Transfer of the murine interleukin-12 gene *in vivo* by a Semliki Forest virus vector induces B16 tumor regression through inhibition of tumor blood vessel formation monitored by Doppler ultrasonography

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To elucidate further the potential of a Semliki Forest virus (SFV) vector *in vivo* for gene therapy, we constructed a vector, SFV-IL12, to transfer murine IL-12 genes into tumors. A single intratumoral injection of established B16 murine melanoma with SFV-IL12 resulted in a significant inhibition of tumor growth, while injection with SFV-LacZ had no effect. This antitumor activity correlated with an increase of IFN γ production, MIG and IP-10 mRNA expression, both at the tumor site and at the periphery. In contrast, no increase in CTL- or NK cell-mediated cytotoxic response could be detected, ruling out the involvement of T and NK cell cytotoxicity. To determine how the transfer of

IL-12 genes induced tumor regression, the antiangiogenic activity of SFV-IL12 was investigated using Doppler ultrasonography (DUS). SFV-IL12 inhibited *in situ* neovascularization within the tumor, without affecting the resistance index of pre-existing intratumoral blood flows. In addition, histological analysis of SFV-IL12-treated tumors showed massive tumor necrosis induced by SFV-IL12 treatment. These data indicate that SFV-IL12 inhibits tumor growth through its antiangiogenic activity, demonstrated for the first time *in vivo* by DUS, and suggest that the SFV vector may be a novel valuable tool in tumor gene transfer.

Keywords: interleukin-12; Semliki Forest virus vector; Doppler ultrasonography; angiogenesis; gene therapy

Introduction

Several studies in animal models have indicated that the expression of various cytokine genes in tumor cells generally results in a dramatic alteration of tumor cell growth and in induction of tumor specific immunity.¹ These studies clearly established that tumor vaccines based on the use of cytokines could be a valuable way of eliciting a specific antitumor response against poorly immunogenic tumors.

Interleukin-12, a heterodimeric cytokine secreted by antigen presenting cells, plays a role in innate immunity and in the early induction of T cell-mediated cytotoxicity.^{2,3} It binds to its receptors on T and NK cells^{4,5} and it promotes the induction of T helper 1 (Th1) response *in vitro* and *in vivo*, leading to cellular-mediated immunity.^{2,6} IL-12 appears to be a physiologic inducer of IFN γ production by T and NK cells.⁷ *In vivo*, recombinant murine IL-12 triggers secretion of IFN γ and increases NK activity.⁸ Systemic and peritumoral injection of IL-12 have been shown to cure or enhance survival of mice

bearing both established subcutaneous tumors and hepatic metastases.⁹ Furthermore, the use of various viral vectors such as adenovirus,^{10,11} retrovirus^{12,13} or vaccinia virus¹⁴ have demonstrated in some specific murine models that gene transfer of IL-12 promoted tumor regression and inhibition of metastases, making the use of this cytokine particularly relevant in cancer therapy.

Recently, a new vector system based on Semliki Forest virus (SFV) was reported.¹⁵ The SFV system is suicidal and characterized by high expression levels and broad host range.¹⁶ This system has already been shown to be effective in recombinant vaccine studies,^{17,18} suggesting that it could be used for *in vivo* transfer of cytokine genes. We have recently reported the construction of the SFV-human IL12 (huIL-12) and its use to express huIL-12 in human tumor cells.¹⁹ The current study was undertaken to validate the use of SFV-IL12 in an *in vivo* murine tumor model and to investigate further the *in vivo* antitumor activity of this cytokine, in particular its effect on intratumoral vascularization using a novel approach based on Doppler ultrasonography (DUS).

Initiation of angiogenic activity is recognized as one of the critical steps in tumor development and metastasis. Above a critical volume of 1–2 mm³, tumor progression is dependent on the ability of tumor cells to promote growth of new capillary blood vessels from the host.²⁰ In

mice models, tumor in a prevascular phase remains in a benign stationary state,²¹ cell proliferation equilibrating with the rate of cell death by apoptosis.²² Moreover, evidence has been provided indicating that inhibition of angiogenesis, by use of antiangiogenic factors or by neutralizing normal pro-angiogenic factors, may be a valuable tool in tumor therapy.^{23–25} Recently, Voest *et al*²⁶ have reported in a model of basic fibroblast growth factor-induced mouse corneal neovascularization that IL-12 could exert a potent antiangiogenic effect, mostly by inducing secretion of IFN γ and an antiangiogenic chemokine, IP-10.

Currently, angiogenesis is evaluated by determining the vascular density using immunohistochemical staining of endothelial cells.²⁷ Although this method has proven its efficacy, it gives an instantaneous picture of vascularization and does not reflect the functionality of blood vessels. In view of increasing clinical research on antiangiogenic treatment, it seems important to use an easy, repetitive, functional and non-invasive means of evaluating angiogenesis in human tumors *in vivo*. Several studies have shown that color DUS is able to detect *in situ* neovascularization in solid human tumors.^{28–32} Recently, we have shown that color DUS was able to visualize vessels of diameter greater than 100 μ m, and thus appropriate for the evaluation of angiogenesis in human malignant melanoma. Indeed, the color DUS results correlated with immunohistochemical staining, as regards vascular density and number of vessels of diameter above 100 μ m.³³ In the current study, we have adapted this method to murine tumors for the purpose of investigating the effect of SFV-IL12 intratumoral injection on tumor vascularization *in vivo*.

In the experiments presented here, we demonstrate the efficiency of *in vivo* IL-12 transfer by SFV vector. This transfer resulted in tumor regression by a mechanism independent from an enhancement of NK and CTL-mediated cytotoxicity, which appears to be associated with an inhibition of tumor blood vessel formation as detected for the first time by a new approach based on the use of Doppler ultrasonography.

Results

IL-12 expression following SFV-IL12 injection of B16 tumor

Initial experiments were performed to assess the infectibility of murine melanoma B16 cells by SFV-IL12. When B16 cells were infected *in vitro* by SFV-IL12 at a MOI of 10, they produced 6 ng of IL-12 per million infected cells in 24 h. The production was maintained for 48 h, after which B16 cells began to die, due to SFV suicidal infection.¹⁹

To assess *in vivo* production of IL-12 using SFV-IL12, B16 tumor-bearing mice were injected intratumorally with 10⁷ IU of recombinant virus per mice, 10 days after tumor cell inoculation. IL-12 could be detected in mice sera only at day 1 (50 ng/ml of IL-12). From day 2 onward, SFV-IL12-treated mice had low IL-12 serum levels comparable to control and SFV-LacZ-treated mice (Table 1). At the tumor site, IL-12 could still be detected 5 days after injection, with a peak expression of 250 ng of total IL-12 within the tumor, at day 1 (Table 1).

Table 1 Production of IL-12 following infection of B16 tumor cells with SFV vector

	Sera (ng/ml) ^a				Tumor (ng) ^b			
	SFV-	LacZ	SFV-	IL-12	SFV-	LacZ	SFV-	IL-12
	mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse
	1	2	1	2	1	2	1	2
Day 1	3	4	46	46	ND	ND	210	302
Day 3	4	4	5	4	ND	ND	90	21
Day 5	4	3	3	4	ND	ND	38	4

Ten-day tumor-bearing mice were treated with a single intratumoral injection of 10⁷ IU of SFV-LacZ or 10⁷ IU of SFV-IL12. Two mice per group (mouse 1 and 2) were killed on days 1, 3 and 5 after SFV treatment and IL-12 concentration was measured in the sera and at the tumor site, using an murine total IL-12 ELISA. Mice injected with control TNE buffer yielded similar results to SFV-LacZ injected mice. ND, non-detectable (<0.1 ng/ml). The experiment was performed twice.

^aConcentration of IL-12 in mice sera is given in ng/ml.

^bTotal IL-12 in tumor homogenate is given in ng.

Single SFV-IL12 intratumoral injection inhibited B16 tumor growth

The antitumoral effect of IL-12 gene transfer using SFV was determined on 10-day established subcutaneous B16 melanoma tumor. Ten days after B16 tumor cell inoculation, tumor-bearing mice were injected intratumorally with 10⁷ IU of SFV-IL12, SFV-LacZ or control TNE buffer. Tumor growth was monitored every 2–3 days. No obvious toxicity was observed in SFV-IL12 treated mice compared with control or SFV-LacZ-treated mice. While SFV-LacZ intratumoral injection did not significantly alter tumor growth, SFV-IL12 injection inhibited tumor growth in all treated mice (70–90% growth inhibition as compared with control mice) (Figure 1). In 50% of the

Figure 1 Inhibition of tumor growth following intratumoral injection with SFV-IL12. Ten-day tumor-bearing mice were treated with a single intratumoral injection of 10⁷ IU per mouse of either SFV-LacZ or SFV-IL12. Experiment was performed twice. Tumor volumes are given as mean of five mice per group. *, Statistical significance compared with control mice injected with TNE buffer. ns, No statistical significance.

mice, SFV-IL12 treatment even induced partial tumor regression (partial regression observed in 11 of 21 treated mice), as compared with an absence of tumor regression in control and SFV-LacZ treated mice. The antitumor response was dose dependent, but optimal effect began with the dose of 10^7 IU of SFV-IL12 per mouse. Repeated injections of SFV-IL12 (every 3 days) were performed to enhance the antitumor effect observed. Although the growth delay was more pronounced than with a single injection, all tumors recovered when SFV-IL12 injections ended (not shown).

SFV-IL12 intratumoral injection did not induce an increase in T or NK cell-mediated cytotoxicity

Experiments performed to examine whether the IL-12 produced by SFV-IL12 construct was biologically active *in vivo* indicated that when SFV-IL12 and control SFV-LacZ virus were injected intraperitoneally into tumor-free C57BL/6 mice, a dose-dependent increase in splenic NK cytolytic activity against YAC-1 target cells (40% of lysis at an effector:target ratio of 200:1) was observed, as compared with splenic cells from mice injected with SFV-LacZ (30% at 200:1) or TNE buffer (20% at 200:1). To assess whether the antitumoral effect of SFV-IL12 could be attributed to a tumor-specific stimulation of cytotoxic immune response, splenic cells were isolated 1 week after SFV injection. No enhancement of tumor-specific CTL or NK cytotoxic activity in SFV-IL12-treated mice could be detected (Table 2).

To investigate further the respective involvement of T and NK lymphocytes in the antitumoral activity of SFV-IL12, nude (T cell-deficient) and beige (NK cell-deficient) B6 mice bearing 10-day established B16 tumors were treated with SFV-IL12. Data in Figure 2 clearly show that in both T- and NK-deficient mice, SFV-IL12 inhibited tumor growth to the same extent as in control immunocompetent C57BL6 mice. This further suggests that the antitumor activity of SFV-IL12 was mediated by a mechanism independent from an increase in CTL- and NK-mediated cytotoxicity.

*SFV-IL12 antitumoral activity correlated with seric and *in situ* IFN γ production*

Because IFN γ is known to be the major mediator of IL-12 activity,^{2,8} we have examined its production following SFV injection. Our data demonstrate that IFN γ could be detected both in the sera and at the tumor site of mice injected with SFV-IL12, but not in control and SFV-LacZ

Figure 2 SFV-IL12 antitumoral activity in nude and beige B6 mice. Ten-day tumor-bearing nude (a) and beige (b) B6 mice were treated with intratumoral injection of 10^7 IU per mouse of either SFV-LacZ or SFV-IL12. Experiment was performed twice. Tumor volumes are given as mean of five mice per group. *, Statistical significance compared with control mice injected with TNE buffer. ns, No statistical significance.

Table 2 Cytotoxic activity of splenic cells isolated from SFV-injected mice

Splenic cells	Target cells	Control				SFV-LacZ				SFV-IL12			
		mouse 1		mouse 2		mouse 1		mouse 2		mouse 1		mouse 2	
		200:1	100:1	200:1	100:1	200:1	100:1	200:1	100:1	200:1	100:1	200:1	100:1
Freshly isolated	Yac-1 ^a	6%	3%	9%	5%	9%	5%	4%	ND	5%	3%	9%	5%
<i>In vitro</i> stimulated	B16 ^b	2%	ND	ND	ND	2%	ND	ND	ND	2%	ND	ND	ND

Ten-day tumor-bearing mice were treated with intratumoral injection of 10^7 IU per mouse of either SFV-LacZ or SFV-IL12. Effector splenic cells from two mice per group (mouse 1 and 2) were sampled 7 days after SFV injection. Cytotoxic activities of: ^afreshly harvested splenic cells (NK-specific target cells YAC-1) and ^btwice *in vitro* B16-stimulated splenic cells (specific target cells B16) were measured in a ⁵¹Cr release assay. ND, non-detectable (<2% of lysis). Two mice per group were used. Experiments were performed twice.

injected mice. While in the sera, IFN γ production was maximal (0.5–2 ng/ml) between day 2 and 3 and could not be detected 5 days after injection, IFN γ secretion lasted for at least 5 days at the tumor site (Table 3). In nude and beige tumor-bearing mice injected with SFV-IL12, IFN γ production could also be detected, both in the sera and at the tumor site (Table 3), further suggesting that the antitumor activity of SFV-IL12 was mediated by a mechanism independent from an increase in CTL- and NK-mediated cytotoxicity, but rather correlated with an increase of IFN γ production.

SFV-IL12 intratumoral injection induced splenic and in situ IP-10 and MIG expression

Recently, IP-10 has been suggested to mediate most IL-12 antiangiogenic activity.²⁶ IP-10 and MIG expression following SFV injection were investigated using semi-quantitative PCR. Our data demonstrate that, 5 days after SFV injection, IP-10 and MIG transcription was increased in splenic cells of mice injected with SFV-IL12, as compared with control and SFV-LacZ injected mice (Figure 3a). At the tumor site, whereas no obvious effect of SFV-IL12 on IP-10 expression could be detected, an increase in MIG mRNA expression level could be observed 5 days after SFV-IL-12 intratumoral injection (Figure 3b).

SFV-IL12 inhibited tumor neovascularization in vivo, as monitored by Doppler ultrasonography

IL-12 has been reported to inhibit angiogenesis in mouse corneal pocket vascularization assay (IL-12 alone) and rhFGF-adsorbed gelatin sponges containing tumor cell model (IL-12 plus IL-2).^{26,34} Therefore, to determine if SFV-IL12 antitumoral effect involved antiangiogenic activity, SFV-treated tumor-bearing mice were monitored for tumor vascularization following color DUS. Repeated DUS examination showed the evolution of tumor vascularization following SFV injection (Figures 4 and 5). At day 10 after B16 cell inoculation and before SFV injection, all mice were found to have few (0–2) intratumoral blood vessels. In control TNE buffer-treated mice, the number of functional intratumoral vessels ranged from two to six

Figure 3 Expression of IP-10 and MIG by splenic cells and at the tumor site in SFV injected mice. Ten-day tumor-bearing mice were treated with intratumoral injection of either control TNE buffer, SFV-LacZ or SFV-IL12 at 10^7 IU per mouse. Five days after SFV injection, the expression of IP-10, MIG and G3PDH mRNA was analyzed by semi-quantitative PCR: (a) in splenic cells, (b) at the tumor site. The zone of linearity specific for each condition (primers and type of extract) was determined and cycles of amplification are as follows: for IP-10: 27 cycles for splenic cells, 25 cycles for tumor homogenates; for MIG: 27 cycles for splenic cells, 30 cycles for tumor homogenates; for G3PDH: 22 cycles for both extracts. G3PDH is shown as a control, assuming its similar level of expression in the various samples. Data from a representative experiment of two are shown.

at day 12 with a sustained increase until the end of examination at day 17. In SFV-LacZ-treated mice, intratumoral blood flows were first visualized slightly later, with a number of intratumoral blood flows ranging from 1 to 5 at day 14 after B16 inoculation, and rising thereafter to the same extent as in control mice. Strikingly, 2 days after SFV injection (day 12), tumor vascularization was comparable in SFV-IL12 and SFV-LacZ-treated mice, as estimated with DUS examination. However, in SFV-IL12-treated mice, intratumoral vascularization stopped or regressed in all mice from day 13 to day 17, and then started again until day 21, where vascularisation was

Table 3 Induction of IFN γ production following infection of B16 tumor cells with SFV-IL12 vector

		Sera (pg/ml) ^a								Tumor (pg) ^b							
		SFV-LacZ				SFV-IL12				SFV-LacZ				SFV-IL12			
		B6		B6		Nude B6		BeigeB6		B6		B6		Nude B6		Beige B6	
		mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse	mouse
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Day 1	ND	ND		708	2243	336	406	966	4565	78	760	5288	5254	1363	842	4143	4037
Day 3	ND	ND		452	997	ND	438	358	1375	150	20	6257	10 514	8895	8795	7317	8439
Day 5	ND	ND		ND	ND	ND	ND	ND	ND	760	165	6719	2672	8674	3990	3215	3134

Ten-day tumor-bearing immunocompetent (B6), T cell-deficient (Nude B6) and NK cell-deficient (Beige B6) mice were treated with a single intratumoral injection of 10^7 IU of SFV-LacZ or SFV-IL12. Two mice per group (mouse 1 and 2) were killed on days 1, 3 and 5 after SFV treatment. IFN γ concentration was measured in the sera and at the tumor site, using a murine IFN γ ELISA. Mice injected with control TNE buffer yielded similar results to SFV-LacZ injected mice. ND, non-detectable (<5 pg/ml). The experiment was performed twice.

^aConcentration of IFN γ in mice sera is given in pg/ml.

^bTotal IFN γ in tumor homogenates is given in pg.

Table 4 Intratumoral RI at various times after SFV injection

	<i>All mice</i>		<i>Control</i>		<i>SFV-LacZ</i>		<i>SFV-IL-12</i>	
	<i>Mean RI</i>	<i>s.d.</i>	<i>Mean RI</i>	<i>s.d.</i>	<i>Mean RI</i>	<i>s.d.</i>	<i>Mean RI</i>	<i>s.d.</i>
Day 1	0.326	0.073	0.36	0.077	0.278	0.056	0.337	0.065
Day 2	0.337	0.074	0.329	0.05	0.332	0.05	0.35	0.109
Day 3	0.345	0.053	0.34	0.045	0.327	0.065	0.374	0.043
Day 4	0.346	0.05	0.348	0.04	0.364	0.053	0.334	0.059

Ten-day tumor-bearing mice were treated with a single intratumoral injection of 10^7 IU of SFV-LacZ or 10^7 IU of SFV-IL12. Mice were examined every day, from 1 to 4 days after SFV inoculation. RI were calculated for each intratumoral blood flow and results are given as the resulting mean $\pm$ s.d. The number of measurements per group varied between five and 15, in two independent experiments with two mice per group.

relatively comparable to control mice at day 12 (Figure 5). Representative evolution of tumor vascularisation over 7 days in a single mouse for each type of treatment are shown in Figure 4.

We also evaluated the effect of IL-12 treatment on a hemodynamic parameter, resistance index (RI), currently used to characterize tumor angiogenesis. Mice were examined daily, from day 1 to day 4 after SFV injection, and RI was measured for every blood flow visualized inside the tumor. Intratumoral RI were found to be significantly lower than peritumoral RI 0.35 ± 0.09 and 0.66 ± 0.09 respectively; $n = 23$; $P = 10^{-8}$), as previously shown.³⁵ However, no significant difference in RI between control, SFV-LacZ and SFV-IL12-treated mice could be detected (Table 3).

SFV-IL12-induced inhibition of tumor vascularisation resulted in massive tumor necrosis

Tumors were resected from mice at day 13 and day 17. The previously demonstrated correlation between the number of blood flows visualized with DUS and the number of vascular structures visualized on standard HES stained tissue section was confirmed.³⁰ At day 17, the histological numbering of blood vessels in SFV-IL12 treated tumor was significantly lower than in control and SFV-LacZ-treated mice, where, in contrast, hypervascular development could be observed (Figure 6). Histological analysis further revealed that, 3 days after SFV injection (day 13), tumor necrosis in SFV-LacZ and SFV-IL12-treated tumor was slightly higher (20–30%) than in control tumors (10–20%), probably as a consequence of cell death induced by SFV injection. However, 7 days after SFV injection, dramatic tumor necrosis could be seen in SFV-IL12-treated tumors (70% compared with 20–30% in control and SFV-LacZ-treated tumors), along with an inflammatory lymphocyte and macrophage infiltration (Figure 6).

Discussion

There is currently much interest in IL-12, in large part because of its demonstrated potent antitumor and anti-metastatic activities in tumor models.⁹ Evidence has been provided indicating that dramatic antitumor effects can be achieved by low-dose and transient IL-12 delivery instead of continuous high-dose treatment.^{36,37} For this purpose, we have constructed a new vector, a Semliki Forest virus (SFV) expressing human IL-12 genes, SFV-

hIL12.¹⁹ The SFV system is suicidal and characterized by broad host range and high expression levels, and is limited to one cell cycle without concomitant expression of any viral structural genes.^{15,16} It has been used in vaccine studies to induce *in vivo* protection against simian immunodeficiency virus (SIV) disease in macaques.¹⁷ We have shown in a previous study that this vector enables transient high IL12 expression,¹⁹ and therefore may provide a valuable tool to examine the effects of IL-12 *in vivo*.

Studies in a variety of models suggest that cytokine gene expression cannot be detected readily after the second administration of first generation adenoviral vectors.³⁸ Interestingly, the SFV vector is less immunogenic since only replication, and no structural, proteins are produced in the host cells.³⁹ Repeated viral injections with SFV-IL12 did efficiently lengthen tumor growth inhibition by IL-12. Indeed, the lack of SFV-LacZ effect on tumor progression also strongly supports the hypothesis of an absence of an antiviral response to SFV intratumoral injection.

Our data clearly show that a single intratumoral injection with SFV-IL12 resulted in significant tumor regression. Evidence has been provided indicating that cytotoxic activity of T lymphocytes and NK cells is often considered to be crucial for IL-12 antitumor activity. Although several reports have shown that the optimal IL-12 antitumor effects were T cell-mediated, in some mice strains, residual IL-12 antitumor activity persists in the absence of T cells.^{9,40} In our model, although IL-12 induced tumor growth inhibition, it did not appear to lead to an enhancement of T and NK cell cytotoxic activity. This is in agreement with the report of Rodolfo *et al*⁴¹ showing that *in vivo* rejection of C26 tumor cells transduced with IL12 genes was not correlated with cytotoxic immune response activation. In addition, noncytolytic IFN γ -producing CD8⁺ T cells have been observed to destroy lung metastases upon adoptive transfer.⁴² This is in agreement with our observation indicating that, following injection with SFV-IL12, B16 tumor growth was similarly inhibited in immunocompetent, NK- and T cell-deficient mice, suggesting that T or NK cytotoxic activity cannot account for the IL-12 antitumor activity. Interestingly, the inhibition of tumor growth correlated with a sustained IFN γ production at the tumor site in C57Bl/6, nude and beige mice. Preliminary results using mice deficient for the IFN γ R (kindly provided by Dr S Huang⁴³) suggest that IFN γ may be crucial to SFV-IL12 antitumoral activity in the B16 model. Indeed, in control

Figure 4 Inhibition of tumor vascularisation monitored by color DUS in SFV-IL12-treated B16 tumors. Ten-day tumor-bearing mice were treated with intratumoral injection of either: TNE buffer (a, b, c, d), SFV-LacZ at 10^7 IU per mouse (e, f, g, h) and SFV-IL12 at 10^7 IU per mouse (i, j, k, l). Mice were examined 1 (a, e, i), 2 (b, f, j), 4 (c, g, k) and 7 (d, h, l) days after SFV injection. Intratumoral blood flows, when detected, are shown with a white arrow. Experiments were performed three times, with two mice per group. Representative results with the same tumor cross-section of a single mouse, followed throughout the experiment in each group are shown.

Figure 5 Inhibition of tumor vascularization following intratumoral injection with SFV-IL12. Ten-day tumor-bearing mice were treated with a single injection of either SFV-LacZ or SFV-IL12 at 10^7 IU per mouse. Evolution of intratumoral blood vessels count visualized by DUS over 10 days after SFV injection is shown. The quantification of the number of vessels was performed by obtaining multiple cross-sections of the tumor. *, Statistical significance compared with control mice injected with TNE buffer. Results are given as the mean of four mice per group, each experiment of two mice per group.

wild-type mice ($\text{IFN}\gamma\text{R}^{+/+}$), SFV-IL12 inhibited B16 tumor growth whereas in mutant $\text{IFN}\gamma\text{R}^{-/-}$ mice treated with SFV-IL12, tumor growth was comparable to SFV-LacZ treated mice. In this regard, it should be noted that $\text{IFN}\gamma$ may act directly on tumor progression, in that it can exert some cytostatic action on tumor cells and delay tumor growth,⁴⁴ without interfering with immunological recognition of tumor cells.

We next examined how IL-12 affects tumorigenesis of B16 melanoma and focused on the effect of IL-12 on angiogenesis.^{26,34} For this purpose, color Doppler ultrasonography (DUS) was adapted to *in vivo* examination of murine tumors, in order to demonstrate directly the antiangiogenic effect of IL-12 on tumor neovascularization and its function. Enumeration of vessels by both DUS and histological analysis revealed that DUS count was slightly lower than classical evaluation of vascularization with histology, most probably due to the fact that DUS makes it possible to visualize functional vessels with a diameter greater than $100\ \mu\text{m}$.³³ Moreover, we cannot exclude the possibility that IL-12 inhibition of angiogenesis affects normal function of vessels, thus preventing their visualization with DUS, but not their detection with histological analysis.

One way of assessing the functionality of blood vessels is by determining RI, which reflects systolic and diastolic velocity. Emoto *et al*⁴⁵ have shown that low resistance to blood flow in vessels within malignant ovarian tumors may be correlated with the endothelial cell activity of the tumor vessels. Our results show that under IL-12-mediated antiangiogenic treatment, RI underwent no significant changes, although a marked inhibition of tumor neovascularisation was observed. This suggests that IL-12 antiangiogenic activity is presumably not related to affected endothelial cell activity, but rather to an indirect

Figure 6 Induction of tumor necrosis following intratumoral injection with SFV-IL12. Ten-day tumor-bearing mice were treated with a single injection of either TNE buffer (a), SFV-LacZ (b) and SFV-IL12 (c) at 10^7 IU per mouse. Tumors were resected 7 days after SFV injection. Representative standard HES staining of paraffin section are given, $\times 50$. Necrotic areas are shown with an asterisk, and blood vessels by an arrow. (a, b) Control and SFV-LacZ-treated tumors exhibit small areas of necrosis and large vessels of diameter above $1\ \text{mm}$. (c) SFV-IL12-treated tumor shows large area of necrosis, along with smaller vessels.

inhibition of blood flow in vessels, maybe by inducing thrombosis. It should be emphasized that inhibition of tumor vascularization began 2 days after SFV injection, correlating with $\text{IFN}\gamma$ production at the tumor site and strongly suggesting a potential role of this cytokine in IL-12-induced inhibition of neovascularization. Histologic analysis further revealed massive tumor necrosis 7 days but not 3 days after SFV-IL12 injection. This necrosis occurred following blood flow inhibition that started 3 days after SFV-IL12 injection, ruling out immune tumor lysis. Whether tumor cells themselves play a role in the inhibition of angiogenesis induced by IL-12 or its downstream mediator $\text{IFN}\gamma$ has yet to be determined.

Recently, mouse matrigel bFGF-induced neovascularization assay demonstrated the lack of involvement of T and NK cell cytotoxicity in the antiangiogenic effect of IL-12 and pointed to the requirement of $\text{IFN}\gamma$ and two

antiangiogenic chemokines IP-10 and MIG, as mediators of this specific IL-12 activity.^{26,46} Indeed in our model, the antitumor effect of IL-12 is correlated with a significant increase of both IP-10 and MIG peripheral expression, as demonstrated in splenic cells. B16 tumor cells do not express IP-10 or MIG constitutively. No obvious effect of SFV-IL12 on IP-10 expression could be detected at the tumor site, but SFV-IL12 injection induced MIG expression 5 days after SFV injection, most probably expressed by lymphocytes or macrophages infiltrating the tumor. Our data suggest that, beside IP-10, MIG may be a second crucial mediator of IL-12 anti-angiogenic effect. Both these factors have recently been shown to mediate potent antiangiogenic activity by themselves. IL-12 may also indirectly stimulate host innate immune mechanisms involving macrophages,¹⁴ responding to IFN γ secretion at the tumor site, since in our model a massive macrophagic infiltration was observed in SFV-IL12-treated tumor 7 days after intratumoral SFV injection. This suggests that macrophages may be involved in IL-12-induced B16 tumor regression.

Taken together, our data suggest that SFV-IL12 injection induces tumor growth inhibition through its antiangiogenic activity and that the SFV vector may provide a novel valuable tool for tumor gene transfer. Based on our findings, it is tempting to speculate that the combination of IL-12 gene transfer with conventional approaches to eradicate residual tumor cells may help in preventing tumor growth and lead to a more rational therapeutic strategy aimed at invasive tumors.

Materials and methods

Mice and cell lines

Specific pathogen-free immunocompetent C57BL/6JRj (B6) mice and T cell-defective nude mice (C57BL/6NRj-nu) were purchased from CERJ (Le Genest St Isle, France). NK cell-defective beige mice (C57BL/6OlaHsd-bg) were purchased from Harlan (Gannat, France). All mice were kept under pathogen-free conditions at the Animal Facility of the Institut Gustave Roussy, Villejuif, France. All experiments were performed in accordance with institutional animal guidelines.

The murine melanoma cell line B16 (B16F1) syngeneic to C57BL/6 mice was grown in DMEM complete medium (supplemented with 10% fetal calf serum and penicillin/streptomycin) (Seromed, Biochrom, Berlin, Germany). BHK-21 c-13 cells (baby hamster kidney cells, ATCC cCL-10) were grown in BHK medium supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, 10 mm HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid).

Cloning of murine IL-12 p35 and p40 subunits in the SFV vector

The pSFV1, pSFV-helper2, pSFV3-LacZ and pSFV3-human IL12 (SFV-hIL12) vectors have been described previously.¹⁹ As a first step to construct the pSFV1-murine IL12 plasmid, encoding biologically active mIL12, mIL12 p35 and p40 cDNAs have both been amplified by PCR using p35/pEF.BOS and p40/pEF.BOS as template, respectively (generous gift of TF Gajewski, Ludwig Institute for Cancer Research, Brussels, Belgium).⁴⁷ The following primers were used: 5'-CCA TGG ATC CAT GTG

TCA ATC ACG CTA CCT C-3' (p35, sense), 5'-GAG CCC GGG TTC AGG CGG AGC TCA GAT AG-3' (p35, antisense), 5'-CCA TGG ATC CAT GTG TCC TCA GAA GCT AAC C-3' (p40, sense) and 5'-GAG CCC GGG CTA GGA TCG GAC CCT GCA GGG A-3' (p40, antisense). Each sense primer contains a *Bam*HI site (bold) while antisense primers contain a *Sma*I site (underlined). *Bam*HI-*Sma*I cut p35 (656 bp) and p40 (1015 bp) PCR products were then inserted independently into pSFV1 plasmid restricted with *Bam*HI and *Sma*I. In the next step the pSFV1/p40 plasmid was cut with *Sma*I and *Hind*III to excise the *Hind*III-*Sma*I 1426 bp fragment that contains the pSFV1 26S promoter sequence upstream of the mIL12 p40 sequence. This fragment was subsequently cloned into pSFV1/p35 opened with *Sma*I. The ligation product was electroporated into SURE bacteria (Stratagene, Heidelberg, Germany) and the clone containing the mIL12 p40 sequence in the sense orientation were screened by restriction analysis.

The packaging of SFV-hIL12 RNA in viral particles has been described.¹⁹ Briefly, *in vitro* transcribed SFV-mIL-12 RNA and SFV-Helper2 RNA were electroporated into BHK21 cells. While SFV-IL12 RNA is packaged into SFV virions, the helper RNA is not, due to lack of the packaging sequence. Moreover, produced particles are noninfectious due to a mutation in the envelope gene. Infectious viral particles were obtained by *in vitro* chymotrypsin.⁴⁸ Viral particles were harvested from the BHK-21 cell culture supernatant by ultracentrifugation and resuspended in TNE buffer (50 mm Tris-HCl pH 7.4, 100 mm NaCl, 0.5 mm EDTA; Sigma, St Louis, MO, USA). Virus was titrated as already described.¹⁹

Activation of SFV virus and *in vitro* infection of B16 tumor cells by SFV-IL12

Activation of the SFV-IL-12 and SFV-LacZ virus was performed with 0.5 mg/ml of chymotrypsin (Sigma) and 10 mm CaCl₂ (Sigma), at room temperature for 30 min and stopped by adding aprotinin (Sigma) at a final concentration of 0.6 mg/ml.

For *in vitro* infection, B16 tumor cells were left to adhere overnight, washed twice with PBS and then covered with the activated virus diluted in medium, at a MOI of 10. Infection was performed at room temperature for 30 min, prolonged by 15 min at 37°C, with gentle tilting from time to time. The virus solution was then removed and replaced by the complete medium for further incubation.

Treatment of B16 tumors with SFV-IL12

Subcutaneous B16 melanoma tumors were established by injection of $0.5-1 \times 10^6$ B16 cells subcutaneously into the right flank of 6- to 8-week-old mice. After 10 days, mice bearing tumor of 100-200 mm³ were injected intratumorally with 10^7 IU of activated SFV-IL12 or SFV-LacZ virus diluted in 50 μ l of TNE buffer per mouse (TNE buffer as control). Five mice per group were monitored for tumor growth using calipers, and tumor volume was calculated with the following formula: volume = $0.5 \times$ longest diameter $\times$ (shortest diameter)².

Cytotoxic activity in splenic cells

Splenic effector cells were obtained by passing mice spleens through a cell filter, followed by a 10-min incubation in NH₄Cl 0.15 M solution (Sigma) to lyse red cells.

For cytolytic assays, effector splenic cells were either used directly in a ^{51}Cr -release cytotoxicity assay or stimulated twice for 5 days with irradiated B16 tumor cells (80 Gy) in the presence of 10 U/ml of IL-2 (Roussel-Uclaf, Romainville, France). B16 cells were used as cytotoxic T cell specific targets and YAC-1 cells as NK cells specific targets in ^{51}Cr -release cytotoxicity assay performed as previously described.¹⁹

Serum and tumor cytokine assay

At the specified time-point, retro-orbital bleed was performed and serum was collected after centrifugation. For quantification of cytokine at the tumor site, mice were killed and tumors were removed and snap-frozen in liquid nitrogen. Frozen tissue was homogenized in 0.5 ml PBS, 100 μM phenylmethylsulfonyl fluoride (PMSF; Sigma), 10 $\mu\text{g}/\text{ml}$ aprotinin (Sigma). The homogenate was then sonicated twice for 10 s, centrifuged for 5 min, and supernatant was stored, as well as mice sera, at -20°C until ELISA dosage. Murine IL-12 concentration in tumor homogenates or mice sera was determined by a total IL-12 ELISA kit (Genzyme, Cambridge, MA, USA). Murine IFN γ concentration was determined using IFN γ ELISA kit (Genzyme).

Semi-quantitative RT-PCR

For analysis of IP-10 and MIG mRNA expression, spleens and tumors were collected from SFV-injected mice 5 days after SFV injection. Splenic and tumor cells were obtained by passing spleens and tumors through a cell filter, followed for splenic cells by a 10 min incubation in NH_4Cl 0.15 M solution (Sigma) to lyse red cells. Total RNA was extracted using a modified guanidium thiocyanate phenol/chloroform method (RNABle; Eurobio, Les Ulis, France). cDNA was prepared by the standard method using reverse transcriptase and an oligo-dT primer (Life Technologies, Cergy Pontoise, France). cDNA were amplified in PCR performed as previously described,⁴⁹ using the following primers: IP-10: 5'-ACC ATG AAC CCA AGT GCT GCC GTC-3' (sense) and 5'-GCT TCA CTC CAG TTA AGG AGC CCT-3' (antisense), MIG: 5'-ACT CAG CTC TGC CAT GAA GTC CGC-3' (sense) and 5'-AAA GGC TGC TCT GCC AGG GAA GGC-3' (antisense) and G3PDH: 5'-GCC ACC CAG AAG ACT GTG GAT GGC-3' (sense) and 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3' (antisense). PCR conditions were as follows: 94°C for 90 s, 94°C for 15 s, 55°C for 30 s, 72°C for 50 s) for the indicated amount of cycles, on a Perkin Elmer apparatus. PCR was performed with various numbers of cycles, to determine the zone of linearity specific for each condition (primers and type of extract). After amplification, PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide (0.01%) and visualized under UV light illumination.

Doppler ultrasonography analysis

To avoid motion artifact from living animals, mice were anesthetized 15 min before each examination, by intraperitoneal injection of 0.2 ml of a mixture of Ketalar (10 mg/ml, Parke-Davis, Courbevoie, France) and Rompun (0.1%, Bayer Pharma, Puteaux, France). During each experiment, two mice per group were monitored. An Esaote-Biomedica AU 4 Idea sonograph (Genoa, Italy) with a 13 MHz high-frequency linear electronic probe was used, with an axial resolution of 200 μm and lateral resolution

of 400 μm with pulsed and color DUS (Doppler frequency 7 MHz). Using a Doppler phantom in silicon (RT 141, Rhône-Poulenc) and a blood analogue, we proved that this sonograph was able to visualize vessels as small as 80 μm in diameter with color Doppler US. The theoretical depth explored was 40 mm. The sonographic examination of each tumor consisted of a morphological study analyzing the structural sonographic pattern and measurement of the thickness and the two transversal perpendicular diameters using electronic calipers (accuracy of 0.01 mm). Color Doppler imaging (PRF = 750 MHz) was used to evaluate intratumoral and peritumoral blood flow, which were recorded on an electronic color printer. The quantification of the number of vessels was performed by obtaining multiple cross-sections of the tumor. This was completed by a spectral analysis by pulsed Doppler (PRF = 1 KHz), with a measurement of systemic and diastolic velocity in each tumor vessels, and of resistance index (RI) calculated as follows: $\text{RI} = (\text{systolic velocity} - \text{diastolic velocity}) / \text{systolic velocity}$.

Histological analysis

Tumors were resected at a specified time, immediately fixed in liquid AFA, a mixture of acetic acid, formalin and alcohol (Carlo Erba Reagenti, Rodano, Italy), embedded in paraffin and sectioned at 4 μm . All blocks were subjected to standard HES staining (hematoxylin, eosin, safran). The analysis comprised quantification of necrosis, estimation of the inflammatory reaction and enumeration of blood vessels with a diameter above 100 μm .

Statistical analysis

Statistical tests were performed using the two-tailed, unpaired Student's *t* test. Results are presented as means $\pm$ standard error of the mean (s.e.m.).

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