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Osteoarthritis and Cartilage (2007) **15,** 475–478
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doi:10.1016/j.joca.2006.11.007

Osteoarthritis and Cartilage

International Cartilage Repair Society

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Brief report
Human parvovirus B19, varicella zoster virus, and human herpesvirus-6 in mesenchymal stem cells of patients with osteoarthritis: analysis with quantitative real-time polymerase chain reaction

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Summary

Objective: To investigate whether there is a possible viral transmission using mesenchymal stem cells (MSCs) in autologous or allogeneic transplantation in the context of osteoarthritis (OA) patients. The presence of parvovirus B19 (B19), varicella zoster virus (VZV), and human herpesvirus-6 (HHV-6) was studied in MSCs from bone marrow of patients with OA and healthy controls.

Methods: MSCs were prepared from bone marrow aspirates obtained from 18 patients undergoing joint replacement as a result of OA and from 10 healthy controls. DNA was extracted from primary MSCs' culture established from these cells and quantitative real-time polymerase chain reaction was performed to analyse the prevalence and viral load of B19, VZV and HHV-6.

Results: The prevalence of total viral DNA among patients with OA was 16.7% (3/18), with a mean viral load of 29.7 copies/μg of DNA. One out of 18 was positive for B19 (viral load, 61.2 copies/μg of DNA), two for VZV (mean viral load, 14.4 copies/μg of DNA), and none for HHV-6. The prevalence of total viral DNA in the control group was 20% (2/10), with a mean viral load of 13.4 copies/μg of DNA. Both positive results were of B19 parvoviruses. There were no statistically significant differences among patients and controls.

Conclusions: This first approach to the viral prevalence in MSCs of bone marrow in OA patients and healthy controls seems to show a very low risk of viral transmission or reactivation in a possible MSCs' transplantation.
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Key words: Osteoarthritis, Virus, Mesenchymal stem cells, Polymerase chain reaction (PCR).

Introduction

Osteoarthritis (OA) is associated with bone and articular cartilage degenerative changes which develop due to the complex interaction of genetic, metabolic, biochemical, biomechanical and inflammatory factors[1,2]. The disease has important social and economic consequences over the increasingly aged occidental populations[3].

Current investigations on mesenchymal stem cells (MSCs) offer a new perspective for bone and cartilage tissue regeneration. MSCs have the capacity to differentiate into a variety of connective tissue cells including bone, cartilage, tendon, muscle, and adipose tissue[4]. These cells are easily isolated from bone marrow and can be expanded in culture through many generations, while retaining their capacity to differentiate when exposed to appropriate signals.

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Received 26 September 2006; revision accepted 21 November 2006.

Several reports have shown that local delivering of autologous stem cells expanded previously in culture might be useful for repairing focal defects in articular cartilage of patients with knee OA[5], as well as in animal models of OA induced by injury to the meniscus[6]. Furthermore, there is growing evidence of the hypoimmunogenic nature of MSCs, a fact that bears key implications in terms of allogeneic therapy. Some reports have described the clinical use of allogeneic donor-mismatched cells with little evidence of host immune rejection. For example, allogeneic bone marrow transplantation in children with brittle-bone disease (osteogenesis imperfecta) resulted in engraftment of donor-derived MSCs and increasing new bone formation[7]. Major disadvantage of allogeneic approaches relates to potential risks of disease transmission from donors to recipients.

It has been described that patients undergoing autologous stem cells' transplants might experience viral infections because of reactivation of the viruses[8,9], and that allogeneic stem cell transplantation is often complicated by herpesvirus reactivation[10]. In this report, we have analyzed the presence and viral load of three viruses that might potentially be transmitted by MSCs from bone marrow: parvovirus B19 (B19), varicella

zoster virus (VZV), and human herpesvirus-6 (HHV-6) in patients with OA and healthy controls using a quantitative real-time polymerase chain reaction (PCR).

Patients and methods

PATIENTS

A total of 18 patients with OA (mean age 74.7 years, range 61–89) and 10 healthy controls with not known history of joint disease (mean age 66.6, range 44–90) were recruited from the Service of Orthopaedic Surgery of Hospital Clínico San Carlos. The diagnosis was based on clinical and radiological criteria. Most patients with OA were treated with analgesic and/or nonsteroidal anti-inflammatory drugs. None of the patients had received corticosteroids or cytostatic drugs during previous months. Patients with systemic inflammatory diseases such as rheumatoid arthritis or spondyloarthropathies were excluded. The study protocol was reviewed and approved by our institutional board, and informed consent was obtained from all the subjects enrolled in the study.

SPECIMENS

Fresh bone marrow aspirates (5–10 ml) were obtained from the distal femur of the patients with OA during total knee replacement surgery and from healthy controls during tissue harvest in the process of multiorganic donation ($n = 3$) or from proximal femur in the surgery for the subcapital fracture of the hip ($n = 7$).

CELL CULTURE

Human MSCs were established from bone marrow aspirates of OA patients and donors according to the method of Pittenger *et al.*[11]. Briefly, the aspirates were washed once with Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Gibco BRL, Paisley, UK) containing 10% fetal bovine serum (FBS) (Sigma–Aldrich, St. Louis, MO), and antibiotic supplements (control medium). The cell-containing fraction was layered onto a 70% percoll solution (Sigma–Aldrich) and centrifuged at 500 *g* for 15 min at 23°C. The low-density fraction was collected, washed once with control medium, and plated into culture dish (100 mm). Cultures were maintained at 37°C in a humidified atmosphere and 5% CO_2. At the end of primary culture, adherent colonies were detached by treatment with 0.25% trypsin containing 1 mM EDTA for 5 min at 37°C, and subsequently replated for continued passaging 1:2. Medium changes were performed twice weekly. Confluent cells (approximately 1×10^6) at the third passage were used for the experiments.

DNA EXTRACTION

DNA was extracted, using Qiagen columns (QIAamp DNA Mini Kit, Qiagen GmbH, Germany), from primary MSCs' culture, established from bone marrow from patients with OA and controls, according to the protocols supplied by the manufacturer.

QUANTITATIVE REAL-TIME PCR

DNAs were analyzed by quantitative real-time PCR for the presence of B19, VZV and HHV-6 genomes, and human β-actin gene as internal control, with the primers, TaqMan probes (Table I), and cycling conditions that we described elsewhere[12]. The mean concentration of the target DNA in each one of the PCRs was 280 ng; all the samples were analyzed in duplicate for each one of the viruses. The quantitative assessment was performed in a Rotor-Gene 3000 real-time cycler (Corbett Research, Sydney, Australia). The sensitivities of the assay were one copy for the detection of HHV-6, two copies for B19, and five copies for VZV.

STATISTICAL ANALYSIS

Categorical variables were presented as frequency distribution and quantitative variables as means ± standard deviation (SD) if they fit a normal distribution, and median [25–75 interquartile deviation, IQD] if distribution was non-normal; odds ratios (OR) are presented with its 95% confidence interval (CI). Differences between groups were analyzed using the two-tailed Student's *t* test and the Mann–Whitney *U* test for normal and non-normal quantitative variables, respectively. The Chi-square test was used to compare categorical variables. A two-sided *P* value of 0.05 was the criterion for statistical significance in all cases.

Results

CULTURE AND PASSAGING OF HUMAN MSCs

Marrow aspirates were obtained from 18 patients undergoing joint replacement due to OA and 10 controls without

Table I
Primers and probes sequences

	Primer/probe	Sequence (5′ → 3′)	Size (bp)
B19	F-primer	TGGCCCATTTTCAAGGAAGT	74
	R-primer	CTGAAGTCATGCTTGGGTATTTTTC	
	Probe	FAM-CCGGAAGTTCCCGCTTACAAC-TAMRA	
VZV	F-primer	GGTGGAGACGACTTCAATAGC	99
	R-primer	CTCGATTTTCCAAGAGAGAC	
	Probe	FAM–CGTAATTCGATCGACCGGGATATCATACTC-TAMRA	
HHV-6	F-primer	CGAAACGCCTACACAGAAT	115
	R-primer	CAAAGCCAAATTATCCAGAGCG	
	Probe	FAM – ATTCCTTCGGGTGTGACG – MGB-DQ	
β-Actin	F-primer	TCACCCACACTGTGCCCATCT	106
	R-primer	GTGAGGATCTTCATGAGGTAGTCAGTC	
	Probe	FAM-ATGCCCTCCCCCATGCCATCCTGCGT-TAMRA	

F-primer = forward PCR primer; R-primer = reverse PCR primer; bp = base pairs.

joint disease. No adherent cells were removed from the dish during medium changes and the subsequent passaging. The cells isolated from these marrow aspirates formed confluent cultures of adherent cells with a fibroblastic morphology in approximately 2 weeks. MSCs were successfully expanded from all these subjects.

STUDY OF B19, VZV, AND HHV-6

The results are shown in Table II. The prevalence of total viral DNA among patients with OA was 16.7% (3/18), with a mean viral load of 29.7 copies/μg of DNA. One out of 18 was positive for B19 (viral load, 61.2 copies/μg of DNA), two for VZV (mean viral load, 14.4 copies/μg of DNA), and none for HHV-6. The prevalence of total viral DNA in the control group was 20% (2/10), with a mean viral load of 13.4 copies/μg of DNA. Both positive results were of B19 parvoviruses. There were no statistically significant differences among patients and controls.

Discussion

Transplanted MSCs carried viruses, which may cause infection in the host, and viral infection of MSCs might also affect the final success of transplantation. Previous reports indicate that patients undergoing autologous stem cells' transplantation can experience viral infections because of virus reactivation[8,9]. In addition, allogeneic stem cell transplantation is often complicated by reactivation of herpesviruses[10]. HHV-6 infection after bone marrow transplantation has been associated in some studies with fever and rash resembling acute graft-vs-host disease, as well as interstitial pneumonitis, encephalitis, cytomegalovirus (CMV) disease, and bone marrow suppression[13]. Several large case analyses could not demonstrate any statistical relationship between HHV-6 infection and such clinical events[14]. However, a correlation between the amount of virus in peripheral blood mononuclear cells before bone marrow transplantation and subsequent viral infection has been previously described[15]. VZV frequently causes severe infection in patients who have undergone bone marrow transplantation. Offidani *et al.*[16] studied the frequency, characteristics, and risk factors associated with VZV infection in 164 patients undergoing autologous peripheral blood progenitor cell transplantation. Roughly, 15% developed VZV infection and the actuarial risk was 10% at 1 year. None of the patients had visceral dissemination or died because of VZV infection, although one-third of the patients developed postherpetic neuralgia. Arnold *et al.*[17] demonstrated that autologous peripheral blood progenitor cells are a potential source of parvovirus infection, which may cause significant disease after autologous bone marrow transplantation. B19 is a rare but clinically significant infection that manifests as refractory anemia during the post-transplantation period. The use of PCR for diagnosis is particularly helpful in immunosuppressed transplant patients who may fail to mount antibodies against B19 during active infection[18].

Herein, we report the study of B19 and other two viruses from *Herpesviridae* family (VZV and HHV-6) in MSCs from bone marrow of OA patients and controls without joint disease. As shown in the Results section, we only found the presence of viral genomes in 3/18 OA patients (16.7%), almost the same prevalence (20%) of viral DNA found in the control group of subjects without joint disease. One sample of MSCs of OA patients and two of controls were positive for B19 DNA. Viral DNA for VZV was detected in two samples of OA patients. On the other hand, HHV-6 DNA was not detected in MSCs of patients and controls, and the viral loads were very low in both groups. Recently, Sundin *et al.*[19] have analyzed MSCs from healthy seropositive donors with PCR for CMV, herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2, Epstein-Barr virus (EBV) and VZV. Their results indicate that viral DNA cannot be detected in healthy seropositive individual and that the risk of herpesvirus transmission by transplantation of MSCs from healthy seropositive donors is low. In other study, Behzad-Behbahani *et al.*[8] examined viral DNA from bone marrow progenitor cells, peripheral blood leukocytes, and plasma sample from 30 allogeneic bone marrow donors, using nested PCR, to detect B19, HHV-6, VZV, human cytomegalovirus (HCMV), and EBV. They cannot detect HHV-6 DNA in any collected specimen by simple PCR. However, B19 and VZV DNA were, respectively, detected in the plasma of four (13.3%) and one (3.3%) of the 30 bone marrow donors but not in bone marrow progenitor cells.

In conclusion, these results provide evidence supporting the very low risk of viral transmission or reactivation in transplantation of autologous or allogeneic MSCs in patients with various disorders requiring tissue regeneration. However, additional studies are needed to confirm these results, since this is the first approach to the viral prevalence in MSCs of bone marrow from OA patients.

Table II
Prevalence and viral load of B19, VZV, and HHV-6 in MSCs of OA patients and controls

	OA patients		Controls		%OA vs %Controls	
	Pos*/*n* (%)	VL†	Pos/*n* (%)	VL	*P*	OR (CI 95%)
Total	3/18 (16.7)	29.7	2/10 (20)	13.4	0.825	0.8 (0.1–8.8)
B19	1/18 (5.6)	61.2	2/10 (20)	13.4	0.236	0.2 (0.1–4.2)
VZV	2/18 (11.2)	14.4	0/10 (0)	–	0.274	–
HHV-6	0/18 (0)	–	0/10 (0)	–	–	–

*Pos = positive samples.
†VL = mean viral load, in copies/μg of DNA.

Acknowledgments

We are indebted to Prof. García-Ruíz JP for her help in MSCs' isolation and culture. We wish to thank Orthopaedic surgeons from the Hospital Clínico San Carlos for providing bone marrow samples. This work was supported by the grants FMMA and FIS 04/1698.

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