Development of sarcomas in mice implanted with mesenchymal stem cells seeded onto bioscaffolds

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Bone marrow-derived mesenchymal stem cells (MSCs) are precursors of bone, cartilage and fat tissue. MSC can also regulate the immune response. For these properties, they are tested in clinical trials for tissue repair in combination with bioscaffolds or injected as cell suspension for immunsuppressant therapy. Experimental data, however, indicate that MSC can undergo or induce a tumorigenic process in determined circumstances. We used a modified model of ectopic bone formation in mice by subcutaneously implanting porous ceramic seeded with murine MSC. In this new model, host-derived sarcomas developed when we implanted MSC/bioscaffold constructs into syngeneic and immunodeficient recipients, but not in allogeneic hosts or when MSC were injected as cell suspensions. The bioscaffold provided a three-dimensional support for MSC to aggregate, thus producing the stimulus for triggering the process eventually leading to the transformation of surrounding cells and creating a surrogate tumor stroma. The chemical and physical characteristics of the bioscaffold did not affect tumor formation; sarcomas developed either when a stiff porous ceramic was used or when the scaffold was a smooth collagen sponge. The immunoregulatory function of MSC contributed to tumor development. Implanted MSC expanded clones of CD4+CD25+ T regulatory lymphocytes that suppressed host's antitumor immune response.

Introduction

Tumor formation is a complex event involving the uncontrolled proliferation of cells and the failure of the antitumor immune response. Tumors are formed when embryonic stem cells are injected in recipient animals (1). It has been reported that also adult stem cells could trigger tumor formation. In particular, human bone marrow-derived mesenchymal stem cells (MSCs) have been shown to favor tumor growth by promoting neangiogenesis (2) and preventing tumor recognition by the immune system (3). They also induce the metastasis of breast cancer cells constituting a stroma delivering a continuous tumorigenic stimulus for metastasis of breast cancer cells (4). On the contrary, other studies have observed that MSC inhibits tumor growth in murine (5) and rat models (6,7).

Abbreviations: GFP-Tg, green fluorescent protein transgenic; FISH, fluorescence in situ hybridization; HA, hydroxypatite; MSC, mesenchymal stem cell; s.c., subcutaneously; Treg, regulatory T lymphocyte.
Surgical procedures

A highly porous ceramic support based on 100% hydroxyapatite (HA) with a Ca/P ratio of 1.66 ± 0.5 and 80 ± 5% porosity (Ensi Pore) (FinCeramica, Faenza, Italy) and a cross-linked collagen sponge (Cynamate biomateriaux, Chaponost, France) were used as bioscaffolds.

After 3 or 4 weeks expansion, MSCs were detached from the dishes with 0.05% (w/v) trypsin in enzyme dilution buffer washed in serum-free medium and resuspended at 2.5 × 10^5 cells per 20 μl fibrinogen (2.5 mg/ml) (Baxter, Milano, Italy). Each aliquot of MSC was then applied on the opposite faces of a heat-sterilized (180°C for 8 h) HA block or collagen sponge. Twenty microliters of thrombin (300 IU/ml) (Baxter) was applied to each scaffold.

After anesthesia, recipient Balb/c, C57Bl/6 and nude mice were subcutaneously implanted with MSC/bioscaffold constructs. More than five animals per group were used for each experiment, repeating experiments at least three times. Similar experiments were performed using MSC derived only from male C57Bl/6 mice and implanted into female C57Bl/6 recipients.

The tumorigenic potential of primary tumors grown in immunocompromised or syngeneic mice was assessed in 4-week-old nude mice. All animals were maintained in a sterile environment. Briefly, parts of primary tumors of ~2 mm of diameter were explanted in sterile conditions from immunocompromised and syngeneic recipients, s.c. implanted into the left flank of five nude mice and observed for 12 weeks. We performed three serial in vivo transplantation passages.

Animals were killed when the growth of a tumor mass was incompatible with the mouse well-being, usually 4 weeks after implantation of MSC/collagen sponge constructs and 3 weeks after implantation of MSC/HA combinations.

As control experiments, 1 × 10^5 MSCs isolated from GFP-Tg mice were s.c. injected as a cell suspension on the back of three nude mice and observed for 12 weeks. Furthermore, 2.5 × 10^5 MSCs isolated from GFP-Tg mice were s.c. injected as a cell suspension on the back of six nude mice and observed for 12 weeks.

In vitro assessment of cell tumorigenic potential

MSC and sarcoma cells were cultured in Coon’s modified F12 medium (Biochrom AG) supplemented with 2 mM L-glutamine, 50 μg/ml penicillin/ streptomycin and 1 ng/ml basic fibroblast growth factor (serum-free medium). As a control, cells were cultured in standard medium. After 8 weeks, plates were washed, fixed with 1% formaldehyde and stained with 1% methylene blue.

Cellular potential for invasiveness of MSC and sarcoma cells was determined using six-well transwell system with a membrane coated with matrigel. Briefly, sarcoma cells and MSCs were seeded into the upper inserts of the chamber coated with 25 μg of matrigel at 2 × 10^5 per insert in serum-free F12 medium. Outer wells were filled with F12 containing 10% fetal calf serum (GIBCO, S.Giuliano Milanese), used as chemoattractant. Cells were incubated at 5% CO2 for 24 h and then non-invasive cells were removed by swabbing top layer. Membranes containing invading cells were stained with hematoxylin for 3 min, washed and mounted on slides. Invading cells contained in the membranes were counted under light microscope at ×32 objective, using a graticule and performing three different random counts per membrane.

Anchorage-independent growth ability of cells was evaluated by a soft agar colony formation assay. Briefly, 1 × 10^5 MG-63 cells, 1 × 10^5 sarcoma cells derived from biopsies obtained from primary tumors grown in immunocompromised or syngeneic recipients and 1 × 10^5 MSC were plated on a 0.6% agarose base in six-well plates in 1 ml of standard medium containing 0.3% agarose. After staining with 0.005% crystal violet (Sigma Chemical Co., St Louis, MO), colonies >50 μm were counted 21 days after plating.

Histological analysis and immunohistochemistry

For histological examination, tumors were surgically removed and fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. Formalin-fixed HA blocks were decalcified. Four micrometer serial sections were stained with hematoxylin and eosin or processed for immunohistochemistry. After fixation, antigen retrieval was heat induced in citrate buffer pH 6.0 and endogenous peroxidases were blocked with 3% H2O2 in water. Non-specific binding was inhibited, by incubating the slides in 10% normal goat serum (Sigma–Aldrich, Milano, Italy). The following primary antibodies were used: rabbit polyclonal anti-GFP antibody (Molecular Probes Europe BV, Leiden, The Netherlands), mouse monoclonal anti-vimentin (clone V9), mouse monoclonal anti-smooth muscle actin (clone 1A4), rabbit anti-desmin (Dako Cytomation, Milano, Italy), rabbit anti-CD14 (Abcam, Cambridge, UK), rat monoclonal anti-mouse CD34 (clone MEC 13.3) (kindly provided by Dr P. Castellani) and rat monoclonal anti-mouse F4/80 (clone A3–1) (Abd Serotec, Milano, Italy).

Negative controls with preimmune serum and positive controls were run in parallel. As a control for the detection of GFP-positive MSC, we performed additional reactions on sections of GFP-Tg (positive controls) or C57Bl/6 (negative controls) mouse tissues.

After extensive washing in Tris-buffered saline, slides were incubated with either biotin-conjugated goat anti-rabbit or anti-mouse (BiSpa, Milano, Italy). After washing, horseradish peroxidase-conjugated streptavidin (BiSpa) was added and incubated for 10 min. Slides were then stained with diamobenzidine chromogen (Lab Vision, Fremont, CA). Counterstaining was performed with hematoxylin. Images were captured by transmitted light microscopy with an Olympus C3030 digital camera and Camedia Master Olympus software.

Fluorescence in situ hybridization analysis

Interphase fluorescence in situ hybridization (FISH) analysis was performed on 4 μm thick slides cut from paraffin blocks. The presence of tumor cells was evaluated in each tissue block on hematoxylin and eosin-stained slides cut before and after the section used for FISH analysis. FISH experiments were carried out as described previously (13) using the a rbinding DNA probe CY3 labeled specific for mouse Y and chromosome painting DNA probe fluorescein isothiocyanate labeled specific for mouse X chromosome (Cambio, Cambridge, UK). Interphase FISH analysis was also performed on isolated nuclei from 15 μm sections (14). More than 50 nuclei from at least five to eight different areas selected for well-preserved cellular and nuclear morphology were examined in each case to ensure representative samples and to avoid a nuclear truncation in paraffin-embedded sections. Only experiments with 90% hybridization efficiency were considered. Histological sections of male mouse tissues were used as technical controls. FISH analysis was performed as recommended by the International System for Human Cytogenetic Nomenclature (15).

In vitro expansion of CD4+CD25+ T regulatory cells

CD4+CD25+ and CD4+CD25– T lymphocytes were isolated from naïve C57Bl/6 mice and were plated at a density of 1 × 10^6 cells in the upper insert of a six-well transwell system (Corning B.V., Schiphol-Rijk, The Netherlands). The lower part of the system was filled with 1 × 10^5 MSC and cultured in presence or absence of 0.4 μM phytohemagglutinin or 1 × 10^5 sarcoma cells. The cultures were maintained for 10 days at 37°C, 5% CO2. After 10 days, at the end of the experiment, cells cultured in the upper inserts were harvested, counted, examined by flow cytometry and used in proliferation assays.

Proliferation assay

We tested proliferation of splenocytes derived from sarcoma-bearing mice to stimulation with 0.2 μM of a mitogenic stimulus, phytohemagglutinin (Sigma), with 5 × 10^5 γ-irradiated syngeneic or allogeneic splenocytes and γ-irradiated syngeneic tumor cells, in the presence or absence of γ-irradiated syngeneic Tregs.

We plated 5 × 10^5 responder cells per well in round-bottomed 96-well plates (Corning BW Lifescience, Pero, Milano, Italy) in proliferation assays. Cultures were incubated for 72 h and pulsed with [3H] thymidine (1.0 μCi per well) (Amersham Biosciences, Milano, Italy) for at least 16 h. Cells were harvested and cell proliferation was evaluated by counting thymidine uptake and the averaged proliferation rate was measured as counts per minute.

In another experimental setting, we tested proliferation of splenocytes derived from mice bearing sarcomas to stimulation with 5 × 10^5 γ-irradiated syngeneic sarcoma cells, in presence or absence of 5 × 10^5 γ-irradiated Tregs in vitro expanded as described previously.

Statistical analysis

Statistical significance of observed differences of tumor formation after in vivo implants among different experimental groups of mice were calculated by Fisher’s exact test. A two-tailed t-test was used to evaluate statistical significance of observed results of the invasion assay, in vitro expansion of CD4+CD25+ Tregs and cell proliferation assays. P-values <0.05 were considered to be statistically significant.

Results

Characterization of MSC

MSC expressed major histocompatibility complex (MHC) class I molecules (average 96% ± 3 SD), but not MHC class II antigens (Figure 1). A large majority of MSC constitutively expressed CD44 (average 97% ± 3 SD), CD106 (average 81% ± 4 SD) and CD140a (platelet-derived growth factor receptor α) (average 96% ± 2 SD) (Figure 1), considered to be markers of progenitor cells of the mesenchymal lineages. The expression of CD45 was always <1%, indicating the absence of contaminating myeloid cells. CD34 was expressed in an average of 21% ± 9 SD. Less than 1% of MSC expressed CD11b.
structs were undifferentiated sarcomas histologically similar to tumors.

In allogeneic mice degenerated into tumors (Fig. 2A). We observed the formation of a tumor in 10/12 (83%) syngeneic recipients and 8/10 (80%) nude mice, whereas none (0/20) of the allogeneic recipients developed a tumor (P < 0.0001) comparing the number of tumors formed in syngeneic or nude animals with the number of tumors formed in allogeneic recipients) (Fig. 2A).

Tumors originated from the implants of MSC/collagen sponges constructed s.c. into syngeneic, immunocompromised (nude) or allogeneic recipients. Syngeneic and nude recipients were not expected to mount an allospecific immune response to the grafted cells, whereas implants into allogeneic, genetically mismatched and not immunosuppressed recipients could elicit a strong immune response. As a control experiment, we injected MSC as cell suspensions s.c. on the back of immunodeficient recipient mice.

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Tumors were defined as undifferentiated sarcomas positive for vimentin and actin and smooth muscle actin (Fig. 2C). Newly formed vessels were evident by a positive staining with anti-CD31 monoclonal antibody (Fig. 2E). Negative and positive controls of immunohistochemistry can be found online in the supplementary Figure 1 (available at Carcinogenesis Online).

Origin of the tumors

To test whether tumor formation was due to the presence of tumorigenic cells in the initial population of MSC and under the assumption that only transformed and immortalized cells can survive and proliferate when cultured in absence of serum, we cultured them in media not supplemented with serum (starvation condition). As a result, we observed that the initial MSC populations did not survive when cultured under starvation conditions for 8 weeks (Fig. 4A). Moreover, cancer cells can infiltrate the surrounding tissues and this property can be assessed when checking the ability of the cells to migrate through pores of the matrigel-coated culture dishes. We found that no cells of the original MSC culture could pass through the matrigel coat, whereas an average of 69% ± 0.04 SD of the cultures were occupied by tumor-derived cells (P < 0.0001) (Fig. 4B). Furthermore, we examined the anchorage-independent colony-forming capacity in soft agar of MSC, sarcoma cells derived from biopsies obtained from primary tumors grown in immunocompromised or syngeneic recipients, and MG-63 cells, a cell line derived from a human osteosarcoma, used as a positive control. After 21 days of culture, both sarcoma cells obtained from primary tumors grown in immunocompromised or syngeneic recipients were able to form colonies of a diameter >50 μm (averaged colony number 24 ± 5 SD and 31 ± 5 SD, respectively) (Fig. 4C). However, they grew with a reduced efficiency if compared with the positive control MG-63 cell line (averaged colony number of tumors formed in syngeneic or nude hosts where they formed new sarcomas histologically similar to the primary tumor (supplementary Figure 2 is available at Carcinogenesis Online). However, the latency time decreased after each in vivo passage; tumor masses were evident ~2 weeks after the first reimplantation passage and 1 week after the second and the third passage.

Tumor development was not observed in any of the allogeneic not immunosuppressed recipient (Fig. 2A), and the exogenous MSC seeded onto the bioscaffold appeared acutely rejected by the second week after implantation (R.Tasso, A.Augello, S.Boccardo, S.Salvi, M.Carida, F.Postiglione, F.Fais, M.Truini, R.Cancedda and G.Pennesi, submitted for publication) (Fig. 3).

Tumor development was not observed in mice receiving MSC as cell suspensions.

Fig. 1. Phenotype of MSC population. Flow cytometric analysis of MSC derived from C57Bl/6 mice and harvested after one in vitro passage. Gray areas represent expression of a specific marker on tested cell populations. The uncolored areas under the black line serve as negative control (cells stained with isotype antibody).

Shown are the results of a representative experiment.
number 58 ± 6 SD) (tumor cells versus MG-63 P < 0.0001) (Figure 4C). As expected, no MSC culture was able to form colonies in soft agar (Figure 4C). Taken together, these results tended to exclude the presence of tumorigenic cells within the MSC population prior seeding and implantation.

Using MSC isolated from GFP-Tg mice, we could determine that sarcomas contained a few clusters of GFP-positive cells of donor origin scattered throughout a mass of GFP-negative cells originated from the recipient’s tissues (Figure 5A). To further confirm that tumors stemmed from the recipient’s tissues, we implanted MSC from male donors seeded onto a bioscaffold into female recipients, sorting the origin of the tumor cells using dual-color FISH with specific probes for the murine Y (labeled in red) and X chromosomes (labeled in green). We analyzed all the sarcoma cells on histological sections and in addition 400 extracted nuclei obtained from 15 μm sections. In all the analyzed nuclei no Y sequences were found. Two chromosome

Fig. 2. Incidence and features of tumors induced by MSC loaded onto bioscaffolds. (A) Histograms show the incidence of tumors induced by MSC/HA implants (left) or by MSC/collagen sponge implants (right) grafted into allogeneic, syngeneic and nude recipients. (B) Gross examination of sarcomas. The left panel shows a representative image of the tumor external appearance, and the right panel shows the internal part of the tumor including massive areas of necrosis (arrowhead) near the HA bioscaffold (asterisk), that is integrated inside the tumor mass. (C) Figure shows a massive angiogenetic process in MSC/collagen combinations implanted in syngeneic recipients at 2 weeks post-implantation (left panel) and the absence of a substantial vascularization in the MSC/collagen constructs implanted into allogeneic recipients at 2 weeks post-implantation (right panel). (D) (left upper panel) Hematoxylin and eosin (H&E) staining of a sarcoma developed in syngeneic recipients (magnification ×500). The insert shows a higher enlargement of the same slide displaying mitotic figures (magnification ×1000); (right upper panel) representative images of sarcomas derived from syngeneic implanted mice immunohistochemically stained with anti-vimentin antibody (magnification ×500); (left bottom panel) with anti-desmin (magnification ×500) and (right bottom panel) anti-α-smooth muscle actin (magnification ×1000). In this latter staining, tumor is negative but the entrapped vessel offers an internal positive control. (E) Anti-CD31 immunohistochemical staining of a sarcoma grown in a syngeneic recipient showing the presence of newly formed vessels (magnification ×20).

Fig. 3. Acute rejection of MSC/porous ceramic combinations grafted in allogeneic immunocompetent recipient. Shown is the histological appearance of grafts of MSC/HA combinations in syngeneic and allogeneic recipients examined at 2 weeks post-implantation. Fibroblastoid cells and new vessels invaded the pores of the scaffold in syngeneic implants (left panel), whereas only necrotic cells are evident within the scaffold of the allogeneic combination (right panel) (hematoxylin and eosin staining; magnification ×20).
X was observed in 57% of cells, whereas aneuploidies of X chromosome were identified in the remaining 43% of tumor cells. These FISH results demonstrated the female origin (then the recipient origin) of tumor cells (Figure 5B) (18). Interestingly, the sarcoma cells showed chromosome instability that is a peculiar feature of undifferentiated solid tumors. A healthy tissue surrounded the sarcoma; composed by small cells inside of an abundant stroma were Y negative and euploid (Figure 5A). The same distribution of CD14+ cells was observed also at later stages of tumor growth, 8–9 weeks after implantation (data not shown). A subpopulation of macrophages expressing the marker F4/80 could promote tumor growth by suppressing host immune reaction (20). To test this possibility, we checked the presence of F4/80+ cells within the early and late stages of tumors. Immunohistochemistry revealed the absence of mononuclear myeloid-derived suppressor cells at the early stage of cancer formation (Figure 5D) and at later stages (data not shown). Positive controls of immunohistochemistry can be found online in the supplementary Figure 3 (available at Carcinogenesis Online).

**Tumor immune escape**

Splenocytes of tumor-bearing mice actively proliferated when in vitro challenged with cells from the autologous tumor in a mixed lymphocyte reaction, suggesting that the immune system of the mice developing the tumors was potentially able to recognize tumor-specific antigens carried by the sarcoma cells (Figure 6A). However, we found that CD4+CD25+ Tregs were present with an averaged incidence of 4.17% ± 0.35 SD in the spleen of tumor-bearing mice, whereas these cells showed an averaged incidence of 1.61% ± 0.45 SD in non-implanted naive syngeneic mice (P < 0.001) (Figure 6B), indicating that the presence of CD4+CD25+ Tregs could have blocked the in vivo antitumor response. Indeed, Tregs isolated from tumor-bearing mice significantly blocked the proliferation of host’s splenocytes challenged with syngeneic tumor cells, but not the proliferation of the same responder cells to a different cellular stimulus (Figure 6A).
MSC expanded clones of tumor-specific Tregs by the action of soluble factors. When we cultured CD4⁺CD25⁻ T lymphocytes on the top of a transwell plate and we stimulated them with MSC and tumor cells put on the bottom part of the plate, we could retrieve CD4⁺CD25⁺ T lymphocytes (Figure 6C) able to suppress the proliferation of splenocytes derived from tumor-bearing mice in vitro challenged with syngeneic tumor cells (Figure 6D). We could not retrieve CD4⁺CD25⁺ Tregs when we stimulated CD4⁺CD25⁻ T lymphocytes (on the top) with MSC alone or MSC and the mitogen agent phytohemagglutinin (on the bottom).

Discussion

Repairing tissues by a tissue engineering approach using exogenous cells seeded onto biomaterials involves actively transforming interactions between three players: (i) the progenitor cells that constitute

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**Fig. 6.** Generation of tumor-specific CD4⁺CD25⁺ Treg by exogenous MSC. (A) The histogram shows the compiled results of proliferation assays where responder splenocytes from tumor-bearing mice were in vitro challenged with a mitogenic stimulus (phytohemagglutinin, PHA), syngeneic or allogeneic splenocytes and syngeneic tumor cells in presence or absence of Treg generated from the same tumor-bearing mouse. An asterisk indicates $P < 0.05$ comparing the proliferation of each condition with the baseline, ‘medium’ bar. (B) Incidence of CD4⁺CD25⁺ T lymphocytes in the spleen of a representative naive mouse (left panel) and in the spleen of a representative syngeneic tumor-bearing mouse (right panel). (C) Incidence of CD4⁺CD25⁺ T lymphocytes generated in transwell cultures of CD4⁺CD25⁻ T cells by MSC alone (left panel) and by MSC + syngeneic tumor cells (right panel). Shown are the results of a representative experiment. (D) Compiled results of proliferation assays where splenocytes from tumor-bearing mice were challenged with syngeneic tumor cells in presence of CD4⁺CD25⁺ T lymphocytes generated in transwell cultures of CD4⁺CD25⁻ T cells and MSC alone, MSC + phytohemagglutinin and MSC + syngeneic tumor cells, respectively. An asterisk indicates $P < 0.01$ comparing the proliferation of each condition with the baseline, medium bar.
the base elements for tissue regeneration and are both the producers and the targets of different growth factors; (ii) the biomaterial that provides the tridimensional scaffold where the engineered tissue develops, and that is gradually reabsorbed, and (iii) the recipient organism supplying the microenvironment allowing a correct tissue development. An engineered tissue should develop and integrate with adjacent host tissues and provide some minimal level of function immediately after implantation that should improve progressively until normal function has been restored (21). When the functional interactions between these three players are not balanced, a functional tissue would not develop and adverse reactions could happen.

A tumorigenic potential of human and mouse MSC was reported in in vitro and in vivo experimental settings using cell populations expanded for five to six passages in plates (22–24). Aneuploid karyotypes were observed in human and mouse MSC cultures after several (P9–P15) in vitro passages (25,26). The spontaneous transformation of MSC could be possibly due to the relative resistance of these cells to the telomerase-dependent mechanisms controlling the cell proliferation (27,28). For these reasons, we decided to use MSC after one passage of in vitro expansion (P1), assuming that a brief cycle of expansion did not allow any MSC to undergo spontaneous transformation into tumor cells. The risk of using this first-round cultured MSC was to have a highly heterogenous cell population including cells with angiogenic and inflammatory properties, such as macrophages and endothelial progenitor cells, able to induce precancerous changes in the tissues. The phenotype of the implanted MSC showed the presence of contaminating macrophages, but not endothelial precursor cells. The CD14+ fraction of macrophages contains cells with different functional activity, including osteoblast activity (29), and we cannot discern which functions were likely to play the implanted CD14+ cells. Moreover, the immunohistochemical analysis of the cancer tissue at the time when the tumorigenic injury was likely to occur, within the 4 weeks after implantation, showed the presence of CD14+ cells at the periphery of the tumor mass and near the areas of necrosis, indicating a more likely function of macrophages as scavenger cells.

The sarcomas observed in the mice implanted with MSC/bioscaffold constructs originated from the recipient’s tissues, thus pointing the attention to the paracrine effect of MSC on other cell populations.

In our experiments, the capacity of MSC to provide a local proliferation stimulus to the adjacent cells was enhanced by the presence of the scaffold creating the tridimensional architecture where the cell could develop into a structure similar to a tumor stroma. It has been established in vitro that growing cells in tridimensional scaffolds create a microenvironment permissive to tumor development (8,30). Both the composition and the stiffness of the scaffold that mimics the extracellular matrix of the tumor stroma have major effects on cell signaling and behavior. Cells can sense the stiffness of the stroma matrix through bidirectional interactions mediated by cell surface integrin receptors and the contractile cytoskeleton. As a consequence, they can alter the functional profile of activated intracellular and extracellular molecular pathways inducing cell proliferation or cytokine production (31,32). Recent reports indicate that MSCs are recruited in large numbers to the stroma of developing tumors (3,33), and there they act enhancing the motility, invasion and metastasis ability of adjacent cancer cells (4).

Others have reported that MSC induced only a transient tumor transformation of adjacent cells when intravenously injected in immunodeficient mice (3,4). On the contrary, our results showed that MSC, when loaded onto bioscaffolds, induced a permanent tumor transformation of adjacent cells, thus creating a tumor niche. Moreover, the composition and stiffness of the scaffold determined the latency of tumor occurrence. Sarcoma developed faster when MSC were loaded onto collagen sponges than onto a stiffer and less resorbable HA block. We can speculate that collagen constitutes a natural component of tumor stroma (34), thus creating a more physiological microenvironment permissive of cancer growth. In alternative, we can think that the superior thickness and slower resorbability characteristic of porous ceramic delays the direct contact between implanted MSC and neighboring cells that constitutes the primary stimulus for tumor formation.

Control of tumor growth by the immune system is a complex issue (35). It has been demonstrated that host immunity can kill tumor cells by the activation of different subsets of natural killer cells and T lymphocytes (36). However, tumor itself and cells in the tumor microenvironment could provide the factors to block this immune reaction. There are evidences that different molecular and cellular pathways induce the differentiation of tumor-specific CD4+CD25+ Tregs able to block the antitumor-specific immune response (37,38). In our experimental model, tumor immune escape could be due to the expansion of specific Tregs that impaired the activation of T lymphocytes against sarcoma cells. The presence of tumor-specific Tregs, spontaneously occurring in different types of human tumors (37,38), could have been enhanced in our system by the implanted MSC (2). The presence of the bioscaffold did not modify the ability of MSC to generate T lymphocytes with suppressing activity (39,40).

In this paper, we reported the tumorigenic effect of the implantation of murine bone marrow-derived MSC in syngeneic animals. It is important to stress that, up to date, none of the patients treated with implants of autologous MSC seeded onto a ceramic bioscaffold for bone repair have been reported to have developed any type of tumors (41). Moreover, there are no published reports of tumor development in any patient implanted with autologous MSC both seeded and not seeded on a scaffold.

The murine model of ectopic bone formation under unloaded conditions is one of the most widely used approaches to study the development of an engineered bone tissue. The classical model involves the use of MSC of xenogeneic origin, i.e. human or sheep, implanted in combination with a bioscaffold into immunodeficient recipient mice. No tumor formation has been thus far reported under these experimental conditions. This discrepancy could be attributed to species-specific susceptibility of cells to tumor transformation determining an impairment of the cross talk between factors released by the donor and recipient cells (42).

Although the observed tumorigenic effect of implanted MSC could be species restricted, our results induce a more critical approach to (i) the use of MSC/bioscaffold constructs, in terms of biological interactions with the recipient; (ii) the source of MSC, autologous or allogeneic and (iii) the selection of patients, using an extreme caution in treating patients with a familial or personal history of malignancies.

**Supplementary material**

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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