laboratory. C57BL/6-EGFP mice were originally from Osaka...
University (Osaka, Japan). All mice were maintained in a specific pathogen-free barrier facility in Institute of Biophysics, Chinese Academy of Sciences.

Cells and cell culture conditions

The IFN-γR- tumor cell line FH32 was derived from IFN-γR- C57BL/6 mice treated with 3-methylcholanthrene (MCA). Tumors were induced following i.m. injection of 0.8 mg of MCA into IFN-γR- mice and, 21–23 wk later, tumors with a diameter of 1.1–1.2 cm were surgically removed. The in vivo tumors were isolated, minced, and incubated in trypsin-EDTA solution (Life Technologies) containing 1 mg/ml collagenase (Life Technologies) overnight at 0°C and then 5 min at 37°C in the presence of 0.1 mg/ml DNase I (Boehringer Mannheim). Primary tumor cells were implanted twice in BALB/c nu/nu mice to minimize the contamination of stromal fibroblasts from primary tumor-bearing mice. The tumor cells were then grafted twice onto IFN-γR- and twice onto IFN-γR+ mice. Single cell suspensions of the passed tumors were prepared and subsequently tumor cells were cloned by limiting dilution. IFN-γ-secreting and IFN-γR-reconstituted tumor cell variants were obtained by transfection and named FH32-IFN-γ and FH32-IFN-γR, respectively. As a control, cells were also transfected with the empty vector pBabe, resulting in the mock-transfected FH32-m cell line. Additionally, the BALB/c-derived plasmacytoma cell line J558L (24), the BALB/c-derived mammary adenocarcinoma cell line TSA (25) and the C57BL/6-derived fibrosarcoma cell line MCA205 (26) were used. All cell lines were cultured in RPMI 1640 medium supplemented with 10% newborn calf serum (HyClone Laboratories).

Mouse embryonic fibroblast (MEF) isolation

Pregnant females were sacrificed at day E14, and each embryo was separated from the placenta. After discarding the brain and other dark red organs, the embryos were minced, suspended in trypsin-EDTA, and incubated with gentle shaking at 37°C for 15 min with 0.1 mg/ml DNase I. Suspended cells were plated in RPMI 1640 medium in the presence of 10% FBS (HyClone Laboratories). The medium was changed the following day, leaving behind the adherent MEFs. In this study, MEF cells with or without IFN-γR expression were isolated from IFN-γR+ or IFN-γR- mice, respectively.

Flow cytometry

To detect the proportions of MEFs in tumor mass, MCA205 tumors grown with 5-fold GFP-positive (GFP+) MEFs were harvested to form single cell suspension at indicated time points. The percentage of GFP+ cells in the prepared cell suspension was analyzed by FACS. Cell surface IFN-γR expression was determined using a rat anti-mouse IFN-γR mAb (GR20; BD Pharmingen). PE-conjugated goat anti-rat IgG (BD Pharmingen) was used as a secondary Ab. To confirm IFN-γ and IFN-γR function, the superantigen from FH32-IFN-γ tumor cells was added to the MCA205 culture and FH32-IFN-γR tumor cells were cultured with or without 50 ng/ml murine IFN-γ (BD Pharmingen) for 24 h. These cells were then stained for MHC class I expression with PE-conjugated mAb anti-H-2Kb (BD Pharmingen) or an isotype-matched control mAb. MEFs subjected to irradiation or treated with 10, 50, 100 ng/ml IFN-γ were cultured for 48 or 24 h in vitro and their proliferation was determined by BrdU incorporation. These cells were harvested, fixed and stained with FITC-conjugated mAb against BrdU (eBioscience) for FACS assay. All samples were analyzed with a BD FACSCalibur System (BD Biosciences).

Tumor growth assay and mouse survival

To detect the presence of stromal fibroblasts in the tumor, 5 × 10^6 MCA205 cells were injected into naive or immunized C57BL/6 mice or C57BL/6-EGFP mice as indicated. When MEFs were used as stromal fibroblasts, MCA205 or J558L tumor cells were mixed with nonirradiated or irradiated MEFs at the indicated numbers in 0.2 ml of PBS, and then s.c. injected into naive mice. In some cases, 1 × 10^5 MEFs were irradiated with 100 Gy of Co and then s.c. inoculated with 2 × 10^5 MCA205 tumor cells. To investigate the effect of IFN-γ on stromal fibroblasts, a mixture of 2 × 10^5 MCA205 tumor cells and 1 × 10^5 FH32-IFN-γR- MEFs s.c. injected into IFN-γR- mice in the left abdominal region, whereas the same amount of FH32-m/FH32-IFN-γR- cells mixed with IFN-γR+ MEFs was injected in the abdominal right region. Tumor growth was monitored every 2–3 days, and mice bearing a tumor of greater than 5 mm in diameter were scored as tumor positive. For immunization of mice, 2 × 10^5 FH32-m tumor cells were irradiated with 100 Gy of Co and inoculated s.c. into mice. Two weeks later, the mice were challenged by s.c. injection of living tumor cells with MEFs as indicated. Neutralization of local IFN-γ was accomplished in immunized IFN-γR+ mice by i.p. injection of 500 μg/mouse rat anti-mouse IFN-γ mAb, resulting in a decrease of tumor growth by 50%. IFN-γ secreting and IFN-γR-reconstituted tumor cell variants were obtained by transfection and named FH32-IFN-γ and FH32-IFN-γR, respectively. As a control, cells were also transfected with the empty vector pBabe, resulting in the mock-transfected FH32-m cell line. Additionally, the BALB/c-derived plasmacytoma cell line J558L (24), the BALB/c-derived mammary adenocarcinoma cell line TSA (25) and the C57BL/6-derived fibrosarcoma cell line MCA205 (26) were used. All cell lines were cultured in RPMI 1640 medium supplemented with 10% newborn calf serum (HyClone Laboratories).

FIGURE 1. Stromal fibroblasts exist in both progressing and regressing tumors. (A) and (B) C57BL/6 mice were s.c. challenged with 5 × 10^5 MCA205 cells. Four days later, cryostat tumor sections were stained with anti-α-SMA mAb to detect stromal fibroblasts. Necrotic (N) regions are indicated. C, Naive C57BL/6-EGFP mice were s.c. challenged with 5 × 10^5 MCA205 cells. Four days later, tumor sections were stained with anti-α-SMA mAb to detect stromal fibroblasts (bottom left) and DAPI for nucleioli (top left). The merged image (bottom right) shows that α-SMA-positive cells colocalized with GFP+ cells. Scale bar represents 50 μm.

Immunostaining and immunoblotting

Frozen sections from isolated tumors were prepared as previously described (11). Immunofluorescence of tissue sections was evaluated on an Olympus FV1000 confocal microscope. The mAbs used for staining were anti-ER-TR7 (Acris Abs), anti-α-smooth muscle actin (α-SMA; Abcam), anti-VEGF-A (Abcam), anti-CD31 (BD Pharmingen), anti-9F1 (BD Pharmingen), isotype-matched control mAbs (BD Pharmingen), and anti-fibroblast specific protein 1 (FSP1) pAb, a research gift from E. G. Neilson (Vanderbilt University, Nashville, TN). Microvessel density was measured according to Weidner’s methods (27). Briefly, three tumors per group were analyzed by immunostaining with anti-CD31 mAb. Two representative sections per tumor were independently evaluated by two authors. The two most vascularized areas within the tumor ("hot spots") were chosen at a low magnification of ×40. The vessels were then counted in a high magnification field of ×200 (or 0.74 mm²). The mean vascular vessel density for CD31 was evaluated as the average of eight counts (two sections, two fields, and two authors).

To detect IFN-γR expression, whole cell lysates were separated by SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and incubated with rabbit anti-mouse IFN-γR mAb GR20 (1/1000; BD Pharmingen), and rabbit anti-mouse β-actin mAb (1/5000; Santa Cruz Biotechnology). Anti-rat or anti-rabbit mAbs conjugated to HRP were used as
secondary Abs, and positive bands were visualized using a chemiluminescent substrate (SuperSignal; Pierce Chemical).

**Cytokine detection**

To detect IFN-γ secretion, the supernatant from FH32 or FH32-IFN-γ cells was collected after a 48 h culture. The concentration of IFN-γ in the cell supernatant was determined using an ELISA kit (BD Pharmingen) according to the manufacturer’s instructions. To detect matrix metalloproteinase (MMP) activities, IFN-γ+ MEFs were plated in 6-well plates and cultured for 24 h in the absence or presence of IFN-γ. The cells were then washed twice with serum-free medium, and 1.5 ml of fresh serum-free medium was added to each well. After 24 h, the conditioned medium was collected, centrifuged to remove any suspended cells, and identified by gelatin zymography to detect the activity of MMPs.

**RT-PCR and real-time quantitative RT-PCR**

MEF RNA was extracted using the TRIreagent kit per the manufacturer’s instructions (Molecular Research Center). Gene expression was detected by RT-PCR and real-time quantitative RT-PCR. To detect IFN-γ, MMP activities, IFN-γ- and IFN-γ+ MEFs, respectively, were plated in 6-well plates and cultured for 24 h in the absence or presence of IFN-γ. RNA was then extracted from these MEFs and cDNA was synthesized. The relative copy number of mRNA encoding β-actin was analyzed in the same sample using primer pairs 5'-TTC TCT GCC CCC CTT GTP TA-3' and 5'-CTC CAC CAT GCC AAG-3' and (antisense) 5'-GAC AGA AAG AAA CCA-3', IFN-γR- and IFN-γR+ MEFs, respectively, were plated in 6-well plates and cultured for 24 h in the absence or presence of IFN-γ. RNA was then extracted from these MEFs and cDNA was synthesized. The relative copy number of mRNA encoding β-actin was analyzed in the same sample using primer pairs 5'-TTC TCT GCC CCC CTT GTP TA-3' and 5'-CTC CAC CAT GCC AAG-3' and (antisense) 5'-GAC AGA AAG AAA CCA-3', IFN-γR- and IFN-γR+ MEFs, respectively, were plated in 6-well plates and cultured for 24 h in the absence or presence of IFN-γ. RNA was then extracted from these MEFs and cDNA was synthesized. The relative copy number of mRNA encoding β-actin was analyzed in the same sample using primer pairs 5'-TTC TCT GCC CCC CTT GTP TA-3' and 5'-CTC CAC CAT GCC AAG-3' and (antisense) 5'-GAC AGA AAG AAA CCA-3', IFN-γR- and IFN-γR+ MEFs, respectively, were plated in 6-well plates and cultured for 24 h in the absence or presence of IFN-γ.

**Analysis of hemoglobin content in tumors**

Hemoglobin content in isolated tumors was estimated according to Drabkin’s cyanmethemoglobin methods (28). Drabkin’s solution (Sigma-Aldrich) containing potassium ferricyanide, potassium cyanide, and sodium bicarbonate was prepared. Tumors were isolated, minced, and homogenized. The hemoglobin present in the tumors was then extracted using 0.1 M ammonia. One milliliter of the sample was added to 5 ml of Drabkin’s solution. Readings were obtained at 540 nm using a spectrophotometer. Hemoglobin values were calculated from a hemoglobin standard curve prepared using serial dilutions of mouse blood. The concentration of hemoglobin in different tumors was expressed as hemoglobin content per microgram of tumor tissue.

**Statistical analyses**

Statistical analysis was performed using a two-tailed Student’s t test. The data were considered as significantly different at a value of \( p < 0.05 \).

**Results**

**Stromal fibroblasts exist in both progressing and regressing tumors**

To investigate the role of stromal fibroblasts during tumor growth and rejection, we first examined a series of immunological staining to check the distribution and other characteristics of these cells in the tumor. Naive mice were inoculated with MCA205 fibrosarcoma cells to harvest tumors in the growing phase. Alternatively, mice were immunized with irradiated MCA205 cells, challenged 14 days later with the same fibrosarcoma cells, and the tumors
were harvested during the regressing phase. Tissue sections prepared 4 days after tumor inoculation or challenge were stained for α-SMA, a key evaluation marker for tumor stromal fibroblasts according to previous studies (6). As shown in Fig. 1A in growing tumors, well-organized α-SMA-positive cells were found lined up around the tumor cell nests and few fibroblasts were found in the central area of the tumor. However, the structure of the α-SMA-positive cells was totally disrupted in the regressing tumors and dispersed clusters of fibroblasts were often detected near the small necrotic regions, suggesting the probable involvement of fibroblasts in tumor rejection (Fig. 1B).

To further confirm that the α-SMA-positive cells were derived from the host but not the tumor cells, we inoculated MCA205 cells into C57BL/6-EGFP transgenic mice. As shown in Fig. 1C, all α-SMA-positive fibroblasts displayed GFP fluorescence, indicating the host origin of these cells in tumors. Further analysis suggested that most of these cells were myofibroblasts because of their large size (diameter and length > 100–200 micron), peripheral localization of nuclei, and multiple nuclei (29). Similar results were obtained when other types of tumors, such as colon carcinoma or plasmacytoma, were analyzed (data not shown).

The α-SMA is also expressed on pericytes and vascular smooth muscle cells (6, 30). To further assess the existence of tumor stromal fibroblasts, another marker, S100A4/FSP1, was applied. The plasmacytoma J558L cells were injected into the naive and immunized syngeneic BALB/c mice. Although the expression pattern of FSP1 was not identical with that of α-SMA (30), FSP1-positive stromal fibroblasts were also detected in both progressing and regressing tumors (see supplemental Fig. S1).3 Additionally, a similar result was obtained with BALB/c-derived mammary adenocarcinoma TSA tumors (data not shown). However, we cannot extrapolate this finding to all other types of tumors. Together, the data show that host-derived fibroblasts compose an important part of the tumor stroma and that the interactions of these cells with other stromal components could be crucial for both tumor growth and tumor rejection.

**MEFs act as stromal fibroblasts and promote tumor growth**

To further evaluate the function of stromal fibroblasts, MEFs were used for co inoculation with tumor cells into experimental mice. Although MEFs are less relevant to cancer than CAFs or tumor-associated fibroblasts, they are used as tumor stromal fibroblasts in previous studies due to their easy isolation and convenient gene-modification (31, 32). C57BL/6-derived MCA205 cells coinjected with syngeneic MEFs achieved a tumor volume of 286.8 ± 58.4 mm³ by day 13, whereas tumor cells alone displayed a tumor volume of only 14.6 ± 7.1 mm³ (Fig. 2A). Moreover, BALB/c-derived plasmacytoma J558L cells coinjected with MEFs generated tumors with a volume of 1811.3 ± 178.5 mm³ by day 16, whereas mice that did not receive MEFs exhibited a tumor volume of only 974 ± 198.9 mm³ (Fig. 2B). Consistent with previous observations showing that stromal cells facilitate cancer progression (33, 34), our results indicate that stromal fibroblasts could promote the tumor growth of different tumor types in different mouse strains.

Stromal fibroblasts may support the survival and proliferation of tumor cells by producing growth factors and chemokines, as well as ECM (6). To determine whether fibroblasts can facilitate tumor progression in a paracrine fashion, MEFs were irradiated by 100 Gy 60Co and then coinjected with MCA205 tumor cells into mice. Irradiated MEFs exhibited nonproliferative properties (see supplemental Fig. S2). The tumors that developed in the presence of irradiated MEFs exhibited similar growth kinetics when compared with the tumors that developed in the presence of their nonirradiated counterparts (Fig. 2C). This observation excludes the possibility that proliferation of MEFs in vivo is responsible for the enhanced growth of MEF-containing tumors, suggesting that they may promote tumor growth via the release of soluble factors. Indeed, in tumors grown with 5-fold GFP+ MEFs, the percentage of GFP+ cells (mainly tumor cells) increased as tumor grew, resulting in continuously decreased proportions of GFP+ MEFs (see supplemental Fig. S3). At day 16, only 1.64 ± 0.3% cells that displayed high GFP intensity (data not shown) left and scattered in the tumor, further confirming that tumor cells, but not MEFs are the major contributor of the progressive tumor growth.

To follow the fate of coinoculated MEFs in vivo, we prepared MEFs from C57BL/6-EGFP transgenic mice and injected these cells s.c. into naive mice together with MCA205 tumor cells. Seventeen days after tumor cell inoculation, GFP+ cells were stained for the fibroblastic marker ER-TR7. As shown in Fig. 2D, most of these cells were ER-TR7-positive, although they were negative for the endothelial cell markers CD31 and 9F1 (see supplemental Fig. S4). Although most of GFP+ cells displayed an appearance of

---

3 The online version of this article contains supplemental material.
myofibroblasts, we cannot exclude that a small proportion of coinjected MEFs have differentiated into other types of cells. Simultaneously, we found that ~20–30% of the endogenous GFP cells having a similar morphology to myofibroblasts dispersed among the GFP+ cells (Fig. 2D). The data indicate that MEF-derived stromal fibroblasts are comparable to those that have developed endogenously.

Establishment of IFN-γR− and competent tumor cell lines

To analyze the interaction between the bone marrow–derived immune cells and tumor stromal fibroblasts, we selected IFN-γ as a model cytokine because it is a key regulator in tumor rejection and is produced by bone marrow–derived cells (1). IFN-γR is expressed by almost all nucleated cells, including tumor cells (18). To exclude the direct effect of IFN-γ on tumor cells, several IFN-γR− tumor cell lines were established from MCA-induced tumors grown in an IFN-γR knockout mouse in the C57BL/6 genetic background (H-2b). One of the cell lines, FH32, was selected for reconstitution of IFN-γR expression, resulting in FH32-γR+ cells. As a control, FH32 cells were also transfected with a mock plasmid, resulting in FH32-m cells. RT-PCR (Fig. 3A) and Western blot analysis (Fig. 3B) showed that FH32-m cells did not express either the IFN-γR mRNA or the protein. In contrast, IFN-γR expression was detected at both the mRNA and protein levels in FH32-γR+ cells. Subsequently, we examined IFN-γR on the cell surface by flow cytometry. Consistent with the previous observation, IFN-γR was detected only on FH32-γR+ cells, but not on FH32-m cells (Fig. 3C). Most importantly, IFN-γR on the surface of FH32-γR+ cells was functional because exposure of these cells to 50 ng/ml IFN-γ efficiently up-regulated their MHC class I expression (Fig. 3D). However, IFN-γ did not change the expression of MHC class I molecules on FH32-m cells, as determined by culturing these cells in the presence of 10, 50, or 200 ng/ml recombinant mouse IFN-γ for 48 h.

Responsiveness of stromal fibroblasts to IFN-γ impairs tumor growth

To address whether endogenous IFN-γ produced by hematopoietic stromal cells (19, 35) acts on stromal fibroblasts in tumor rejection, we coinoculated FH32-m tumor cells with either IFN-γR− or IFN-γR+ MEFs into IFN-γR knockout mice (IFN-γR− mice). IFN-γR− mice were used to exclude any effects of IFN-γ on host-derived stromal cells. IFN-γR− and IFN-γR+ MEFs were generated from the corresponding mice. Only IFN-γR+ MEFs, as tumor stromal fibroblasts, could be the target of IFN-γ in this coinjection model. Because IFN-γ-secreting immune cells infiltrated into local tumor sites 14 days after immunization, we first immunized 8 IFN-γR− mice with irradiated FH32-m cells. Two weeks later, these mice were s.c. challenged with a mixture of viable FH32-m tumor cells and IFN-γR+ or IFN-γR− MEFs at contralateral abdominal sides. Compared with the 100% tumor incidence of FH32-m inoculated with IFN-γR− stromal fibroblasts at day 10, none of the FH32-m tumors containing IFN-γR− fibroblasts had grown. The tumor volume of FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR+ stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3, whereas FH32-m with IFN-γR− stromal fibroblasts achieved a tumor volume of 381.9 ± 114.6 mm3. The tumor volume was expressed as the mean ± S.D. for 10 mice. *p < 0.05. **p < 0.01. (Fig. 4). Importantly, treatment with an anti-IFN-γ neutralizing Ab (R4-6A2) in both groups greatly diminished this difference (Fig. 4A). Furthermore, coinjection of the IFN-γ receptor competent FH32-γR tumor cells with the IFN-γR− or IFN-γR+ MEFs demonstrated similar results to those obtained with FH32-m tumor cells (data not shown).
Seventeen days later, the hemoglobin content in tumors was measured using the Drabkin’s procedure. Hemoglobin content of tumors containing IFN-γR−MEFs at contralateral abdominal sides of naive or IFN-γR−mice. The growth of tumors containing IFN-γR−stromal fibroblasts was significantly retarded compared with those containing IFN-γR−stromal fibroblasts (p < 0.05) (Fig. 4B). Thus, the action of exogenous IFN-γ on stromal fibroblasts also inhibits the tumor growth promoted by these cells.

Effect of IFN-γ on stromal fibroblasts induces angiostasis in the tumor

These findings encouraged us to further investigate how IFN-γ acts on stromal fibroblasts to impede their ability to promote tumor growth. Our previous data based on bone marrow chimeras showed that IFN-γ acts on nonhematopoietic tumor stromal cells and induces angiostasis (10, 11). In this study, we wondered whether IFN-γ impairs tumor angiogenesis via its direct effect on stromal fibroblasts in this model. The concentration of hemoglobin in the tumors was quantified to reflect the abundance of blood vessels. As shown in Fig. 5A, the FH32-m tumors containing IFN-γR−stromal fibroblasts had 34.48 ± 1.36 μg/mg and 36.5 ± 1.14 μg/mg hemoglobin, whereas the hemoglobin concentration of FH32-m tumors containing IFN-γR−stromal fibroblasts was only 22.86 ± 1.08 μg/mg and 9.39 ± 1.41 μg/mg, respectively. Again, the difference was due possibly to the effect of endogenous IFN-γ on IFN-γR−MEFs in tumors because it diminished greatly after the neutralization of IFN-γ activity in vivo (Fig. 5B).
Immunostaining using anti-CD31 Ab was also performed on sections of these tumors to detect the vasculature intensity. Consistent with our results, an elevated intensity of CD31 staining was observed in FH32-m tumors containing IFN-γR−/H9253 stromal fibroblasts (~24 ± 3.6 vessels per high power field) (Fig. 5C), but not in FH32-m tumors containing IFN-γR+ stromal fibroblasts (only 8 ± 1.5 vessels per high power field) (Fig. 5D). Therefore, the findings strongly suggest that IFN-γ inhibits tumor-induced angiogenesis by acting on stromal fibroblasts.

**IFN-γ down-regulates stromal fibroblast-derived VEGF**

We examined subsequently how IFN-γ acts on stromal fibroblasts to mediate its effect for angiostasis. It is well known that stromal fibroblasts are a source of ECM-degrading proteases such as the MMPs, which are crucial for initiating tumor angiogenesis (38–40). The activity of MMPs produced by MEFs was determined in the presence or absence of IFN-γ signaling by zymography. Both IFN-γR+ and IFN-γR− MEFs secreted comparable MMP2, but undetectable amounts of MMP9 in vitro (Fig. 6A). In the presence of different doses of mouse IFN-γ, no significant difference in MMP2 activity was detected (Fig. 6B).

Both SDF-1 and VEGF-A are mainly produced by stromal fibroblasts and have been shown to play a central role in tumor neoangiogenesis (7–9). The mRNA of these two genes could be detected in cultured MEFs by reverse transcriptase PCR (data not shown). To investigate whether IFN-γ alters the production of SDF-1 or VEGF-A in MEFs, IFN-γR+ MEFs and IFN-γR− MEFs were stimulated with IFN-γ for 24 h in vitro and the mRNA of both genes was quantified by real-time quantitative PCR. No significant differences in SDF-1 mRNA expression were detected between IFN-γR− MEFs and IFN-γR+ MEFs with or without IFN-γ stimulation (Fig. 6C). However, the mRNA of VEGF-A in IFN-γR− MEFs was clearly down-regulated 53% by following IFN-γ treatment. As a control, IFN-γ was unable to affect VEGF-A expression in IFN-γR+ MEFs (Fig. 6D). To further analyze the VEGF-A secretion by stromal fibroblasts in vivo, we injected GFP+ MEFs together with tumor cells into naive mice. Immunostaining showed that GFP+ stromal fibroblasts were also positive.
for VEGF-A in the tumor (Fig. 6E). Importantly, the number of VEGF-A-positive cells was drastically decreased in FH32 tumors with IFN-γR−/− stromal fibroblasts as compared with those with IFN-γR−/− stromal fibroblasts in the immunized IFN-γR−/− mice (Fig. 6F). In summary, the results indicate that IFN-γ inhibits tumor angiogenesis by down-regulation of the stromal fibroblast-derived VEGF.

Discussion

In the present study, we coinkjected tumor cells and MEFs to investigate the functional switch of stromal fibroblasts in different tumor microenvironments, especially in response to IFN-γ stimulation. Although MEFs have been analyzed in tumor models, previous studies have used immortalized gene-deficient MEFs as fibroblasts or tumor cells to assess the function of certain genes without knowing the fate of MEFs in vivo (32, 41). Our results strongly suggest that MEFs can be used as stromal fibroblasts to promote tumor growth and can express fibroblast markers when coinjected with tumor cells. Subsequently, by coinoculating IFN-γR-competent and IFN-γR-deficient MEFs with tumor cells into immunized IFN-γR−/− knockout mice, we were able to examine the antitumor mechanism of IFN-γ in the tumor-stroma interaction. The data confirm our previous observation that IFN-γ acts on non-hematopoietic stromal cells to retard tumor progression and further demonstrate that the action of IFN-γ on stromal fibroblasts is sufficient for this effect by inducing angiostasis.

Fibroblasts, the major cell type present in tumor stromal compartments, exert different functions in tumor development when analyzed in different models. Most studies have shown that stromal fibroblasts play important roles during tumor growth, angiogenesis, and metastasis (9, 35, 42–45). These cells favor tumor progression by producing increased amounts of growth factors for adjacent tumor cells and by actively contributing to tumor neo-vascularization (9, 46). In addition, stromal fibroblasts often secrete elevated levels of ECM-degrading proteases such as MMP2 and MMP3, allowing cancer cells to cross tissue boundaries and escape from the primary tumor sites during metastasis (39, 40). In contrast, other studies have shown that stromal fibroblasts have an inhibitory effect on tumor growth when interacting with some cytokines (47). The reason for these contrary results remains unknown. In the present study, we showed that stromal fibroblasts were able to promote tumor growth and this was greatly impaired in the presence of IFN-γ. Our results suggest that stromal fibroblasts can be friends or foes with regard to tumor growth, and the outcome is at least partly dependent on immune mediators present in the tumor microenvironment.

The emerging role of stem cells within the tumor stroma has been emphasized in recent years (45, 48). Previous studies astutely used mesenchymal stem cells as potential precursors of the tumor stroma to analyze their association with malignant cells (48). However, which types of tumor stromal cells, endothelial cells or fibroblasts, can be derived from mesenchymal stem cells remains unknown. In our experiments, MEFs were isolated from mid-gestation mouse embryos (E14) and had the potential to differentiate mainly into fibroblasts or myofibroblasts (49). After differentiation, they expressed the fibroblast specific marker ER-TR7 and in some cases, α-SMA. Therefore, MEFs may be suitable as progenitor cells of stromal fibroblasts for evaluation of the function of these cells during tumor development.

IFN-γ is a key cytokine in tumor immunity; however, the target and underlying mechanism of IFN-γ during the antitumor response is still not completely clear. Our previous data based on bone marrow chimeras showed that IFN-γ acts on nonhematopoietic tumor stromal cells and induces angiostasis (10, 11). In the present study, we further characterized the antitumor mechanism of IFN-γ in a specific circumstance that only stromal fibroblasts can respond to this cytokine. In immunized hosts, IFN-γ is produced by hematopoietic cells such as CD4+ Th1 cells, CD8+ T cells, and NK cells (19, 35). Our results demonstrate that the effect of such endogenous IFN-γ on stromal fibroblasts down-regulates their VEGF production, which is necessary for angiostasis. These results reflect a complex interaction between hematopoietic cells, nonhematopoietic stromal components and tumor cells.

Although VEGF has been reported as an important tumor angiogenesis factor in many studies, evidences about its source in the tumor microenvironment are still limited. Previous studies have shown that VEGF promoter activity is restricted to stromal fibroblasts in both spontaneous and transplant tumor models (7), indicating that fibroblasts present in tumors may be the major producer of VEGF and therefore be crucial for tumor angiogenesis. In this study, we show that stromal fibroblasts present in tumors express VEGF at both the RNA and protein levels, confirming the previous observations. Furthermore, our data demonstrate that IFN-γ, as a major antitumor effector molecule, could down-regulate VEGF production by acting on fibroblasts, the nature key producer of VEGF.

It is accepted by more and more researchers that stromal fibroblasts play an essential role in tumor development. Although most studies focus on the promoting effect of stromal fibroblasts, there are also evidences for the opposite. In this study, we demonstrate for the first time to our knowledge that the function of stromal fibroblasts is altered by their response to a cytokine in the tumor microenvironment. The IFN-γ, secreted by immune cells or gene-modified tumor cells, acts on stromal fibroblasts and alters their promoting effect on tumor growth, reflecting a cross-talk between stromal fibroblasts and immune counterparts in tumor stroma. These findings uncover a novel mechanism of tumor-stroma interaction with specific emphasis on the role of IFN-γ, providing clues for stroma-targeted antitumor therapy.

Acknowledgments

We thank Chun Chun Liu for flow cytometry analysis, Jinhua Zhang for immunostaining, and Zhiguang Li and Jing Jiang for helpful discussion.

Disclosures

The authors have no financial conflict of interest.

References


