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Tumor risk by tissue engineering: cartilaginous differentiation of mesenchymal stem cells reduces tumor growth

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Summary

Objective: Implantation of autologous chondrocytes (AC) is a promising option for the treatment of cartilage defects, but problems with cell harvesting, dedifferentiation, or the donor age limit the clinical outcome. Mesenchymal stem cells (MSC) gain much interest because of their simple isolation and multipotential differentiation capacity along with their immunosuppressive properties. The latter might introduce tumor manifestation. The influence of undifferentiated and chondrogenically differentiated MSC or AC on tumor growth and metastasis formation was investigated in a murine melanoma model.

Methods: Allogeneic melanoma cells and either syngeneic MSC (C3H10T1/2, transduced with enhanced green fluorescent protein gene) or AC were co-injected at a distance of 3 cm into the contra lateral groins of five mice/group, and evaluated macroscopically and histologically after 4 weeks.

Results: Undifferentiated MSC migrated to the tumor site and induced strong tumor growth and metastasis formation. Even avital MSC promoted tumor growth and spreading, but insignificantly without detectable MSC at the tumor site. Chondrogenically differentiated MSC did not migrate and had a significantly lower impact on tumor growth and spreading; AC had no measurable influence on melanoma cells.

Conclusions: Our data suggest that differentiation of MSC reduces MSC-dependent promotion of latent tumors and that native AC do not introduce any increased risk of tumor growth. The question of how far MSC should be differentiated prior to clinical application should be addressed in further studies.

Key words: Tissue engineering, MSC, Cartilage, ACI, Tumor growth, Cell labeling.

Introduction

Articular cartilage is frequently injured as a result of sports related trauma, but it has very limited capacity for repair due to its avascular nature. Therefore, treatment of focal cartilage defects is very challenging. There are several approaches to manage this problem as it causes pain and functional disability. At present, autologous chondrocyte implantation (ACI) is one of the most favored treatment options[1,2]. Autologous chondrocytes (AC) are harvested from the patients by a cartilage biopsy, expanded *ex vivo* and are reimplanted into the cartilage lesion. This procedure could be shortened and the complication rate might me minimized, if artificial cartilage engineered by mesenchymal stem cells (MSC) is used. A preoperative cartilage biopsy would be no longer necessary. This potential usage of MSC for cartilage repair is intensively investigated in several animal models[3–5].

There are different sources described for MSC, e.g., bone marrow, umbilical cord tissue, peripheral blood and placenta[6–10]. These cells can be separated from other tissues by their adherence characteristics and surface markers, and expanded more than 10^4-fold without loss of their multipotential differentiation capacity[11,12]. MSC are identified by the absence of CD34 and CD45 hematopoietic cell markers. They stain positive for CD90, endoglin/CD105 and vascular cell adhesion molecule-1 (VCAM-1/CD106) and SH3[9,10]. MSC express the major histocompatibility complex (MHC) class I but do not express MHC class II, B7-1/2, CD40 or CD40L molecules. A number of cytokines and regulatory molecules that play important roles in the proliferation and maturation of hematopoietic stem cells is also produced[13,14]. The C3H10T1/2 cell line applied for our experiments is bone marrow derived and has already been characterized regarding its mesenchymal stem cell quality[15].

MSC isolated from bone marrow aspirates have got multipotential differentiation capabilities and are the excellent candidates in tissue engineering (TE), but these cells also exhibit powerful immunosuppressive effects, about 200-fold compared to immunosuppression achieved by common immunosuppressants (e.g., cyclosporine A)[16]. For this reason, MSC are already used for clinical application in treatment of graft versus host disease (GVHD) after bone marrow transplantation with success[17,18]. Primary MSC are capable of homing to the bone marrow and survive in the long term[19,20]. Unfortunately, these systemic immunosuppressive effects could also result in a high risk of tumor manifestation that is found in patients receiving immunosuppressants[21–24]. Some studies could already show a consecutive tumor growth promotion after implantation of undifferentiated MSC in animal models, whereas tumor induction has only been described for embryonic stem cells (ESC)[25–28].

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Therefore, the aim of this study was to clarify whether bone marrow derived MSC (C3H10T1/2 cell line) with or without further cartilaginous differentiation, as well as mature AC utilized for ACI could display side effects favoring tumor growth in a murine melanoma model. This animal model allows to estimate the influence of MSC and other cells on B16 melanoma cell proliferation within the period of 4 weeks, when these cells are injected simultaneously into the contra lateral groins.

Our study is based on a melanoma model which has been described previously: we support findings showing the characteristic high malignancy of B16 melanoma cells by syngeneic subcutaneous inoculation in C57BL/6 mice[29]. Additionally, the baseline of tumor growth in the control group was determined after allogeneic melanoma cell inoculation in C3H/He mice (baseline $\leq$ 30 µl of tumor volume), and subsequently co-injection of allogeneic melanoma cells and undifferentiated MSC showed a strong MSC-dependent increase in melanoma growth and metastases formation, as it has been shown previously[26]. The MSC utilized for our experiments were transduced with enhanced green fluorescent protein (EGFP) gene carrying the retroviral vector pBabePuro[30]. Thus, it was possible to show any migration of MSC to the tumor site and their metastases. Our study shows that chondrogenic differentiation of MSC reduces MSC-dependent promotion of latent tumors, and that native AC (normally used for ACI) do not introduce any increased risk for tumor promotion.

Material and methods

The experiments done in this study were approved by the regional government of Schleswig-Holstein (Germany), department of farming, environment and country places.

ANIMALS

Two different strains of mice were used according to their MHC antigen disparity: C3H/He and C57BL/6. Mice were bred in the central animal husbandry of the University Hospital Schleswig-Holstein, Campus Kiel, Germany. They were housed in the facilities of the Victor-Hensen-Haus and cared for according to the Laboratory Animal Care Guidelines. Thirty two adult animals aged 8–12 weeks were used and distributed among seven experimental groups [five animals (or two)/group, see Table I]. Three mice (C3H/He) aged 2 weeks were used for isolation of AC.

CELL CULTURE

The murine C3H10T1/2 MSC line and the B16-BL6 melanoma cell line were kindly given by Mrs. Danièle Nöel (INSERM U475, Montpellier, France). These cells were each cultured in complete Dulbecco Modified Eagle Medium (DMEM, Sigma, Germany) supplemented with 10% fetal calf serum (FCS, Seromed, Germany), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml amphotericin (Seromed, Germany). Any *in vitro* contamination of the different cells lines was avoided.

EGFP-GENE TRANSDUCTION IN MSC

We successfully performed the stable fluorescent labeling of the MSC with a retroviral system (pBabePuro). The retroviral vector consists of an internal ribosome entry site (IRES) element, an EGFP gene and a puromycin resistant gene for later selection of effectively transduced MSC. MSC were best transduced after incubation with the retroviral particles for 2 days. Not transduced cells were killed with 300 µg/ml puromycin (Seromed, Germany) within 4 days. MSC cell clones with high EGFP production were selected after punctual trypsinization from the monolayer culture (>99% EGFP-positive cells) and expanded for the animal experiments [Fig. 1(A, B)].

CARTILAGINOUS DIFFERENTIATION OF MSC AND RT-PCR

For cartilaginous differentiation 750,000 EGFP-gene-transduced C3H10T1/2 MSC were cultured in three-dimensional high density pellets at the bottom of an Eppendorf cap (Sarstedt, Germany) as described by Kurz *et al.*[31]. The differentiation medium contained the chondrogenic differentiation supplements: 0.1 mM dexamethasone and 10 ng/ml murine transforming growth factor (TGF-ß1) (Sigma, Germany). These pellets were exposed to 5% oxygen over a period of 10 days under otherwise normal culture conditions. The morphogenic genotype of the cells was confirmed by RT-PCR for aggrecan and collagen type II. Expression of collagen type II was negative in undifferentiated MSC, while there was a clear signal in cartilaginous differentiated MSC as well as in AC [Fig. 1(C)]. The EGFP production by MSC was not changed after cartilaginous differentiation (not shown). Prior to injection the cells were isolated from the pellets by collagenase treatment and subsequent filtration through a nylon mesh; cell viability and number were evaluated as described below.

RNA was isolated from the cell cultures using the Qiagen RNeasy Mini-Kit according to the manufacturer's instructions. Lysed samples were homogenized using the QIAshredder spin columns (Quiagen). Isolated RNA was determined for quantity and quality spectrophotometrically (260 and 280 nm). cDNA was

Table I

Statistical analysis of the detected primary tumor volumes 4 weeks after treatment using the student's t *test. The group B16-ALLO/- is the reference group with allogeneic mice (ALLO: C3H/He) and B16-BL6 melanoma cells: tumors with a volume of up to 30 µl had been found in this group and therefore been defined as background with a tumor incidence of 0%. The promotion of tumor growth was highly significant in syngeneic animals (SYN: C57BL/6) and in allogeneic animals (ALLO) receiving syngenic undifferentiated MSC (isolated from C3H/He). Cartilaginous differentiation of MSC (MSC-C) decreased the promotion of tumor growth, and even more the injection of MSC-AV. The impact of differentiation was clearly observed when native autologous chondrocytes (AC: isolated from C3H/He) were co-injected into the murine melanoma model. EGFP: cells were transduced with EGFP gene. Non-transduced MSC that were labeled with the fluorescent cell-tracker CM-DiI also favored tumor growth and metastasis formation. SD = standard deviation*

	Group name	Tumor volume [µl], $n = 5$ animals/group					Mean volume [µl]	SD [µl]	Incidence [%, based on tumor volume]		Student's *t* test ($\alpha < 0.05$)
		1	2	3	4	5			Tumor	Metastasis	*P* value
Injection of B16 melanoma cells alone	B16-ALLO/-	12	30	0	0	0	18	13	0	0	Control
	B16-SYN/-	112	178	96	150	148	137	33	100	100	<0.0001
Co-injection of B16 melanoma cells with MSC	B16-ALLO/ MSC-EGFP	105	250	200	186	60	160	76	100	80	<0.003
	B16-ALLO/ MSC-EGFP-AV	32	74	0	16	0	24	31	40	40	0.316
	B16-ALLO/ MSC-C-EGFP	60	105	36	48	30	56	30	80	0	0.012
	B16-ALLO/ MSC-CM-DiI	96	68				82	20	100		
Co-injection of B16 melanoma cells with AC	B16-ALLO/ AC	22	7	16	0	0	9	10	0	0	0.937