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Experiment Oral Pathology

Effect of bone marrow transplantation on the immunolocalization of p53, hMSH2, and hMLH1 proteins on oral mucosa

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OBJECTIVE: Considering that hMSH2, hMLH1 and p53 are important in maintaining genomic stability of the oral mucosa epithelium, the purpose of the present study was to investigate the immunolocalization of these proteins in the epithelium of the oral mucosa of patients submitted to bone marrow transplantation (BMT) compared with controls.

MATERIALS AND METHODS: Twenty-one samples of lip biopsies from BMT recipients were retrieved. Twenty samples of normal lower labial mucosa associated with mucocele in non-transplanted patients were included as control group. The streptavidin–biotin complex stain was used to detect the human DNA mismatch repair proteins hMSH2, hMLH1 and p53 protein.

RESULTS: The main findings demonstrated that the mean number of suprabasal epithelial cells positive for MSH2 was statistically higher than the control group. The immunostaining of hMLH1 and p53 at the basal and suprabasal epithelial layers were statistically higher in the oral labial mucosa of the BMT patients compared with controls.

CONCLUSION: The present study shows that oral epithelial cells of BMT patients show increased immunolocalization of the DNA repair related proteins.

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Keywords: BMT; P53; hMSH2; hMLH1; oral mucosa; immunohistochemistry

Introduction

Bone marrow transplantation (BMT) has been used increasingly throughout the world as a therapeutic modality in recent years for patients diagnosed with various malignant and non-malignant haematological diseases, including acute and chronic leukaemia's, aplastic anaemia, myelodysplasic syndromes, severe combined immunodeficiency, lymphomas, and selected solid tumours. BMT involves the ablation of the abnormal or malignant cells with high-dose chemotherapy, with or without total body irradiation followed by infusion with healthy myeloproliferative cells (Storn and Thomas, 1983; Deeg *et al*, 1984; Bortin and Rimm, 1989; Lishner *et al*, 1990; Donato *et al*, 1998). The infusion may consist of syngenic bone marrow cells from a genetically identical twin, allogeneic bone marrow or stem cells from HLA-matched sibling donors, or autologous bone marrow or stem cells harvested from the patients and then reinfused.

Many oral complications such as oral mucositis, oral infections and graft-*vs*-host-disease (GVHD) may occur following BMT. GVHD is a common complication in patients who are treated with allogeneic BMT. It develops as transplanted immunocompetent donor T lymphocytes attack various recipient tissues because of differences in major and minor histocompatibility antigens (Lum *et al*, 1981; Sullivan, 1986). The diagnosis and staging of chronic GVHD (c-GVHD) can be made from specimens obtained by labial mucosa and minor salivary glands biopsies 100 days after transplantation (Schubert *et al*, 1999). The histopathological features of labial mucosa and minor salivary glands biopsies reflect the systemic state of c-GVHD (Nakamura *et al*, 1996). Secondary malignancies, including lymphoma, granulocytic sarcoma, may develop after BMT (Deeg and Witherspoon, 1993; Boivin *et al*, 1995; Deeg *et al*, 1996; Otsubo *et al*, 1997; Abdelsayed *et al*, 2002). Recently, some cases of oral squamous cell carcinoma have been reported and it has been regarded as developing secondary to GVHD, sequelae of treatment with cytotoxic medications or immunosuppressive therapy (Lishner *et al*, 1990; Otsubo *et al*, 1997; Jansisyanont *et al*, 2000; Abdelsayed *et al*, 2002).

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The human DNA mismatch repair (hMMR) system plays an important role in reducing mutation maintaining genomic stability. It corrects base–base mismatches and short insertions/deletions generated as a consequence of DNA replication errors and homologous recombination (Kolodner, 1996). MMR genes, including *hMSH2*, *hMLH1*, *hMSH3*, *hMPS1*, *hPMS2* and *GTBP*/*hMSH6* are very important in distinguishing and repairing misparing and slippage errors in DNA synthesis (Modrich and Lahuer, 1996). The mismatch is detected by two complexes, the hMSH2–GTBP/hMSH6 and the hMSH2–hMSH3 heterodimers (Acharya *et al*, 1996) after interaction with hMLH1–hPMS2 heterodimers, an endonuclease is activated and incises the newly synthesized DNA strand that contains the mutation (Bellacosa *et al*, 1999). Recently, it has been suggested that inactivation of the MMR gene *hMLH1* is an important mechanism on the tumourigenesis of a subset of head and neck squamous cell carcinomas (Liu *et al*, 2002).

TP53 has a key role in the control of the cell cycle, the maintenance of genomic stability, cell differentiation, and apoptosis. Increased p53 expression in response to DNA-damage promotes transcription of the *WAF1*/*CIP1* gene, whose protein product p21, causes growth arrest through inhibition of the cyclin/cyclin-dependent kinase complex that is required for G1 to S transition (Levine *et al*, 1991; Cox, 1997; Prives and Hall, 1999). Mutations of the *p53* gene usually result in a product with an increased half-life that can sometimes be demonstrated immunohistochemically. Under certain conditions, the normal p53 protein can also be retained in the tissue by, for example, binding to other proteins or due to some defect in the normal degradation pathway. In this way, it can be detected by immunohistochemistry. In the latter instances, this 'retained' wild type protein is inactive, either due to blocking by another protein or due to partial degradation (Nylander *et al*, 2000). Alterations of the p53 gene are the most common genetic changes found in human malignant tumours.

Despite MMR genes and *p53* could be important in maintaining genomic stability of the oral mucosa epithelium; it has not been investigated in the oral mucosa of BMT patients. This fact coupled with the evidence that p53 protein stabilization is implicated on oral carcinogenesis prompted us to investigate the immunolocalization of the proteins p53, hMSH2 and hMLH1 in the epithelium of the oral mucosa of patients submitted to BMT.

Material and methods

The material and methods were approved by ethical committee of Universidade Federal de Minas Gerais (UFMG).

Tissue samples

According to the protocol of the Bone Marrow Transplantation Unit of Hospital das Clínicas, UFMG, biopsy samples of lower lip are performed at 100 days after transplantation in order to diagnose and stage GVHD in oral mucosa and salivary glands (Nakamura *et al*, 1996; Schubert *et al*, 1999). The staging of GVHD at the oral mucosa and salivary glands is described elsewhere (Gomez *et al*, 2001; Souza *et al*, 2004). From this universe, 21 samples (mean age 33.47 years, ranged from 14 to 54 years old) of oral labial mucosa tissues with no or minimal inflammatory cells were included in the study. (Table 1). Twenty samples (mean age

Table 1 Clinical features of the patients submitted to BMT

Patients	*Age (years)*	*Gender*	*Basic disease*	*Transplant type*	*Donor*	*Cell font*
1	12	Female	ALL	Allograft	Brother	Bone marrow
2	36	Male	AA	Allograft	Sister	Bone marrow
3	48	Male	CML	Allograft	Brother	Bone marrow
4	33	Female	AML	Allograft	Brother	Peripheral stem cells
5	39	Male	AMM	Allograft	Brother	Bone marrow
6	44	Male	CML	Allograft	Sister	Peripheral stem cells
7	21	Male	AA	Allograft	Brother	Bone marrow
8	46	Female	AA	Allograft	Brother	Bone marrow
9	31	Male	CML	Allograft	Brother	Peripheral stem cells
10	24	Male	ALL	Allograft	Brother	Peripheral stem cells
11	41	Female	Myelodisplasia	Allograft	Sister	Peripheral stem cells
12	14	Female	CML	Allograft	Brother	Peripheral stem cells
13	19	Male	AA	Allograft	Brother	Bone marrow
14	33	Male	AML	Allograft	Sister	Peripheral stem cells
15	47	Male	CML	Allograft	Brother	Peripheral stem cells
16	31	Male	CML	Allograft	Brother	Bone marrow
17	33	Male	ALL	Allograft	Sister	Peripheral stem cells
18	20	Male	CML	Allograft	Brother	Bone marrow
19	15	Male	AA	Allograft	Brother	Bone marrow
20	42	Male	CML	Allograft	Sister	Bone marrow
21	54	Female	SAA	Allograft	Brother	Peripheral stem cells

ALL, acute lymphoblastic leukaemia; AA, aplastic anaemia; CLL, chronic lymphocytic leukaemia; AMM, agnogenic myeloid metaplasia; CML, chronic myeloid leukaemia; SAA, severe aplastic anaemia.

36.05 years, ranged from 6 to 67 years old) of normal lower labial mucosa adjacent to mucocele in non-transplanted patients were included as control group. Both groups were paired by age, sex and contained absent/mild inflammatory infiltrate at the epithelium and connective tissue.

Patient protocol

Patients were prepared for transplantation according to the following protocol. Patients with aplastic anaemia received busulphan (1 mg kg^{-1} of body weight) on first day and cyclophosphamide (50 mg kg^{-1} of body weight) during following 4 days. Patients with other diseases received busulphan (4 mg kg^{-1} of body weight) during 4 days and cyclophosphamide (60 mg kg^{-1} of body weight) during following 2 days. Methotrexate and cyclosporine were used for immunosupression after transplant procedure to prevent acute GVHD. None of the patients was using corticoid at the time of oral mucosa biopsy, nevertheless, patients with aplastic anaemia were using cyclosporine 11.6 mg kg^{-1} day^{-1} while the patients with other diseases were using 7.2 mg kg^{-1} day^{-1}.

Immunohistochemical method

hMSH2, hMLH1 and p53 proteins staining were performed by the LSAB kit (DAKO, Carpinteria, CA, USA). Briefly, 4 μm sections were dewaxed in xylene and hydrated with graded ethanol. Removal of formolic pigment was performed. Endogenous peroxidase was blocked by incubating sections in 6% (v/v) H_2O_2/methanol. Slides were placed in 10 mM citrate buffer (pH 6.0) and heated to 96°C in a steamer for 30 min and incubated with the primary antibody for 18 h at 4°C. The primary serum used were anti-hMSH2 (Clone G219-1129; Pharmingen, San Diego, CA, USA) diluted 1:100, anti-hMLH1 (Clone G168-15; Pharmingen) diluted 1:100, and anti-p53 (Clone DO7; DAKO) diluted 1:75 in 20 mmol l^{-1} Tris–HCl buffer (pH 7.4) containing 0.9% NaCl. After washing in the Tris–HCl buffer, sections were incubated for 30 min at room temperature with biotinylated multi-link swine anti-goat, mouse and rabbit immunoglobulin. Sections were washed and incubated for 30 min at room temperature with prediluted streptavidin-peroxidase conjugated. The peroxidase activity was visualized by applying 0.01% diaminobenzidine tetrahydrochloride and 0.03% H_2O_2. Sections were counterstained with Meyer's haematoxylin and mounted in Permount^{TM} (Fischer Scientific, New Jersey, USA). Negative controls consisted of omission of the primary or the secondary antibody or primary incubation in the presence of non-immunized rabbit serum instead of the primary antibody. Immunoreactions were independently analysed by two investigators unaware of the clinical data.

Cell quantification and statistical analysis

The immunolocalization of hMSH2, hMLH1 and p53 proteins were quantitatively analysed. Epithelial cells were considered to be positive if there was any staining of the nucleus, regardless of staining intensity. Cell counting was performed in six high-power microscopic fields (400×), which comprised most of the epithelial tissue. The total number of basal epithelial cells along the six microscopic fields and the number of basal cells stained with each of the antibodies were assessed. The same procedure was done with the suprabasal epithelial cells. As percentage does not conform normal distribution, the Mann–Whitney test was used for statistical analysis. The values were considered significantly different when the *P*-value was less than 0.05.

Results

Positive labelling for hMSH2, hMLH1 and p53 proteins was mainly detected in the basal and intermediate epithelial layers (Figures 1–3). The mean percentage of basal and suprabasal epithelial cells in the experimental and control groups were given in Table 2. While increased immunostaining of basal epithelial cells positive for MSH2 was observed in the BMT group, it was not statistically different from the control group.

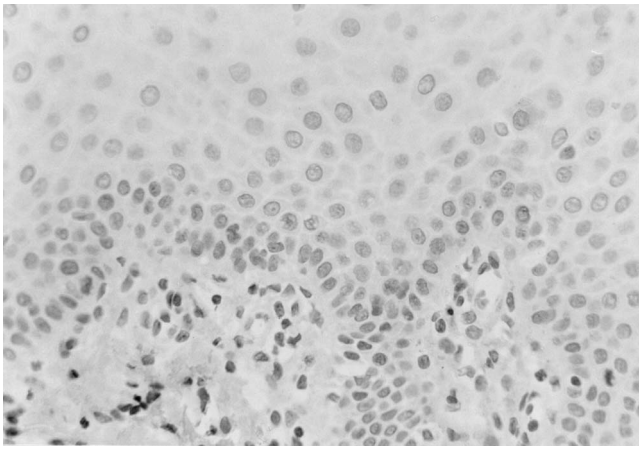

Figure 1 Positive labelling for p53 in the basal epithelial layer of oral mucosa epithelium of BMT patient (streptavidin–biotin amplified system, ×400)

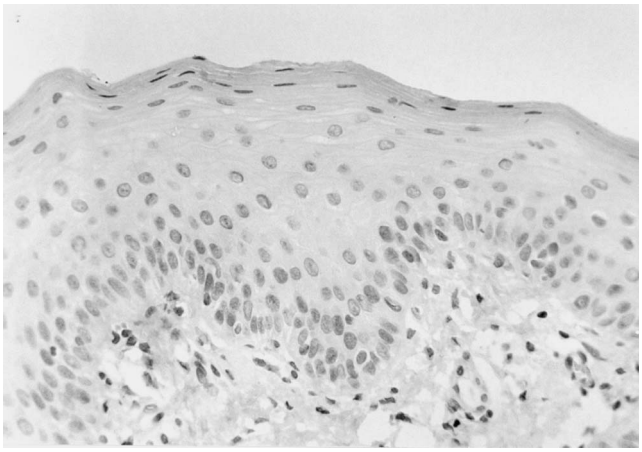

Figure 2 Basal and suprabasal positive labelling for hMSH2 in oral mucosa epithelium of BMT patient (streptavidin–biotin amplified system, ×400)

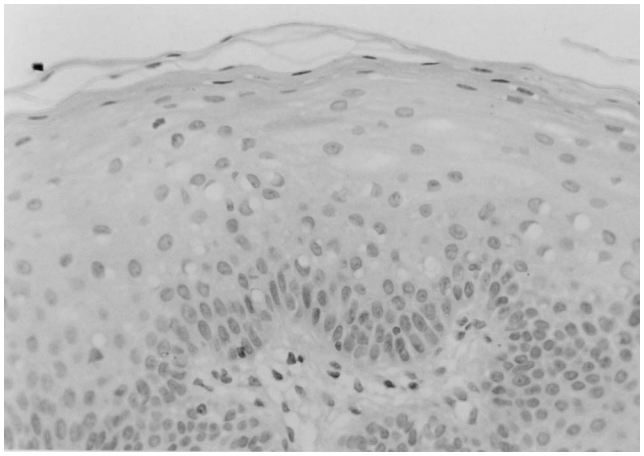

Figure 3 Basal and suprabasal positive labelling for hMLH1 in oral mucosa epithelium of BMT patient (streptavidin–biotin amplified system, ×400)

Table 2 Percentage of hMSH2, hMLH1 and p53 positive cells in the epithelium of oral labial mucosa of BMT patients and non-transplanted healthy controls

	BMT		*Control*		
	Mean	*Median*	*Mean*	*Median*	*P*-value
hMSH2					
Basal	34.3	74	17.3	73.9	n.s.
Suprabasal	20.7	76.8	9.8	68.8	$P = 0.026$
hMLH1					
Basal	70.2	71.1	60.6	65.4	$P = 0.046$
Suprabasal	71.5	75.7	58.7	66.0	$P = 0.018$
P53					
Basal	34.3	34.2	17.3	15.3	$P = 0.02$
Suprabasal	20.7	16.5	9.8	5.7	$P = 0.011$

n.s., Non-significant.

However, the mean number of suprabasal epithelial cells positive for MSH2 was statistically higher than the control. The immunostaining of hMLH1 at the basal and suprabasal epithelial layers was statistically higher in the oral labial mucosa of the BMT patients compared with controls. The same observation was obtained with p53, where increased immunolocalization of it was detected in the oral labial mucosa of BMT group.

Discussion

BMT is frequently the treatment of choice for several malignant and non-malignant haematological diseases. Although most of the secondary malignancies after BMT arise in the haematopoietic tissue, non-haematologic malignancies also occur sporadically. Recently, some cases of oral squamous cell carcinoma associated with c-GVHD have been published (Lishner *et al*, 1990; Curtis *et al*, 1997; Jansisyanont *et al*, 2000). The development of secondary cancers after BMT may be associated with some potential factors including the preoperative regimens of chemotherapy and total body irradiation, c-GVHD, and prolonged immunosuppressive treatment after transplantation (Deeg and Witherspoon, 1993; Boivin *et al*, 1995; Deeg *et al*, 1996; Otsubo *et al*, 1997; Abdelsayed *et al*, 2002).

Several genetic events initiate the progression of normal oral mucosa to squamous cell carcinoma. *p53* is a tumour suppressor gene located at the chromosome 17p considered to be the guardian of the genome. When there is DNA damage, *p53* is activated and induces growth arrest as well as cell death. Mutations of the *p53* tumour suppressor gene play an important role in this process (Raybaud-Diogène *et al*, 1996). Due to its short half-life, the p53 protein is hard to detect in normal tissue. However, occasional positive cells can be detected in normal tissue, especially in the basal layer of the epithelium. In our study, the normal oral mucosa presented positive cells for p53 in a similar pattern as described previously (Dowell and Ogden, 1996; Oijen *et al*, 1999). While positive staining for p53 may be correlated with genetic mutation, the wild protein can also be retained in the tissue by, for example, binding to other proteins or by some defect in the normal degradation pathway, and can therefore be identified by immunohistochemistry (Nylander *et al*, 2000).

In the present study, to avoid the interference of inflammation on the immunolocalization of MMR and p53 proteins, only labial lip biopsies with minimal inflammatory infiltrate were included. Although all the samples from the BMT group were clinically normal oral mucosa, increased immunolocalization of p53 at the basal and suprabasal epithelial layers was found when compared with normal controls. As it was already stated, this could be caused by *p53* mutation, binding of the wild protein to other proteins or stress effect due to DNA damage induced by BMT regimen. Although previous study has demonstrated that oral squamous cell carcinoma in post-transplant patients has low level of *p53* mutation and increased HPV infection (Zhang *et al*, 2002), further analysis is necessary to delineate the participation of HPV on p53 protein immunolocalization in BMT patient's oral mucosa. The increased immunostaining of the MMR system proteins, hMSH2 and hMLH1, in the BMT group indicates the presence of DNA damage probably caused by BMT regimen (busulphan, cyclophosphamide, cyclosporine, etc.). This DNA damage may be related to the increased p53 immunolocalization.

In conclusion, the present study shows that oral epithelial cells of BMT patients show increased immunolocalization of the DNA repair related proteins. Further studies are necessary to delineate the importance of p53 pathway and of the MMR system in oral squamous cell carcinoma tumourigenesis in BMT patients.

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