J Oral Pathol Med (2004) 33: 13–6

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Vascular endothelial growth factor (VEGF) and chronic graft-versus-host disease (cGVHD) in salivary glands of bone marrow transplant (BMT) recipients

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BACKGROUND: The purpose of this study was to compare the immunolocalization of vascular endothelial growth factor (VEGF) in salivary glands of bone marrow transplant (BMT) recipients with normal controls and between the different stages of chronic graft-versus-host disease (cGVHD). In addition, the impact of the immunolocalization of VEGF on the survival rate of BMT patients was investigated as well.

METHODS: Labial salivary glands obtained at the day 100+ from 36 consecutive patients, who underwent BMT, were included in the study. The streptavidin–biotin–peroxidase complex stain was used to detect VEGF in the salivary glands. Time of death after BMT was displayed by means of the Kaplan–Meier method for the following parameters: age and gender of the patients, donor gender, acute GVHD, cGVHD staging at the labial salivary glands, primary disease, platelet and neutrophils counts on day of biopsy, stem cell, oral mucositis, parenteral nutrition, oral lichenoid lesions of GVHD, conditioning regimen and immunolocalization degree of VEGF in labial salivary glands. The data were initially analyzed by means of the log-rank test and then included in the Cox's proportional hazard model.

RESULTS: No differences on the immunolocalization of VEGF in the labial salivary glands of BMT recipients and control group or between the different stages of glandular cGVHD were noted. Both univariate and multivariate analysis of the survival rate showed significance of 5% only for platelet count over 100×10^9/l on the day of biopsy and male donor gender.

CONCLUSIONS: Platelet count over 100×10^9/l and male donor gender are positive predictive factors on the survival rate after BMT. In addition, the immunolocalization of VEGF in salivary glands is not altered in BMT recipients at day 100+ and is not influenced by the stage of cGVHD.

J Oral Pathol Med (2004) **33**: 13–6

Keywords: GVHD; salivary glands; VEGF

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Accepted for publication June 4, 2003

Introduction

Bone Marrow Transplant (BMT) is being used as a therapy for hematologic diseases and other disorders (1, 2). The major cause of morbidity and mortality in patients undergoing BMT is graft-versus-host disease (GVHD), mainly in acute form, when symptoms occur before day 100+, or in a chronic form, after this time (3). Chronic GVHD (cGVHD) commonly appears with oral manifestations subsequent to BMT. These manifestations include oral lichenoid lesions, mucosal atrophy, erythema, ulcers, and xerostomia (4, 5). Mucosal healing problems are associated with GVHD and complications of conditioning regimen (1, 2, 6).

Vascular endothelial growth factor (VEGF) is a potent multifunctional cytokine that exerts several important actions on vascular endothelium (7). The biological effects of VEGF are to induce mitogenesis and migration of endothelial cells, and permeabilize the local microvasculature (8, 9). The expression of VEGF in normal human salivary glands and its secretion into saliva of healthy individuals suggest an important role for this cytokine in the maintenance of homeostasis of mucous membranes (10, 11). Moreover, salivary VEGF permeabilize intraglandular capillaries and thus participate in the regulation of saliva production itself (11). VEGF is considered the most important mediator of angiogenesis during wound healing, and this growth factor has been related to tissue repair, including oral mucosa (12–14).

Considering that cGVHD can cause severe damage to salivary glands that may lead to altered expression of this factor in the saliva, together with the absence of data regarding this subject in the literature, the purpose of the present study was to investigate the impact of BMT on the immunolocalization of VEGF in the salivary glands. In this study, the immunolocalization of VEGF in the salivary glands of BMT recipients was compared to normal controls and was analyzed according to the different levels of GVHD severity. Finally, the impact of the immunolocalization of VEGF on the survival rate of BMT patients was investigated as well.

Materials and methods

The material and methods have been approved by the ethical committee of Universidade Federal de Minas Gerais.

Sample selection

Thirty-six consecutive samples of labial lip biopsies from BMT recipients obtained at day 100+ were retrieved from the files of the Oral Pathology Service, Universidade Federal de Minas Gerais. The labial lip biopsies were performed for GVHD staging at the salivary glands. Thirty-six samples of mucocele biopsies presenting salivary glands from non-transplanted patients were included as control group. The two groups were matched by age and intensity of the inflammatory infiltrate associated with the salivary glands as described below.

Glandular staging of GVHD

The GVHD staging in each sample from the BMT group was performed by the evaluation of the intensity of the inflammatory infiltrate and degenerative changes in accini and ducts of the salivary glands under light microscopy. The samples were classified as mild, moderate or severe according to the count of inflammatory cells in the accini on six microscopic fields (400×). The samples were distributed into three groups according to the total count of inflammatory cells: mild, when inflammatory cells were from 30 to 140; moderate, when there were 141 to 250 cells; and severe in samples with more than 250 cells. The same staging was performed on the control group.

Immunohistochemical methods

The immunohistochemical reactions were performed as described elsewhere (15). Briefly, the tissue blocks were cut at 3 μm and subjected to the biotin–streptavidin-amplified system. As formalin fixation and paraffin wax embedding interfere with immunocytochemical detection of some antigens, microwave stimulation was undertaken to overcome these problems (16). Different protocols of microwave antigen retrieval were tested. Improved immunoreactions were obtained submitting the slides to a microwave (700 W)/citrate buffer (pH 6.0, 10 mM) pre-treatment for 15 min (3×5 min) and then left to cool for 20 min. The sections were then immersed in 3% methanol–hydrogen peroxide solution for 10 min to block endogenous peroxidase activity and incubated with anti-VEGF (rabbit, polyclonal; Biogenex, San Ramon, USA; 17) during 18 h at 4°C with 1% bovine serum albumin. After washing in 20 mM Tris–HCl buffer (pH 7.4) containing 0.19 M NaCl, the sections were (i) incubated at room temperature with biotinylated multilink swine antirabbit immunoglobulin (Dako, Carpinteria, CA, USA) diluted 1 : 150 in Tris–HCl for 30 min, (ii) washed with Tris–HCl two times for 10 min, (iii) incubated for 30 min with horseradish peroxidase-conjugated streptavidin (Dako) diluted 1 : 100 in Tris–HCl, (iv) washed with Tris–HCl two times for 10 min, (v) incubated for 3 min with 0.01% diaminobenzidine tetrahydro-chloride (Sigma, St Louis, MI, USA) and 0.03% H_2O_2 in 20 mM Tris–HCl buffer at pH 7.4, and (vi) rinsed in distilled H_2O for 10 min and counterstained with haematoxylin. Negative controls included sections incubated with non-immune serum at the same concentration as the specific antisera and sections incubated with other antibodies with the same isotype but not specific to VEGF.

Quantitative analysis

The immunolocalization of VEGF was semiquantitatively evaluated using a scale of 1+ to 4+ (+ = 1–25%, ++ = 26–50%, +++ = 51–75%, ++++ = >75% of total accini positive). Six high power fields (400×) were chosen according to the areas almost fully filled by glandular accini and ducts. The same scale was used for the control group. All the sections were independently assessed by the two observers.

The Mann–Whitney test was used to compare immunolocalization of VEGF between labial salivary glands from transplanted and control groups. The Kruskal–Wallis test was used to compare the immunolocalization of VEGF in labial salivary glands between the different stages of glandular GVHD.

Survival analysis

The medical records of the patients were reviewed. Time of death after BMT was displayed using Kaplan–Meier plots for the following parameters: age and gender of the patients, donor gender, acute GVHD, clinical diagnosis of GVHD, labial salivary gland stage of GVHD, primary disease, platelet and neutrophil counts at day 100+, stem cells, oral mucositis, parenteral nutrition, oral lichenoid lesions of GVHD, conditioning regimen and immunolocalization degree of VEGF in labial salivary glands. The results of Kaplan–Meier plots were initially compared by the log-rank test. The factors that showed $P \leq 0.2$ were included in the Cox's proportional hazard model. Statistical significance was set as $P < 0.05$. The cut-off limit of analysis was 24 months.

Results

Immunohistochemical staining

Positive immunolabeling for VEGF was observed mainly in the basal portion of glandular accini, glandular ducts and some inflammatory cells (neutrophils and plasma cells). This pattern of immunoreaction was observed in the salivary glands of transplanted and non-transplanted patients (Fig. 1). The semiquantitative analysis of VEGF in both groups did not show statistical difference. Also, no statistical difference was found regarding the semiquantitative analysis of VEGF on the salivary glands with mild, moderate and severe forms of GVHD.

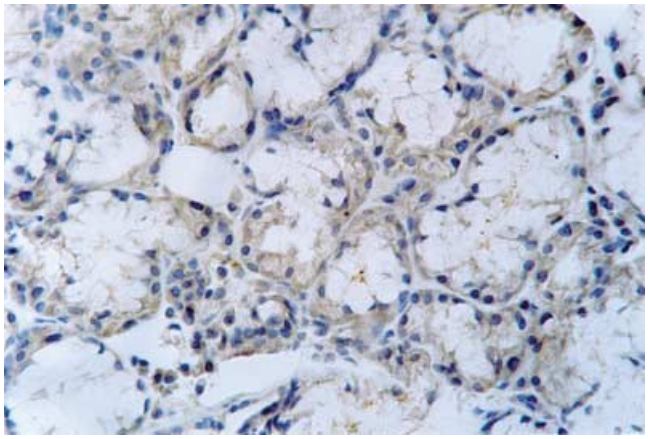

Figure 1 Immunolocalization of VEGF in the basal portion of glandular accini and in some inflammatory cells of a patient affect by cGVHD (streptavidin–biotin-amplified system, 400×).

The baseline characteristics of living and dead patients and the univariate analysis of the clinical parameters analyzed by the log-rank test are given in Tables 1 and 2. The male donor gender ($P = 0.042$) and platelet counts over 100×10^9/l ($P = 0.0009$) were statistically significant as positive predictive factors to survival of BMT recipients. The mean survival time in the group with platelet counts over 100×10^9/l (884.8 days) was higher than the mean of the group with platelet count below 100×10^9/l (520.7 days).

All parameters with P-value <0.2 at the log-rank test were included in Cox's proportional hazard model. The results of multivariate analysis confirmed that both parameters – platelets count over 100×10^9/l ($P = 0.001$) and male donor gender ($P = 0.009$) – were significant.

Table 1 Baseline characteristics of living and dead patients

	Patients		
Parameters	*Dead*	*Living*	*P-value (log-rank test)*
Recipient gender			
Male	3	18	
Female	5	13	0.416
Donor gender			
Male	2	18	
Female	5	11	0.042
Age of patient (years)			
>30	4	16	
≤30	4	20	0.733
Primary disease			
Chronic myeloid leukemia	4	20	
Acute myeloid leukemia	2	5	
Aplastic anemia	1	2	
Other	1	1	0.291
Stem cell			
Marrow bone	1	8	
Peripheral blood	7	20	0.336
Conditioning regimen			
Hematologic diseases	7	26	
Aplastic anemia	1	2	0.829
Platelets number			
$>100 \times 10^9$/l	3	24	
$\leq 100 \times 10^9$/l	5	4	0.0009

Table 2 Baseline characteristics of living and dead patients

	Patients		
Parameters	*Dead*	*Living*	*P-value (log-rank test)*
Acute GVHD			
Yes	1	8	
No	7	20	0.297
Chronic GVHD			
No	6	17	
Mild	1	10	
Moderate/severe	1	1	0.379
Oral lichenoid lesions			
Yes	3	10	
No	5	18	0.969
Immunolocalization degree of SG			
2	3	8	
3	3	10	
4	2	10	0.794
Salivary gland GVHD			
Mild	3	13	
Moderate	4	11	
Severe	1	4	0.89
Parenteral nutrition			
Yes	4	7	
No	4	21	0.101
Oral mucositis			
Absence/mild	3	14	
Moderate/severe	5	14	0.567

Discussion

Pammer et al. (9) reported that the considerable quantities of VEGF found in normal human saliva suggest an important role for this cytokine in the maintenance of the homeostasis of mucous membranes. Although the expression of VEGF in salivary glands has been investigated (9–11), the impact of BMT on its immunolocalization in salivary glands was not established. Our results revealed no statistical difference in VEGF staining between normal salivary glands, used as controls, and glands with cGVHD. In addition, no statistical difference on its immunolocalization was noted between the different stages of cGVHD in the salivary glands. These data reveal that at day 100+, the immunostaining of VEGF at salivary glands is not altered in BMT recipients. However, prospective studies evaluating VEGF in the saliva since the day of the transplantation are imperative to clarify the importance of VEGF in the oral mucosa homeostasis during BMT.

It has been demonstrated that salivary duct glands when associated with inflammation become positive for VEGF (9). In the present study, positive immunolabeling was observed in the ducts of the salivary glands of the control group associated with inflammation and cGVHD. In addition, inflammatory cells also secrete VEGF. Therefore, although salivary gland parenchyma destruction occurs at cGVHD, these sources of VEGF may help to permeabilize intraglandular capillaries stimulating the production of saliva.

Both univariate and multivariate analyses showed that the variable platelet count on the day of biopsy over 100×10^9/l and male donor gender were positive independent prognostic variables for post-transplant survival. Nevertheless, the immunolocalization of VEGF in salivary glands was not a predictive factor for survival rate on BMT recipients. The results related to platelet counts reinforced our previous study with other group of patients submitted to BMT (18). Our findings related to male donor gender are in accordance to previous studies showing that all female donor for a male recipient is a risk factor for GVHD (19, 20).

In conclusion, the present study shows that platelet count over 100×10^9/l and male donor gender are positive predictive factors on the survival rate after BMT. In addition, the immunolocalization of VEGF at the salivary glands is not altered in BMT recipients at day 100+ and is not influenced by the stage of cGVHD.

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Acknowledgements

This study was supported in part by grants from PRONEX, PADCT, FAPEMIG, and CNPq. Dr RS Gomez is research fellow of CNPq.