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ORIGINAL ARTICLE

Microchimerism in labial salivary glands of hematopoietic stem cell transplanted patients

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OBJECTIVE: Microchimerism has been extensively investigated in autoimmune diseases, which display similarities with graft-*vs*-host disease. This study was conducted to investigate the presence of microchimerism in minor salivary glands of hematopoietic stem cell transplanted patients, one of the targets of graft-*vs*-host disease.

METHODS: Labial salivary glands biopsy specimens from 11 stem cell transplanted patients were analysed. The samples were grouped in control (five specimens from a female-to-female transplantation) and study group (five glands from male-to-female transplantation). One male transplanted patient was used as a positive control. Fluorescence *in situ* hybridization with Y-chromosome probe and immunofluorescence with anticytokeratin AE1/AE3 and CD45 were used to identify Y-chromosome positive glandular epithelial cells from allogeneic hematopoietic stem cell transplanted patients.

RESULTS: In the study group, all samples were positive to Y-chromosome and cytokeratin AE1/AE3, in agreement with the pattern exhibited by male labial salivary gland. None of the samples from control group were positive to Y-chromosome despite being positive to cytokeratin AE1/AE3. Positivity to CD45 was not relevant.

CONCLUSION: Microchimerism in the labial salivary glands of sex-mismatched stem cell transplanted patients is a real phenomenon. Further studies are necessary to elucidate the impact of this phenomenon on the clinical status of stem cell transplanted patients.

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Keywords: microchimerism; Y-chromosome; minor salivary glands; hematopoietic stem cell transplant; GVHD

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Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) has been used with increasing frequency for treatment of neoplastic and non-neoplastic diseases (Gomez *et al*, 2004; Imanguli *et al*, 2008). Many complications may occur following HSCT, but graft-*vs*-host disease (GVHD) remains as the leading cause of morbidity and mortality in HSCT recipients (Souza *et al*, 2004; Fraser *et al*, 2006; Imanguli *et al*, 2008). GVHD manifests as acute or chronic forms, with diagnosis criteria based on characteristic symptoms and signs that are not associated with a temporal definition (Filipovich *et al*, 2005). Chronic GVHD (cGVHD) can affect a wide variety of organs but most commonly involves skin, oral, vaginal and conjunctival mucosa, salivary and lachrymal glands and the liver (Filipovich *et al*, 2005; Fraser *et al*, 2006; Imanguli *et al*, 2008). cGVHD develops in approximately 40–70% engrafted patients and it can persist for months or years, urging for long-term multidisciplinary care (Fraser *et al*, 2006).

Allogeneic HSCT leads to the development of a condition called 'mixed chimerism' on the recipient, which consists on the presence of circulating donor and recipient hematopoietic cells (Jaksch *et al*, 2005). The monitoring of chimerism may indicate the risk of extensive GVHD, rejection and graft fail (Balon *et al*, 2005; Jaksch *et al*, 2005). Microchimerism has been defined as a chimaera or mixture of a small number of cells or DNA from different individuals coexisting within tissues and peripheral blood (Ando and Davies, 2003). It has been demonstrated after organ transplantation, including bone marrow, by identification of the concomitant persistence of donor and recipient cells. The donor cells may initiate GVHD and also have an important role in the induction of host tolerance to the grafted tissue (Triulzi and Nalesnik, 2001; Ando and Davies, 2003).

Microchimerism has been investigated in a variety of autoimmune conditions to clarify its possible role on their onset. Correlations of Y-chromosome microchimerism to Sjögren syndrome were investigated by determining the presence of Y-chromosome in salivary glands

(Aractingi *et al*, 2002; Endo *et al*, 2002; Kuroki *et al*, 2002). Nevertheless, the role of maternal-foetal Y-chromosome microchimerism in the pathogenesis of Sjögren syndrome remains an issue of controversy.

Bone marrow stem cells are believed to evolve into cells of all dermal lineages, such as hepatocytes, skeletal myocytes, cardiomyocytes, neural, endothelial, epithelial and even endocrine cells (Maria *et al*, 2007). The plasticity of adult bone marrow derived cells was questioned by *in vitro* studies suggesting that fusion between donor and host cells results in mimicking of transdifferentiation (Terada *et al*, 2002; Ying *et al*, 2002). Epithelial tissue chimerism after HSCT was demonstrated by investigation of Y-chromosome microchimerism of cheek scraps and colon samples (Tran *et al*, 2003; Spyridonidis *et al*, 2004).

Considering that microchimerism in sex-mismatched recipients of HSCT is a real phenomenon in different tissue, but was not yet proved in labial salivary glands, we performed an investigation to verify the possibility of its occurrence in labial salivary glands of male-to-female HSCT recipients.

Materials and methods

The study was approved by the local ethics committee.

Labial salivary gland samples

Eleven samples of labial salivary glands (LSG) from eleven patients submitted to HSCT were obtained from lower lip biopsies performed at 100th day after transplantation to diagnose and stage cGVHD in oral mucosa and salivary glands (Nakamura *et al*, 1996; Schubert *et al*, 1999), according to the protocol of the Bone Marrow Transplantation Unit of Hospital das Clínicas, UFMG. Fresh tissue samples were stored in Tissue-Tek (Sakura Finetek, Torrance, CA, USA) solution at −80°C. These samples comprised of five LSG from female-to-female HSCT (control group), five samples of LSG from male-to-female HSCT (study group) and one sample from a male patient submitted to HSCT to serve as a positive control. Female patients had no history of bearing male child. Samples were examined by qualified professionals from the Service of Oral Pathology at UFMG to determine the stage of cGVHD, as described elsewhere (Gomez *et al*, 2001; Souza *et al*, 2004). Experiments were performed blindly and the results of the staging were only retrieved from the files after the experiments. The frozen specimens of labial salivary glands were submitted to sequential 7 μm cryostat sections. Three sections from each sample were fixed with 4°C acetone (Merck KGaA, Darmstadt, Germany) and another section was submitted to Haematoxylin-eosin (HE) to verify morphology preservation and adequacy to study; two sections were reserved for immunofluorescence procedure and another was reserved for the FISH procedure.

Fluorescent in situ hybridization (FISH)

Fluorescent *in situ* hybridization was performed with slight modifications and adaptations to the protocol established by manufacturer of the probe and the protocol described elsewhere (Buño *et al*, 2003).

The sections were fixed by incubation with Carnoy's solution (methanol: acetic acid 3:1 by volume) at room temperature twice for 5 min. The slides were incubated in Eppendorf Mastercycler Gradient 5331 (Eppendorf AG, Hamburg, Germany) with *in situ* adapter (Eppendorf AG, Hamburg, Germany) at 90°C for 20 min and then washed in 2X saline sodium citrate at 37°C. The sections were dehydrated by crescent ethanol bath series of 1 min each. The samples received treatment with pepsin solution 0.005% (Zymed laboratories, San Francisco, CA, USA) with HCl 0.01N at 37°C for 1 h and were dehydrated in alcohol. The probe solution was prepared with 0.5 μl of Vysis CEP Y Satellite III Spectrum Green (Abbott Molecular Inc., Des Plaines, IL, USA), 3.5 μl of Vysis CEP Hybridization Buffer (Abbott Molecular Inc.) and 1 μl of purified water for each sample, centrifuged, vortexed and centrifuged again. The tube was placed in a 73°C water bath for 5 min and then placed on a 48°C slide harmer. Reactions were performed in two sections of each sample. 5 μl of probe mixture was applied to the section, coversliped and sealed with rubber cement. For each case, a negative control, in which the probe was omitted, was included. Sections were incubated for 5 min at 75°C for denaturation, and transferred to humid chamber at 42°C to overnight incubation. After the incubation, sections were submerged in a solution of 0.3% NP-40 (Sigma-Aldrich Corp, St. Louis, MO, USA) at 75°C for 2 min, followed by 1 min in a solution of 0.1% NP-40 at room temperature. The samples were counterstained in 4′, 6-diamino-2-phenylindole, dihydrochloride (DAPI) (Sigma-Aldrich Corp.) diluted 1:300 in PBS-BSA 2%, rinsed twice and mounted with Hydromount (National Diagnostics Inc., Atlanta, GA, USA) at room temperature.

Immunofluorescence reactions

Two sequential sections from each sample were submitted to immunofluorescence reaction with cytokeratin to prove epithelial nature of the positive cells for Y-chromosome microchimerism and to CD45 to exclude the possibility of donor-derived leukocytes in close contact to the epithelial cells. The sections were rinsed five times with PBS for 3 min, fixed in acetone (Merck KGaA, Darmstadt, Germany) for 10 min, rinsed with PBS, incubated with PBS/BSA 2% at room temperature for 40 min and finally incubated overnight with AE1/AE3 (Dako, Carpinteria, CA, USA) and CD45 (Dako) monoclonal antibodies at a ratio of 1:40. After rinsing with PBS, samples were incubated with PBS/BSA 2% for 30 min and then with anti-mouse Texas Red antibody 50 μl of solution (2.5 μl antibody/250 μl of PBS-BSA) for 50 min. The samples were then rinsed with PBS and counterstained with DAPI. The slides were mounted with Hydromount. Negative controls, in which the primary antibody was omitted, were included for all samples.

Confocal microscopy

The images were obtained with Zeiss 1024 laser scanning confocal using LSmix programme with Zeiss Axiovert 100 (Carl Zeiss, Germany) with oil immersion objective

(60X, 1.2 NA). UV lasers (488 nm) or argon/krypton laser were used to excite the prepared tissues (trough 363 nm, 488 nm or 543 nm) and the emitting light was selected by using filters (505/30 to FITC and LP 560 to PE). Z series was performed with an aim to demonstrate the nuclear staining to Y-chromosome.

Five fields of each sample were obtained by confocal microscopy utilizing specific wave lengths for FITC, PE e DAPI (obtained with mercury lamp) and the images were stored in DVD for further analyses. The processing and analyses of images were made by mean of LSmix, Adobe Photoshop 7.0 and Image Tool.

Images analysis

Only samples exhibiting nuclear staining were considered Y-chromosome positive, as previously established (Kuroki *et al*, 2002; Buño *et al*, 2003; Metaxas *et al*, 2005; Ferlicot *et al*, 2010). The epithelial nature of Y-chromosome positive cells was confirmed by immunofluorescence with anticytokeratin AE1/AE3 in the 7 μm serial sections.

Patients' information

Primary disease, type of transplant, labial salivary glands cGVHD staging, donor relation and age at HSCT were retrieved from medical and dental files (Table 1).

Table 1 Female patients' distribution by primary disease, type of HSCT, labial salivary gland cGVHD staging, donor relation and age at HSCT

Sample	*Group*	*Primary disease*	*Cell source*	*cGVHD*	*Donor*	*Patient's age*
1	Control	AA	BM	Absent	Sister	26
2	Control	CML	PBSC	Moderate	Sister	51
5	Control	AML	PBSC	Absent	Sister	16
6	Control	CML	BM	Mild	Sister	26
8	Control	AML	PBSC	Mild	Sister	25
3	Study	CML	PBSC	Mild	Brother	46
4	Study	CML	BM	Mild	Brother	53
7	Study	AML	PBSC	Absent	Brother	44
9	Study	CML	BM	Mild	Brother	40
10	Study	CML	PBSC	Absent	Brother	56

AA, aplastic anaemia; BM, bone marrow; CML, chronic myeloid leukaemia; PBSC, peripheral blood stem cells; AML, acute myeloid leukaemia; HSCT, hematopoietic stem cell transplantation; cGVHD, chronic graft-*vs*-host disease.

Results

Extensive positive fluorescence for Y-chromosome was detected in the sample from the male positive control (Figure 1a). All five samples from the study group (male-to-female HSCT) were positive for Y-chromosome microchimerism (Figure 1b). No positivity for Y-chromosome was observed in any of the five samples from female-to-female HSCT control group. Immunofluorescence for cytokeratin AE1/AE3 was observed in all 11 samples (Figure 1c), whereas a very low positivity to CD45 was verified, unrelated to epithelial cells. Thus, these results confirmed the epithelial nature of the specimens with Y-chromosome positivity. Table 2 summarizes the findings, including cGVHD staging retrieved from the files.

Discussion

We demonstrated that microchimerism does occur in minor salivary glands of female patients receiving HSCT from male donors. As HSCT patients are submitted to transfusion several times before and after HSCT, other sources of microchimerism different from male donors or male-child bearing history were impossible to exclude

Table 2 Female patients' distribution for FISH Y-chromosome and anticytokeratin AE1/AE3 positivity, labial salivary gland cGVHD and donor gender

Sample	*Group*	*Y-chromosome*	*Cytokeratin AE1/AE3*	*cGVHD*	*Donor*
1	Control	−	+	Absent	Sister
2	Control	−	+	Moderate	Sister
5	Control	−	+	Absent	Sister
6	Control	−	+	Mild	Sister
8	Control	−	+	Mild	Sister
3	Study	+	+	Mild	Brother
4	Study	+	+	Mild	Brother
7	Study	+	+	Absent	Brother
9	Study	+	+	Mild	Brother
10	Study	+	+	Absent	Brother

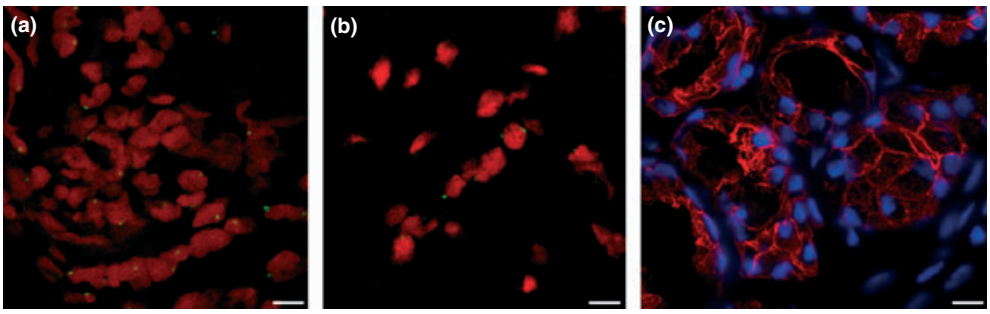

Figure 1 (**a**) Male positive control. Green points represent positivity for Y-chromosome on epithelial glandular cells with nucleus counterstained with DAPI (red). (**b**) Female patient that received a sex-mismatched HSCT (study group). Positivity for Y-chromosome (green dots) on epithelial glandular cells with nucleus counterstained with DAPI (red). (**c**) Anticytokeratin AE1/AE3 immunofluorescence reaction exhibiting epithelial nature of the cells (red) and nucleus counterstained with DAPI (blue). Bar, 10 μm

(Dzik, 1995; Bianchi *et al*, 1996; Kuroki *et al*, 2002; Yan *et al*, 2005). However, we included a female-to-female control group, in which the recipients had also undergone similar transfusion regimen. As we did not observe a positive staining for Y-chromosome in this group, it is likely that the microchimerism observed in the male-to-female group is indeed due to HSCT.

All male-to-female samples evaluated in this study were positive for Y-chromosome microchimerism but while three of the patients presented mild cGVHD the other two did not present cGVHD at the time of sample collection (100 days of transplantation). Nevertheless, cGVHD could occur before, during or after this time (Filipovich *et al*, 2005). These findings brought the possibility of Y-chromosome microchimerism positivity that could precede the fibrotic changes of acini (Sawaya *et al*, 2004; Spyridonidis *et al* (2004); Ogawa *et al*, 2005), as tissue damage enhances the possibility of epithelial microchimerism (Borue *et al*, 2004; Spyridonidis *et al*, 2004).

The microchimerism of Y-chromosome identified in the nuclei of epithelial glandular cells by FISH and anticytokeratin AE1/AE3 probably represent transdifferentiation of hematopoietic donor tissue into epithelial glandular cells, as previously suggested by *in vivo* studies (Tran *et al*, 2003; Spyridonidis *et al*, 2004; Brittan *et al*, 2005; Metaxas *et al*, 2005) and not cell fusion as postulated by *in vitro* studies (Terada *et al*, 2002; Ying *et al*, 2002). Furthermore, the very low positivity of CD45 cells not related to the epithelial cells excludes the possibility of donor-derived leukocytes in close contact with epithelial cells.

In spite of the presence of the microchimerism of Y-chromosome in labial salivary glands of male-to-female HSCT, the relation of these findings with damaged tissues in regeneration processes remains unsolved, as observed in previous studies (Borue *et al*, 2004; Spyridonidis *et al*, 2004; Dubernard *et al*, 2008, 2009; Gadi, 2010).

Further studies with a larger series of cases are necessary to establish the possible correlation of the degree of Y-chromosome microchimerism with cGVHD staging of labial salivary glands as well as to verify the possible impact of this phenomenon on evolution of these patients.

Conclusion

The microchimerism of Y-chromosome in salivary glands from sex-mismatched HSCT female patients is a real phenomenon.

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Author contributions

The authors contributed equally to the research and manuscript handling.

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