Mesenchymal Stem Cells: Potential Precursors for Tumor Stroma and Targeted-Delivery Vehicles for Anticancer Agents

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Background: **High concentrations of interferon beta (IFN-β) inhibit malignant cell growth *in vitro.* However, the therapeutic utility of IFN-β *in vivo* is limited by its excessive toxicity when administered systemically at high doses. Mesenchymal stem cells (MSC) can be used to target delivery of agents to tumor cells. We tested whether MSC can deliver IFN-β to tumors, reducing toxicity. *Methods:* Human MSC were transduced with an adenoviral expression vector carrying the human IFN-β gene (MSC-IFN-β cells). Flow cytometry was used to measure tumor cell proliferation among *in vitro* co-cultures of MSC-IFN-β cells and human MDA 231 breast carcinoma cells or A375SM melanoma cells. We used a severe combined immunodeficiency mouse xenograft model (4–10 mice per group) to examine the effects of injected MSC-IFN-β cells and human recombinant IFN-β on the growth of MDA 231- and A375SM-derived pulmonary metastases *in vivo* and on survival. All statistical tests were two-sided. *Results:* Co-culture of MSC-IFN-β cells with A375SM cells or MDA 231 cells inhibited tumor cell growth as compared with growth of the tumor cells cultured alone (differences in mean percentage of control cell growth: −94.0% [95% confidence interval {CI} = −81.2% to −106.8%; *P*<.001] and −104.8% [95% CI = −82.1% to −127.5%; *P*<.001], respectively). Intravenous injection of MSC-IFN-β cells into mice with established MDA 231 or A375SM pulmonary metastases led to incorporation of MSC in the tumor architecture and, compared with untreated control mice, to prolonged mouse survival (median survival for MDA 231–injected mice: 60 and 37 days for MSC-injected and control mice, respectively [difference = 23.0 days (95% CI = 14.5 to 34.0 days; *P*<.001]; median survival for A375SM-injected mice: 73.5 and 30.0 days for MSC-injected and control mice, respectively [difference = 43.5 days (95% CI = 37.0 to 57.5 days; *P*<.001]). By contrast, intravenous injection of recombinant IFN-β did not prolong survival in the same models (median survival for MDA 231–injected mice: 41.0 and 37.0 days for IFN-β–injected and control mice, respectively [difference = 4 days, 95% CI = −5 to 10 days; *P* = .308]; median survival for A375SM-injected mice: 32.0 and 30.0 days for IFN-β–injected and control mice, respectively [difference = 2 days, 95% CI = 0 to 4.5 days; *P* = .059]). *Conclusions:* Injected MSC-IFN-β cells suppressed the growth of pulmonary metastases, presumably through the local production of IFN-β in the tumor microenvironment. MSC may be an effective platform for the targeted delivery of therapeutic proteins to cancer sites. [J Natl Cancer Inst 2004;96:1593–1603]**

The utility of many biologic agents for cancer therapy is often limited by their short half-life or excessive toxicity. For example, interferon beta (IFN-β) has potent antiproliferative *(1,2)* and proapoptotic *(3–5)* effects against many types of malignant cells *in vitro.* However, these observations have not translated into successful clinical therapy: results of most clinical trials of IFN-β have shown that it has minimal activity for the treatment of human malignancies *(6–10).* These disappointing results may be due to the fact that the concentrations of IFN-β that are required to inhibit the growth of malignant cells *(1–5)* are substantially higher than the serum levels of IFN-β that are achievable in patients after systemic administration of IFN-β at the maximally tolerated dose *(11–13).* Consequently, systemic administration of IFN-β has not resulted in adequate drug levels in tumors *(14,15)* and has failed to exert antiproliferative and proapoptotic effects against malignant cells *in vivo (13).*

We have developed a therapeutic strategy that uses mesenchymal stem cells (MSC) as cellular vehicles for the targeted delivery and local production of biologic agents in tumors *(16).* MSC are bone marrow–derived non-hematopoietic precursor cells *(17,18)* that contribute to the maintenance and regeneration of connective tissues through engraftment. MSC can be obtained from bone marrow aspirates, expanded *in vitro,* and genetically modified for therapeutic strategies *in vivo.* However, it has become evident that *in vivo* engraftment is not only an intrinsic function of MSC but also depends on appropriate external signals produced by the tissue microenvironment *(16,19,20).* These signals are related to the hierarchic organization of tissues with regard to their proliferative and differentiation potentials *(21).* Tissues that have a high spontaneous turnover, such as skin or gut, continuously replace terminally differentiated epithelial cells from compartments of undifferentiated precursor cells and stem cells. This process involves the proliferation of undifferentiated precursor cells present *in situ* or possibly their migration from other sites in the organism *(21–23).* By contrast, the turnover of connective tissues is low, and their proliferative potential becomes apparent only when the demand for new functional connective cells increases, such as during wound healing or tissue regeneration after injury. These conditions are

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characterized by an increased turnover of connective tissues that can possibly also mediate engraftment of bone marrow–derived MSC. Indeed, MSC have been shown to contribute to tissue regeneration and to the formation of fibrous scars at the sites of injury *(19,23).*

Tumors are composed of malignant tumor cells and nonmalignant benign cells. The "benign" tumor compartment includes blood vessels, infiltrating inflammatory cells, and stromal fibroblasts. Stromal fibroblasts provide structural support for malignant cells and influence the behavior and aggressiveness of cancers *(24).* The formation of tumor stroma closely resembles wound healing and scar formation *(25).* Malignant cells induce *de novo* formation of connective tissue in order to provide enough stroma to support cancer growth *(26,27).* We proposed that signals that mediate increased turnover and proliferation of connective stromal cells in tumors may also mediate the engraftment and proliferation of MSC in tumors *(16).* MSC engrafted in tumors could potentially serve as delivery vehicles to target anticancer agents to malignant cells. We tested this hypothesis by examining the effects of MSC transduced with adenovirus expressing human IFN-β (MSC-IFN-β) against metastatic MDA 231 breast carcinomas and A375SM melanomas in a mouse xenograft model. We wanted to investigate the direct inhibitory effects of IFN-β on tumor cells *in vivo,* without interference by its immunomodulatory and anti-angiogenic properties. To this end, we tested the effects of human IFN-β, which is species-specific and therefore would not be expected to influence any residual severe combined immunodeficiency (SCID) mouse immune cells or tumor endothelial cells of murine origin *(28).*

Materials and Methods

Isolation of MSC and Cell Culture

MSC were isolated from the bone marrow of 10 healthy donors who were undergoing bone marrow harvest for use in allogeneic bone marrow transplantation. All bone marrow donors provided written informed consent, and this study was conducted according to institutional guidelines under an approved protocol. Bone marrow was subjected to centrifugation (700*g* for 15 minutes at 4 °C), as described *(18)* over a Ficoll–Hypaque gradient (Sigma, St. Louis, MO) to separate mononuclear cells, which were resuspended in alpha-minimal essential medium (α-MEM) containing 20% fetal bovine serum (Gibco BRL, Rockville, MD), L-glutamine, and penicillin–streptomycin (Flow Laboratories, Rockville, MD) and plated at an initial density of 1×10^6 cells/cm^2. Three days later, the cultures were washed with phosphate-buffered saline (PBS) to remove nonadherent cells, and the remaining monolayers of adherent cells were cultured in fresh medium until they reached confluence. The cells were harvested by trypsinization (0.25% trypsin with 0.1% EDTA), subcultured at densities of 5000–6000 cells/cm^2, and used during the third or fourth passages for experiments. The resulting cells reacted positively with SH2, SH3, and SH4 antibodies, which detect CD105 (Endoglyn) and CD 73 *(18),* two antigens co-expressed on MSC (a generous gift from Dr. Robert Dean, Osiris, Baltimore, MD), when analyzed by FACScan flow cytometry (Becton-Dickinson, San Jose, CA) (data not shown). The cells also differentiated into adipocytes, chondroblasts, and osteoblasts in commercially available differentiation assays (BioWhittaker, Walkersville, MD) (data not shown). These assays confirmed that we obtained MSC.

Human melanoma A375SM and breast cancer MDA 231 cells were a gift from Dr. I. Fidler (Department of Cancer Biology, M. D. Anderson Cancer Center, Houston, TX). The cells were maintained in α-MEM containing 10% fetal calf serum (FCS), sodium pyruvate, nonessential amino acids, L-glutamine, vitamin solution (Life Technologies, Grand Island, NY), and penicillin–streptomycin. Human kidney 293 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS and penicillin–streptomycin.

Adenoviral Vectors and MSC Transduction

We used the AdEasy Adenoviral Vector System (Qbiogene, Carlsbad, CA) to create adenoviral vectors that expressed either *Escherichia coli* β-galactosidase (β-gal) or human IFN-β. Briefly, we subcloned the gene for β-gal into the *Not*I and *Hin*dIII sites of an adenovirus shuttle vector that contains a cytomegalovirus (CMV) promoter (pShuttle CMV) to create pShuttleCMV(β-gal). A plasmid containing the gene for human IFN-β (hIFN-β; obtained from InvivoGen, San Diego, CA) was digested with *Cla*I, and the 3′ overhangs were filled in using DNA polymerase to achieve blunt ends. This product was further digested with *Bgl*II to release a 570-base pair fragment containing the human IFN-β gene, which was then subcloned into the *Bgl*II and *Eco*RV sites of pShuttleCMV to create pShuttleCMV(hIFN-β). Both plasmids were sequenced to verify correct reading frames and DNA sequences and then digested with *Pme*I, dephosphorylated using calf-alkaline phosphatase, extracted twice with buffer-saturated phenol–chloroform (1:1, vol/vol), and admixed with *Pac*I-digested pAdEasy-1 (Qbiogene), an adenoviral backbone plasmid bearing a gene for kanamycin resistance. These two plasmid combinations (pShuttleCMV(β-gal)/pAdEasy-1 and pShuttleCMV(hIFN-β)/pAdEasy-1) were electroporated into *E. coli* BJ5183 cells, according to the manufacturer's protocol. The bacteria were plated on kanamycin-containing agar plates, kanamycin-resistant clones were picked, plasmid DNA was extracted, and clones containing the gene product for β-gal or IFN-β were identified by restriction enzyme digestion. Four clones containing each recombinant adenoviral plasmid plus gene product (β-gal or IFN-β) were identified, and theses clones were expanded in a 3-mL bacterial culture. We isolated plasmid DNAs from these cultures and used Fugene6 transfection reagent (Roche, Indianapolis, IA) to transfect them into human kidney 293 cells, according to the manufacturer's instructions. The transfected cells were incubated for 18–20 days to allow homologous recombination to occur and recombinant adenoviral plaques to form; recombinant plaques were then picked and eluted in 5 m*M* Tris–HCl (pH 7.8). These supernatants were used to infect 293 cells grown in 24-well culture plates, and the resulting recombinant virus was rescued from the cultures. We performed two rounds of viral amplification; recombinant adenoviruses that expressed human IFN-β(identified with the use of an enzyme-linked immunosorbent assay; Fujirebio, Tokyo, Japan) or β-gal (as detected by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal]) were then used to transduce MSC. MSC were incubated with each adenovirus at a multiplicity of

infection of 3000 for 2 hours. MSC transduced with adenovirus expressing human IFN-β (i.e., MSC-IFN-β) produced 3×10^4 to 4×10^4 IU of IFN-β per 10^6 MSC during the first 24 hours after infection, and MSC transduced with adenovirus expressing β-gal (i.e., MSC-β-gal) produced X-gal–positive staining in greater than 90% of the MSC during the first 24 hours after infection.

Cell Proliferation Assay

Monolayers of MDA 231 or A375SM cells were washed with PBS and the cells were harvested by trypsinization, resuspended in RPMI-1640 medium containing 10% FCS at a concentration of 1.5×10^4 cells/mL, plated into 96-well plates at 3000 cells (200 μL) per well, and incubated overnight at 37 °C to allow the cells to adhere to the plates. We then added fresh medium containing 0–10 000 IU/mL recombinant IFN-β (Avonex, Biogen, Cambridge, MA). Eight wells were used for each dilution. Nine wells from one 96-well plate were subjected to the MTS assay, which measures the number of viable cells (Promega, Madison, WI), at the time of initial addition of IFN-β. Absorbance (at 490 nm) at that time point was denoted as OD_{Day0}. Media with IFN-β were changed daily in the other plates, and the assay was read again after 5 days (OD_{Day5}). In this assay, absorbance at 490 nm is proportional to the number of viable cells in the well. Results were expressed as the percentage of control cell growth $(OD_{Day5} - OD_{Day0})/(OD_{control} - OD_{Day0}) \times 100$, where $OD_{control}$ corresponded to absorbance measured for wells that received medium without IFN-β on day 5.

Flow Cytometry Analysis of MDA 231 Cell or A375SM Cell Co-Cultures With MSC *In Vitro*

A375SM cells (5×10^4 cells per well) and MDA 231 cells (10^5 cells per well) were plated in six-well plates alone or mixed with MSC or MSC-IFN-β cells, respectively, at a ratio of 10 A375SM cells or MDA 231 cells to 1 MSC-Gal or MSC-IFN-β cell. One plate was used for each experimental condition. After 5 days, the cells were trypsinized, counted, and fixed with 70% ethanol. The cells were then labeled with phycoerythrin (Sigma), and the DNA content of the cells was analyzed with a FACScan flow cytometer *(16)*. The relative numbers of MSC or MSC-IFN-β (diploid cells) and A375SM or MDA 231 tumor cells (aneuploid cells) were determined by using ModFit software, version 2.0 (Verity Software House, Topsham, ME). Results were expressed as the percentage of control cell growth: (the number of tumor cells co-cultured with MSC or with MSC-IFN-β on day 5 – the number of tumor cells co-cultured on day 0)/(the number of tumor cells cultured alone on day 5 – number of tumor cells cultured alone on day 0) × 100.

Determination of A375SM Cell Death

A375SM melanoma cells were plated in 75-cm^2 flasks at a density of 300 000 cells per flask and incubated for 24 hours to allow them to adhere to the flask. Recombinant IFN-β was then added to each flask at a concentration of 1000 IU/mL. Medium was changed daily, and fresh IFN-β was added daily for 3 days. Cells were then harvested by trypsinization, counted, and stained with propidium iodide (PI). Dead cells stained PI-positive because of loss of cell membrane integrity. Percentages of dead and living cells were analyzed on a FACScan flow cytometer.

Mouse Xenograft Model

Female C.B-17 SCID mice (6 weeks old) were obtained from Harlan (Indianapolis, IN). Mice were used according to approved institutional protocols. Mice were injected intravenously in the lateral tail vein with 2×10^6 MDA 231 or A375SM tumor cells suspended in 200 μL of PBS. In preliminary experiments, we determined that all mice injected with 2×10^6 MDA 231 or 2×10^6 A375SM cells developed macroscopic tumor nodules in their lungs at 8 days after tumor cell injection (data not shown).

Determination of Effect of MSC-IFN-β, MSC-Gal, and Recombinant IFN-β on MDA 231 Tumor Weight in Mouse Lung

Eight days after MDA 231 tumor cell injection (as above), the mice started treatment with recombinant IFN-β (100 000 IU injected every other day by subcutaneous injections for the whole duration of the experiment [n = 4]), MSC-IFN-β (three doses of 10^6 cells given weekly by intravenous injections [n = 4]), or MSC-Gal (three doses of 10^6 cells given weekly by intravenous injection [n = 4]). Mice injected with MDA 231 tumor cells alone (n = 4) and healthy mice with no tumor cell injection (n = 4) served as controls. Mice were killed by asphyxiation with CO_2 30 days after tumor cell injection. We measured the weight of whole lungs in all groups of mice and used whole lung weight as a surrogate endpoint of MDA 231 tumor burden in the lung and to assess the effect of MSC-IFN-β, MSC-Gal, and recombinant IFN-β on tumor growth. Lungs and other organs from mice injected with MSC-Gal were also used for histochemistry studies described below.

Survival Analysis

Eight days after MDA 231 cell injection (see above), the mice started treatment with recombinant IFN-β (100 000 IU injected subcutaneously every other for the duration of the experiment [n = 10]), MSC-IFN-β (three doses of 10^6 cells given weekly by intravenous injections [n = 8]), or MSC-Gal (three doses of 10^6 cells given weekly by intravenous injections [n = 5]). Mice injected with MDA 231 tumor cells only, with no further treatment, served as controls (n = 10).

Eight days after A375SM tumor cell injection (as above), the mice started treatment with recombinant IFN-β (40 000 IU injected subcutaneously daily for the duration of the experiment [n = 10]), MSC-IFN-β IV (three doses of 10^6 MSC-IFN-β cells given weekly by intravenous injections [n = 8]), or MSC-IFN-β SC (three doses of 10^6 cells given weekly by subcutaneous injections [n = 5]). Mice injected with A575SM tumor cells only with no further treatment served as controls (n = 10). All mice were followed daily until death. None of the mice had to be sacrificed because of excessive bleeding, open wound infection, moribund status, or prostration with weight loss of more than 25% of initial body weight, as specified in our protocols.

Tissue Processing and Imaging Studies

Eight days after MDA 231 tumor cell injection, the mice (n = 5) received MSC-Gal (three doses of 10^6 cells given weekly by intravenous injection). The mice were killed by asphyxiation with CO_2 at 30 days after tumor cell injection. Healthy mice (n = 5) with no tumors received MSC-Gal (three doses of 10^6 cells given weekly by intravenous injection) and were used as

controls. Lungs and other organs from both groups of mice were embedded in Tissue TEK OTC compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −80 °C. Frozen tissue was sectioned (6- to 8-μm-thick sections), mounted onto slides, and stained with hematoxylin–eosin or for β-gal as described below. Images were captured with the use of an Axioplan2 microscope (Carl Zeiss, Thornwood, NY) equipped with a charge-coupled device camera (Hamamatsu, Bridgewater, NJ) and processed using Adobe Photoshop software, version 5.0 (Adobe Systems, San Jose, CA).

Histochemistry

Whole lungs were incubated with 2% X-gal (Sigma) in 1 *M* $MgCl_2$, 30 m*M* potassium ferricyanide, and 30 m*M* potassium ferrocyanide overnight, and refixed in 10% neutral-buffered formalin. The tissues were then dehydrated with ethanol and, after minimal exposure to xylene, embedded in paraffin, sectioned (5 μm thick), and mounted on slides. The sections were de-paraffinized through minimal exposure to xylene and decreasing concentrations of ethanol and counterstained with eosin or Nuclear Fast red (Arcturus, Mountain View, CA), according to the manufacturer's instructions. Alternatively, slides with sections of frozen tissues were fixed with cold acetone : ethanol (1 : 1 vol/vol) for 20 minutes and stained with X-gal.

Measurement of IFN-β Concentration in Mouse Plasma

Mice with established MDA 231 lung metastases were injected intravenously (n = 5) or subcutaneously (n = 5) with 10^6 MSC-IFN-β cells or subcutaneously with 40 000 IU (n = 5) or 100 000 IU (n = 5) of recombinant IFN-β. Beginning 1 day after MSC-IFN-β injection or 1 hour after recombinant IFN-β injection and at appropriate intervals thereafter, we collected 200 μL of blood from each mouse into heparinized capillary tubes through tail vein incisions. Blood was immediately centrifuged (700*g* for 5 minutes at 4 °C) to remove cells, and plasma was collected and stored at −80 °C. We used an enzyme-linked immunosorbent assay (Fujirebio, Tokyo, Japan) and a National Institutes of Health (NIH) standard for IFN-β1a provided by the manufacturer to determined plasma concentrations of IFN-β. The NIH standard of IFN-β1a allowed us to compare serum levels (in IU/mL) from our study with the data published in the literature.

Statistical Methods

We initially used the Kruskal–Wallis test to assess the statistical significance of overall differences in lung weights between all treatment groups at day 30. Because the results of the Kruskal–Wallis test showed that the difference between MSC-IFN-β–treated and control mice was statistically significant ($P = .004$), we used the Wilcoxon rank sum test to perform pairwise comparisons of treatment effect on lung weight between all groups. Survival was measured from the day of MDA 231 cell or A375SM cell injection until the day of death. For the survival data, the log-rank test was used to assess differences in survival among the four treatment groups. Because this overall test showed that the difference between MSC-IFN-β–treated and control mice was statistically significant for both tumor models ($P<.001$), pairwise log-rank tests were performed. All statistical tests were two-sided; a *P* value of less than .05 was considered statistically significant. Statistical analyses were performed by using either SAS, version 8.2 (SAS, Cary, NC) or Statistica, version 7.0, software (StatSoft, Tulsa, OK).

Results

Effects of Recombinant IFN-β and MSC-IFN-β Cells on MDA 231 Breast Carcinoma and A375SM Melanoma Cells *In Vitro*

We examined the effect of recombinant IFN-β on the growth of MDA 231 breast carcinoma cells and A375SM melanoma cells by using the MTS assay, which measures cell viability as a percentage of the growth of control cells. Recombinant IFN-β inhibited the growth of both cell lines in a concentration-dependent fashion (Fig. 1, A and B). High concentrations of recombinant IFN-β (i.e., ≥200 IU/mL) led to negative values for A375SM cells and therefore suggested that higher concentrations of IFN-β induced death in these tumor cells. We tested whether this was the case and found that A375SM cells incubated with 1000 IU/mL of IFN-β had more propidium iodide–positive (i.e., apoptotic) cells than A375SM cells incubated in the absence of IFN-β (Fig. 1, C). Next, we investigated whether co-culturing the tumor cells with MSC that were transduced with adenovirus expressing IFN-β (i.e., MSC-IFN-β cells) would inhibit tumor cell growth. MDA 231 and A375SM cells were each co-cultured with uninfected MSC or MSC-IFN-β cells at a 10 : 1 ratio. Compared with MDA 231 cells cultured alone, MDA 231 cells co-cultured with MSC-IFN-β cells displayed statistically significantly reduced growth (difference in mean percentage of control cell growth = −94%, 95% CI = −81.2% to −106.8%; $P<.001$), whereas MDA 231 cells co-cultured with uninfected MSC were not growth inhibited (difference in mean percentage of control cell growth = 35.7%, 95% CI = 23.7% to 47.7%; $P = .063$). Growth of A375SM cells co-cultured with MSC-IFN-β cells was also statistically significantly inhibited compared with the growth of A375SM cells alone (difference in mean percentage of control cell growth = −104.8%, 95% CI = −82.1% to −127.5%; $P<.001$). By contrast, the growth of A375SM cells co-cultured with uninfected MSC was enhanced compared with the growth of A375SM cells alone (difference in mean percentage of control cell growth = 60.1%, 95% CI = 28.6% to 91.6%; $P = .031$) (Fig. 1, D and E). These data suggest that MSC-IFN-β cells inhibited the proliferation of these tumor cells and, because these effects were observed *in vitro,* this inhibition did not require additional components of the host immune system.

Effects of Systemically Administered MSC-IFN-β Cells and Recombinant IFN-β on the Growth of MDA 231 Cell–Derived Lung Metastases *In Vivo*

We next examined the *in vivo* antitumor activity of MSC-IFN-β cells by using an SCID mouse xenograft model. We injected MDA 231 cells intravenously into the tail veins of the mice to establish pulmonary metastases. Eight days later, we began treating the mice with intravenous injections of 10^6 MSC-IFN-β cells at weekly intervals for 3 weeks (n = 4). Control mice received either no treatment (n = 4) or intravenous injections of 10^6 MSC-Gal cells at weekly intervals for 3 weeks (n = 4). An additional group of mice (n = 4) was treated with subcutaneous injections of 100 000 IU of recombinant IFN-β every other day from day 8 after tumor cell

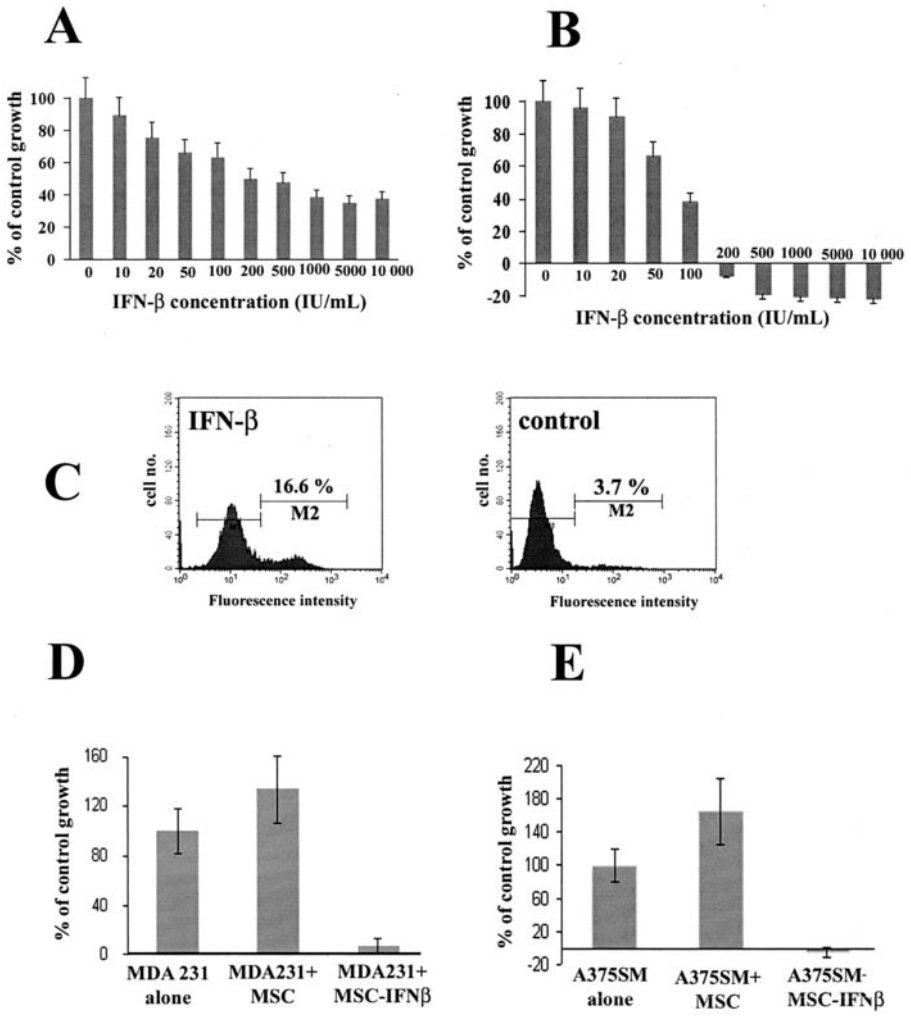

Fig. 1. Effect of recombinant interferon beta (IFN-β) and mesenchymal stem cells expressing human IFN-β (MSC-IFN-β) on proliferation of human breast carcinoma MDA 231 cells and melanoma A375SM cells *in vitro.* MDA 231 cells (**A**) and A375SM cells (**B**) were cultured in the presence of increasing concentrations of IFN-β. The effect of IFN-β is expressed as the percentage of growth for control cells cultured in medium lacking IFN-β. Results are expressed as mean values; **error bars** correspond to 95% confidence intervals. **C**) Induction of A375SM cell death by IFN-β. A375SM cells were cultured for 3 days in medium containing (**left panel**) or lacking (**right panel**) 1000 IU/mL IFN-β. Cell death was quantified by propidium iodide staining and flow cytometry. Propidium iodide–positive cells are in the areas shown by the bars labeled M2. MDA 231 cells (**D**) or A375SM cells (**E**) were co-cultured with human MSC or MSC-IFN-β cells in 10:1 ratio for 5 days. Cells were counted and the relative numbers of aneuploid tumor cells and diploid MSC in the co-cultures were determined by flow cytometry. Results are expressed as the mean percentage of cell growth for MDA 231 cells or A375SM cells that were cultured alone; **error bars** correspond to 95% confidence intervals.

injection until the end of the experiment. A group of healthy mice (n = 4) that received no cell injections served as the referent for measurement of normal lung weight. Thirty days after tumor cell injection, we measured the weight of whole lungs.

The mean lung weight of mice injected with MDA 231 tumor cells was statistically significantly greater than the mean lung weight of healthy mice (mean lung weights: 0.977 g versus 0.413 g; difference = 0.564 g, 95% CI = 0.426 g to 0.702 g, $P = .021$) (Fig. 2, B; Table 1). Much of this weight difference was due to the tumor tissue occupying substantial portions of the lungs of the mice injected with the tumor cells (Fig. 2, A). Therefore, we used whole lung weight as a surrogate endpoint of tumor burden in lungs and to assess efficacy of treatment (Fig. 2, B; Table 1). Mice injected with tumor cells and treated intravenously with MSC-IFN-β cells had statistically significantly smaller lungs than control untreated mice injected with tumor cells only (mean lung weights: 0.408 g versus 0.977 g; difference: −0.569 g, 95% CI= −0.446 g to −0.692 g; $P = .021$). By contrast, the mean weight of lungs from mice treated with recombinant IFN-β was not statistically significantly different from that of untreated control mice injected with tumor cells only (1.09 g versus 0.977 g; difference = 0.113 g, 95% CI = 0.014 to 0.246 g; $P = .083$). The mean lung weight for mice treated with MSC-Gal cells was also not statistically significantly different from that of untreated mice with tumors (1.125 g versus 0.977 g; difference = 0.148 g, 95% CI = −0.019 to 0.315 g; $P = .081$). These findings suggest that intravenous administration of MSC-IFN-β cells inhibits the growth of tumors in lungs, whereas systemically administered recombinant IFN-β (at the dose used) or intravenously injected MSC-Gal cells does not (Table 1).

Effects of MSC-IFN-β, MSC-Gal, and Recombinant IFN-β on Survival of Mice With MDA 231 Breast Carcinomas and A375SM Melanomas

We examined whether the various treatments improved the survival of mice with preestablished pulmonary metastases derived from MDA 231 cells or A375SM cells (Fig. 3, A; Table 2). Mice were treated with recombinant IFN-β, MSC-IFN-β cells, or MSC-Gal cells, as described above and followed until death due to lung tumors. Among mice bearing MDA 231 cell–derived pulmonary metastases, those treated with MSC-IFN-β cells lived statistically significantly longer than untreated mice (median survival: 60 days versus 37 days, difference = 23 days, 95% CI = 14.5 to 34 days; $P<.001$), whereas mice treated with MSC-Gal cells (median survival: 36 days) or with systemically administered IFN-β (median survival: 41 days) did not (difference in median survival: −1 day; $P = .509$ and 4 days; $P = .308$, respectively) (Fig. 3, A; Table 2).

To examine the effects of the various treatments on the survival of mice with preestablished pulmonary metastases de-

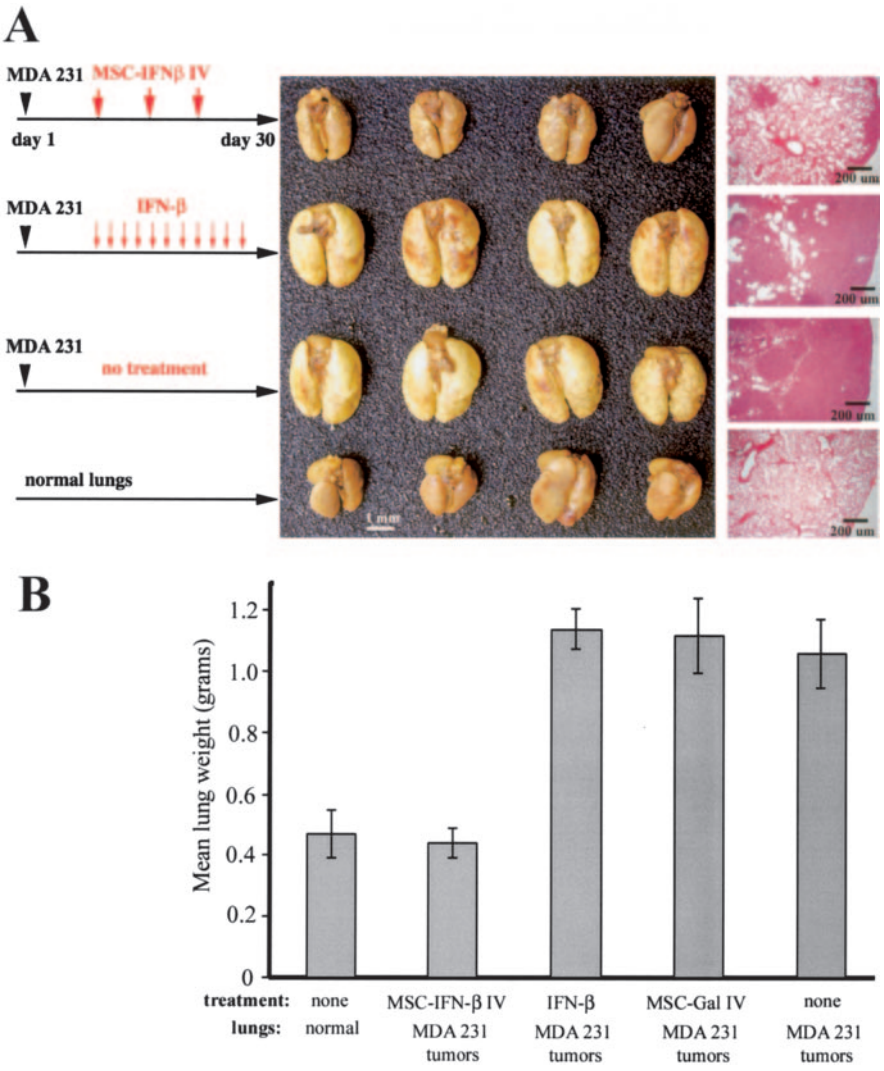

Fig. 2. Effects of systemically administered mesenchymal stem cells expressing human IFN-β cells (MSC-IFN-β) and recombinant IFN-β on the growth of MDA 231 and A375SM lung metastases *in vivo.* **A**) Mice with established MDA 231 pulmonary metastases were treated with three weekly intravenous (IV) injections of MSC-IFN-β cells or MSC expressing β-galactosidase (MSC-Gal). An additional group of mice received subcutaneous injections with 100 000 IU of IFN-β every other day for the duration of the experiment. Control mice with established MDA 231 pulmonary metastases received no treatment. All mice were killed on day 30 after tumor cell injection. Treatment schedules are shown on the **left portion of panel A.** Lungs were weighed and either stained with X-gal (mice injected with MSC-Gal only) or fixed in Bouin's solution, photographed, and examined histologically. All untreated control mice developed large tumors in their lungs that could be easily distinguished from the normal lungs of healthy mice, as shown in **middle and right portion of panel A. B**) Lung weights after treatment with MSC-IFN-β, MSC-Gal, or IFN-β compared with those of untreated mice with MDA 231 metastasis. Lung weights of healthy animals with no tumors are included for comparison. Each group consisted of four mice. The results are expressed as mean values; **error bars** correspond to 95% confidence intervals.

rived from A375SM cells, we injected mice with A375SM cells through the tail vein, and 8 days later, we began daily subcutaneous injections with 40 000 IU of recombinant IFN-β, weekly intravenous injections of 10^6 MSC-Gal cells for 3 weeks, or no treatment. The mice were followed until death and the survival times were compared between the treatment groups by using the log-rank test (Fig. 3, B; Table 3). Among mice bearing A375SM cell–derived pulmonary metastases, those treated with MSC-IFN-β cells survived statistically significantly longer than untreated control mice (median survival: 73.5 days versus 30 days, difference = 43.5 days, 95% CI = 37 to 57.5 days; $P<.001$). By contrast, survival of mice injected with recombinant IFN-β was not statistically significantly different from that of untreated control mice (median survival: 32 days versus 30 days, difference = 2 days, 95% CI = 0 to 4.5 days; $P = .059$) (Table 3).

In this experiment, we also included as a control group mice bearing A375SM cell–derived pulmonary metastases that were subcutaneously injected with MSC-IFN-β cells (n = 5). We wanted to examine whether production of IFN-β at a site distant from the location of the tumor could affect survival. We assumed that intravenously injected MSC-IFN-β cells would be distributed through the circulation to tumors, where they would engraft and affect malignant cell growth through a paracrine

Table 1. Effect of treatment on mouse lung weight*

Tumor cells injected	Treatment (route)	Mean lung weight, g (95% CI)	Difference in mean lung weight, g (95% CI)	*P*	Difference in mean lung weight, g (95% CI)	*P*	Difference in mean lung weight, g (95% CI)	*P*	Difference in mean lung weight, g (95% CI)	*P*
MDA 231	Recombinant IFN-β (SC)	1.09 (1.025 to 1.155)	0.68 (0.64 to 0.72)	.021	0.116 (0.068 to 0.164)	.083	−0.032 (−0.098 to 0.034)	.884	(referent)	
MDA 231	MSC-IFN-β (IV)	0.408 (0.358 to 0.458)	−0.005 (−0.022 to −0.032)	.773	−0.569 (−0.446 to −0.692)	.021	−0.717 (−0.794 to −0.64)	.020	−0.685 (−0.669 to −0.701)	.021
MDA 231	MSC-Gal (IV)	1.125 (1.002 to 1.248)	0.712 (0.707 to 0.717)	.020	0.148 (0.142 to 0.152)	.081	(referent)			
MDA 231	None	0.977 (0.847 to 1.107)	0.564 (0.426 to 0.702)	.021	(referent)					
None	None	0.413 (0.236 to 0.590)	(referent)							

*The Kruskal–Wallis nonparametric test was used to assess the overall difference of lung weights. Because the Kruskal–Wallis test was statistically significant ($P = .004$), the Wilcoxon rank-sum test was used to perform pairwise comparisons of treatment effect on lung weight between groups of mice. CI = confidence interval; IFN-β = interferon beta; SC = subcutaneous injection; MSC = mesenchymal stem cells; IV = intravenous injection; Gal = β-galactosidase.

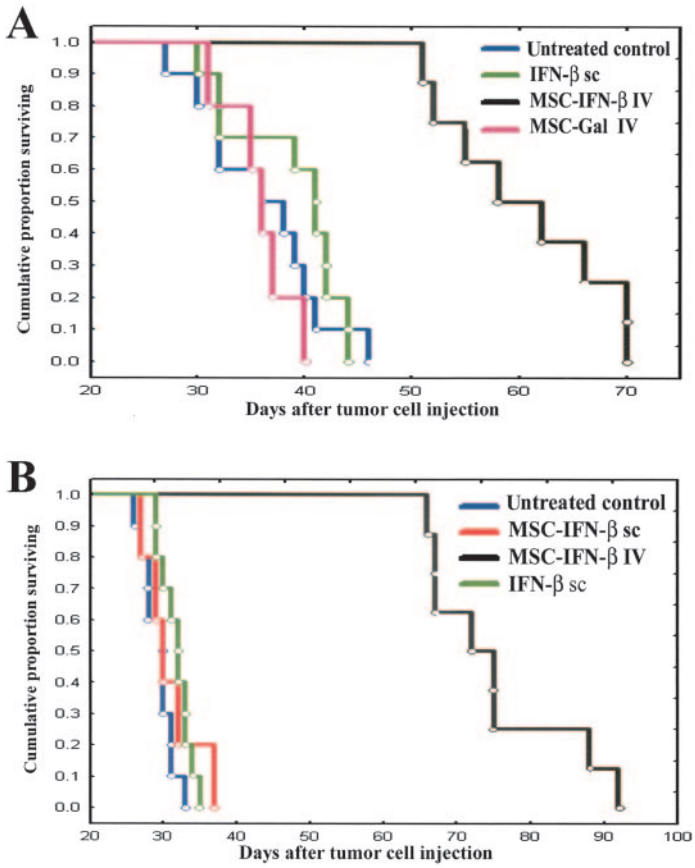

Fig. 3. Survival analysis. **A**) Survival of mice with established pulmonary metastases of MDA 231 carcinoma intravenously injected with three doses of 10^6 mesenchymal stem cells expressing human IFN-β (MSC-IFN-β) IV [n = 8]) or MSC-Gal (MSC-Gal IV [n = 5]). An additional group of mice received subcutaneous (SC) injections with 100 000 IU of IFN-β every other day for the duration of the experiment (IFN-β SC [n = 10]). Control mice with established pulmonary metastases derived from MDA 231 cells received no treatment (untreated control [n = 10]). **B**) Survival of mice with established pulmonary metastasis of A375SM melanoma intravenously injected with three doses of 10^6 MSC-IFN-β (MSC-IFN-β IV [n = 8]), subcutaneously injected with three doses of 10^6 MSC-IFN-β (MSC-IFN-β SC [n = 5]), or subcutaneously injected daily with 40 000 IU of IFN-β (IFN-β SC [n = 10]). Control mice with A375SM pulmonary metastases received no treatment (untreated control [n = 10]). Survival was measured from the day of MDA 231 cell or A375SM cell injection until the day of death.

mechanism of locally produced IFN-β. By contrast, subcutaneously injected MSC-IFN-β cells would reside only at the injection site and affect tumors by producing IFN-β systemically released into the circulation. The survival of mice treated with subcutaneously injected MSC-IFN-β was not statistically significantly different from that of untreated control mice (median survival: 30 days versus 30 days, difference = 0 days, 95% CI = −3 to 4 days; $P = .557$). Mice treated with intravenously injected MSC-IFN-β lived statistically significantly longer than mice treated with subcutaneously injected MSC-IFN-β (median survival: 73.5 days versus 30 days, difference = 43.5 days, 95% CI = 35 to 56 days; $P < .001$). Our results indicate that intravenously injected MSC-IFN-β but not subcutaneously injected MSC-IFN-β prolonged survival, supporting our hypothesis that the local production of IFN-β in tumors is critical for the observed effect to occur.

Table 2. Effect of treatment on the survival of mice bearing MDA 231 pulmonary metastases*

Treatment group	Median survival, days (95% CI)	*P*	*P*	*P*
MSC-Gal IV	36 (37 to 42)	.509	.051	(referent)
MSC-IFN-β IV	60 (52 to 70)	<.001	<.001	.001
IFN-β SC	41 (32 to 42)	.308	(referent)	
Untreated	37 (32 to 40)	(referent)		

*The log-rank test was used to assess the statistical significance of differences in survival among the four treatment groups. Because this overall test was statistically significant for both tumor models ($P < .001$), pairwise log-rank tests were performed. CI = confidence interval; MSC = mesenchymal stem cells; Gal = β-galactosidase; IV = intravenous injection; IFN-β = interferon beta; SC = subcutaneous injection.

MSC Engraftment *In Vivo*

To confirm that injected MSC engrafted in tumors, we intravenously injected mice that did or did not carry MDA 231 xenograft tumors with three weekly doses of 10^6 MSC-Gal cells and traced the progeny of those cells by histochemical staining of mouse lungs and other organs for β-gal. Histochemical staining was performed 14 days after the last doses of MSC-Gal cells were administered; representative images are shown in Fig. 4. We observed numerous X-gal–positive cells in MDA 231 pulmonary tumors (Fig. 4, B). These cells had formed colonies (average of 4 colonies per section, 95% CI = 2 to 6 colonies) and were incorporated into the tumor architecture (Fig. 4, B), suggesting that MSC can reach the extravascular space and contribute to the development of tumor connective stroma. We suspect that each X-gal–positive colony originated from a single (or very few) MSC that proliferated *in situ,* presumably under the influence of signals from the surrounding microenvironment *(16).*

By contrast, we found that healthy mice (i.e., mice not injected with MDA 231 cells) that were injected intravenously with MSC-Gal cells had only very rare single X-gal–positive cells (average of 0.4 cell per section, 95% CI = 0 to 0.8 cell) scattered in their lungs (Fig. 4, C). These X-gal–positive cells showed no signs of proliferation or integration into the normal lung parenchyma. No other examined organs (i.e., liver, spleen, kidney, or muscle) from either group of mice showed signs of MSC-Gal cell engraftment (Fig. 4, D), and only very rare single X-gal–positive cells (average of 2 cells per section, 95% CI = 0 to 4 cells) were observed in the liver. These results suggest that

Table 3. Effects of different treatments on survival of mice with A375SM metastasis in lungs*

Treatment group	Median survival, days (95% CI)	*P*	*P*	*P*
MSC-IFN-β IV	73.5 (67 to 88)	<.001	<.001	<.001
IFN-β SC	32 (30 to 33)	.059	.424	(referent)
MSC-IFN-β SC	30 (27 to 33)	.557	(referent)	
Untreated	30 (28 to 31)	(referent)		

*The log-rank test was used to assess the statistical significance of differences in survival among the four treatment groups. Because this overall test was statistically significant for both tumor models ($P < .001$), pairwise log-rank tests were performed. CI = confidence interval; MSC = mesenchymal stem cells; IFN-β = interferon beta; IV = intravenous injection; SC = subcutaneous injection.

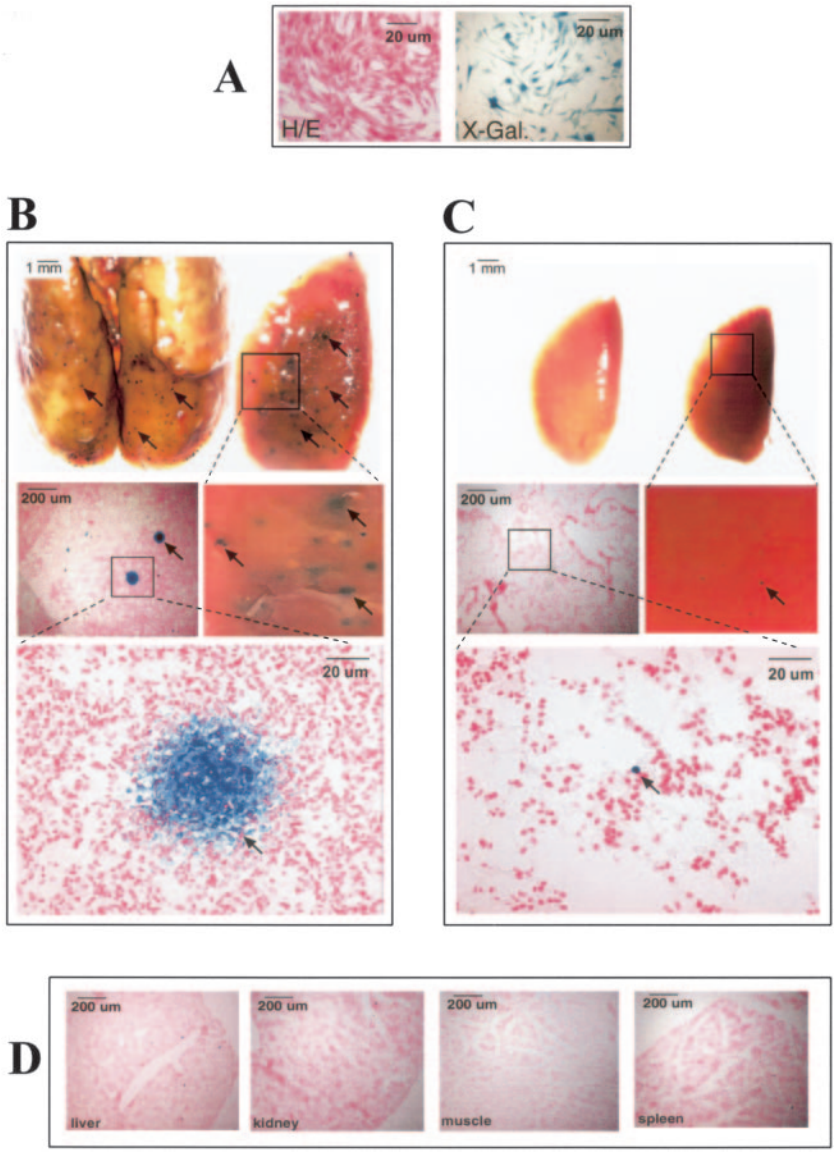

Fig. 4. Selective engraftment of mesenchymal stem cells (MSC)-Gal in MDA 231 pulmonary metastases. **A**) MSC transduced with β-galactosidase–expressing adenoviral vector (MSC-Gal) were cultured *in vitro* and stained with hematoxylin–eosin (H/E; **left panel**) or for β-galactosidase with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; **right panel**). Three weekly doses of 10^6 MSC-Gal were injected intravenously into mice with established MDA 231 pulmonary metastases (**B** [n = 5]) or into healthy mice (**C** [n =5]).The mice were killed 14 days after the last dose was administered, and their lungs, livers, kidneys, spleens, and muscle tissue were removed. Whole lungs were first stained with X-gal (**top panels of B and C**) and then sectioned and mounted on slides (**lower panels of B and C**). **D**) Livers, kidneys, spleens, and muscles were sectioned, mounted on slides, and stained with X-gal. We examined 10 sections from each organ. **B**) MDA 231 tumors in lungs contained numerous colonies of X-gal–positive cells (**arrows**). **C**) Very few single X-gal–positive cells were detected in normal lung from healthy mice (**arrows**). **D**) X-gal–positive cells were not detected in spleens, kidneys, or muscles of mice with lung tumors. A few X-gal–positive cells were observed in the liver of mice with lung tumors.

the tumor microenvironment plays a critical role in the successful engraftment and proliferation of MSC *in vivo.*

Plasma Levels of IFN-β After Administration of Recombinant IFN-β or MSC-IFN-β Cells to Mice

The activity of human IFN-β is species-specific; thus, human IFN-β does not substantially affect murine cells *(28).* Accordingly, the clinically relevant dose of human IFN-β in mouse xenograft models cannot be determined on the basis of drug tolerance in mice. However, because IFN-β toxicity in patients is associated with high IFN-β levels in serum *(11,12),* it is possible that serum concentrations of IFN-β in mice could serve as a surrogate marker of the toxicity that might be expected in humans. We therefore examined plasma levels of IFN-β in mice with MDA 231 cell–derived pulmonary metastases at various times after the mice had received subcutaneous injections of recombinant human IFN-β. We found that mice injected with 40 000 IU of recombinant human IFN-β had mean serum IFN-β levels of 156 IU/mL (95% CI = 128 to184 IU/mL) after 2 hours, 29 IU/mL (95% CI = 11.5 to 46.5 IU/mL) after 6 hours, and 0 IU/mL (95% CI = 0 to 0 IU/mL) after 24 hours. Mice injected with 100 000 IU of recombinant human IFN-β had mean serum IFN-β levels of 192 IU/mL (95% CI = 152 to 232 IU/mL) after 2 hours, 61 IU/mL (95% CI = 0 to128 IU/mL) after 6 hours, and 16 IU/mL (95% CI = 0 to 40 IU/mL) after 24 hours (Table 4). We also examined serum levels of human IFN-β in mice after a single subcutaneous or intravenous injection of 10^6 MSC-IFN-β cells. Subcutaneously injected MSC-IFN-β resulted in mean serum IFN-β levels of 47.2 IU/mL (95% CI = 21.7 to 72.7 IU/mL) after 1 day, 7.7 IU/mL (95% CI = 1.0 to 14.3 IU/mL) after 3 days, and 1.4 IU/mL (95% CI = 0.6 to 2.1 IU/mL) after 6 days. Intravenous injection of MSC-IFN-β resulted in mean serum IFN-β levels of 14 IU/mL (95% CI = 4.8 to 24.3 IU/mL) after 1 day, −0.48 IU/mL (95% CI = −0.5 to 1.4 IU/mL) after 3 days, and 1.0 IU/mL (95% CI = 0.1 to 2.0 IU/mL) after 6 days (Table 4). Mean serum IFN-β levels in mice injected subcutaneously with MSC-IFN-β were not statistically significantly different from those in mice injected intravenously with MSC-IFN-β at 1 day (difference = 32.6 IU/mL, 95% CI = 5.32 to 59.9 IU/mL; $P = .07$), at 3 days (difference = 7.2 IU/mL, 95% CI = 0.5 to 13.9 IU/mL; $P = .1$), or at 6 days (difference = 0.33 IU/mL, 95% CI = −0.84 to 1.5 IU/mL; $P = .81$) after cell

Table 4. IFN-β concentration in mouse plasma after administration of recombinant human IFN-β or MSC-IFN-β cells*

Treatment	Dose	Route	Mean IFN-β concentration (95% CI), IU/mL		
Recombinant IFN-β			***After 2 hours***	***After 6 hours***	***After 24 hours***
	40 000 IU	SC	156 (128 to 184)	29 (11.5 to 46.5)	0 (0 to 0)
	100 000 IU	SC	192 (152 to 232)	61 (0 to 128)	16 (0 to 40)
MSC-IFN-β cells			***After 1 day***	***After 3 days***	***After 6 days***
	10^6 cells	SC	47 (21.7 to 72.7)	7.7 (1.05 to 14.3)	1.4 (0.6 to 2.1)
	10^6 cells	IV	14 (4.8 to 24.8)	0.5 (−0.5 to 1.4)	1.0 (0.1 to 2.0)

*Mice (five animals per group) with established MDA 231 tumors in lungs were injected subcutaneously (SC) with recombinant interferon beta (IFN-β) or subcutaneously or intravenously (IV) with mesenchymal stem cells expressing human IFN-β (MSC-IFN-β) cells. Each mouse received a single injection. Plasma concentrations of IFN-β were measured by an enzyme-linked immunosorbent assay for all mice at the indicated times after treatment. The mean plasma concentration of human IFN-β for mice that received no treatment (n = 5) was 0.4 IU/mL (95% CI = 0 to 1.2 IU/mL).

administration. However, given our finding that intravenously injected but not subcutaneously injected MSC-IFN-β inhibited A375SM tumor growth in lung (Fig. 3, B; Table 3), we conclude that the observed antitumor effect of intravenously injected MSC-IFN-β is not associated with an increase of IFN-β levels in serum. Overall, these results provide further evidence that intravenously injected MSC-IFN-β cells inhibit the growth of malignant cells by local production of IFN-β in the tumor microenvironment and that this effect occurs even though systemic plasma concentrations of IFN-β are low.

Discussion

We showed that recombinant IFN-β as well as MSC that expressed IFN-β (MSC-IFN-β cells) directly inhibited proliferation of human tumor cells *in vitro.* Importantly, MSC-IFN-β cells that produced IFN-β locally in the tumor microenvironment inhibited malignant cell growth *in vivo,* whereas systemically administered recombinant IFN-β did not. We found that inhibition of tumor cell growth by MSC-IFN-β cells (given as three weekly doses) was not permanent. This lack of a sustained effect likely reflects the fact that adenoviral transgenes do not integrate into the genomes of transduced MSC and that transgene copy number per cell declines as MSC-IFN-β cells proliferate in tumors. Sustained inhibition of tumor cell proliferation may be achievable through the use of MSC that are stably transfected with a plasmid that expresses IFN-β under the control of conditional promoter.

Our results suggest that the antitumor activity of MSC-IFN-β cells is associated not with the serum concentration of IFN-β but rather with the engraftment of MSC in tumors and the local effects of the IFN-β they produce on malignant cells. Therefore, selective engraftment of MSC in tumors is an important issue for further clinical development of this method. We did not observe engraftment of intravenously administered MSC in any of the healthy organs we examined (i.e., lung, liver, spleen, kidney, and muscle). These findings are consistent with earlier reports that systemically administered MSC poorly engraft in healthy tissues *(32).* Jiang et al. *(33)* have reported extensive engraftment of multipotent adult progenitor cells (MAPC), which are similar to and can copurify with MSC. However, MAPC differ from the MSC used in our study. MAPC can differentiate into a wide range of epithelial cell types and can engraft in lung, skin, blood, gut, and liver *(33).* By contrast, the differentiation potential of MSC is restricted to cells with mesodermal or connective tissue phenotypes: osteoblasts, chondroblasts, adipocytes, myocytes, and fibroblasts *(17,18).* This limited differentiation potential of MSC likely prevents them from engrafting in epithelial tissues, which have high spontaneous cell turnover. Future clinical trials of this method should consider the possibility that MSC may engraft at sites of tissue injury and thus exclude patients who have undergone surgery or invasive procedures or have had infections *(23).* In addition, the selectivity of MSC for other cancer metastases will require careful investigation.

Even if MSC-IFN-β cells engraft selectively in tumors, the IFN-β they produce could still be released into the circulation and contribute to toxicity by affecting other organs. In this regard, we demonstrated that intravenous administration of MSC-IFN-β cells results in a peak mean IFN-β serum level of 14.0 IU/mL. This value is similar to the mean (± standard deviation) maximal IFN-β serum concentration of 12.3 ± 3.9 IU/mL reported in humans after subcutaneous administration of IFN-β at the maximally tolerated dose of 18×10^6 IU *(11,12).* Therefore, intravenous administration of MSC-IFN-β at a dose that inhibits malignant cell growth may also be tolerated in a clinical setting. These findings are again consistent with the known rapid metabolism and short half-life of IFN-β in circulation and support its physiologic role as a paracrine regulator of cell fate that acts locally in tissues.

It has been postulated that IFN-β is an important inhibitor of cell growth during development *(31,34)* and oncogenesis *(35).* IFN-β can reverse the oncogenic potential of transformed tumorigenic cells in experimental systems *(36,37),* and recent data have also suggested that IFN-β has a role in the regulation of apoptosis in malignant cells *(3–5).* Although the molecular mechanisms that are responsible for growth inhibition and elimination of tumor cells by IFN-β await clarification, results of numerous studies have implicated a variety of IFN-regulated genes, such as members of the interferon regulatory factory gene family, PKR, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L, in these processes *(3–5,38).* Several reports have suggested that abnormally low expression of IFN-β in the tissues surrounding certain tumors contributes to unregulated cell growth and malignant potential *in vivo (39,40).* Therefore, therapy with MSC-IFN-β cells could be used to increase IFN-β expression in tumors and surrounding tissues and to control the growth of malignant cells. In fact, the potential clinical application of MSC-IFN-β cells could be broad, because many types of malignancies are sensitive to the antiproliferative or proapoptotic effects of IFN-β *in vitro (1–3,5).* Moreover,

MSC may be effective carriers for other biologic molecules, such as the tumor necrosis factor family of proteins *(41)* and anti-angiogenic molecules *(42)*. These agents have shown potential promise as cancer therapies in many experimental systems, but their clinical application has suffered from pharmacologic limitations similar to those seen for IFN *(41,42)*.

Overall, we have demonstrated that MSC engrafted in tumors may act as precursors for stromal cells and can serve as cellular vehicles for the delivery and local production of biologic agents. This approach may overcome the extensive metabolism and toxicity associated with some biologic agents and could serve as a versatile tool for manipulating the extracellular milieu of malignant cells.

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NOTES

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