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Chronic GVHD in minor salivary glands and oral mucosa: histopathological and immunohistochemical evaluation of 25 patients

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BACKGROUND: Graft-vs.-host disease (GVHD) is the major cause of morbidity and mortality in patients undergoing allogeneic Bone Marrow Transplantation (BMT). The aim of our study was to identify the most relevant histological features for diagnosis of chronic Graft-vs.-Host Disease (cGVHD) in oral mucosa and minor salivary glands of 25 patients, as well as to evaluate the immunophenotype of the inflammatory cells.

METHODS: Sixteen patients that were submitted to allogeneic BMT but did not present cGVHD were selected as a control group. The sections were studied on H & E and CD68, CD45, CD4, CD8, CD20 staining.

RESULTS: The most frequent histologic findings in oral mucosa at the day of diagnosis of cGVHD were: hydropic degeneration of the basal layer of the epithelium, apoptotic bodies, lymphocytic infiltration, and focal or total cleavage between the epithelial and connective tissue. In the labial salivary glands (LSG), lymphocytic infiltration, acinar loss and fibrosis were the main alterations. Cytotoxic CD8-T cells and macrophages were predominant both in the epithelium and connective tissue, as well as in minor salivary glands.

CONCLUSIONS: Histological features were useful in the diagnosis of oral cGVHD. It is suggested that CD8-T cells and macrophages play important role in the pathogenesis of the disease.

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Keywords: bone marrow transplantation; CD4; CD8; CD20; CD45; CD68; oral mucosa; pathology; salivary gland

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Introduction

Bone marrow transplantation (BMT) has been used with increasing frequency in the treatment of patients with several hematologic disorders. Graft-vs.-host disease (GVHD) is the major cause of morbidity and mortality in patients undergoing allogeneic BMT (A-BMT) (1).

Depending on the time of disease onset GVHD is divided into acute GVHD (aGVHD), when clinical manifestations appear within 100-day post-transplantation, or chronic GVHD (cGVHD), if the manifestations occur later on (2–4).

The spectrum of clinical findings of cGVHD includes liver dysfunction, pulmonary fibrosis, lichenoid or sclerodermatous skin changes, oral and gastrointestinal mucosal changes, and reduced production of tears and saliva (5).

Erythema, mucosal atrophy, lichenoid changes, mucositis, xerostomia, and infections are common oral findings in cGVHD (1, 6). Pain associated with oral mucositis may be debilitating, leading to dysphagia and total cessation of oral hygiene measures (6).

Oral clinical examination and lip biopsy have been proposed as valuable screening tests for cGVHD diagnosis approximately 3 months after transplantation, due to the high incidence of oral mucosa involvement and the high predictive value, nearly 100% (7–10).

The histological examination of oral mucosa reveals epithelial atrophy with apoptotic bodies, hydropic degeneration of the basal cells, interface mucositis, and a subepithelial lymphocyte infiltrate (1, 11). The LSG may show diffuse/periductal lymphocyte infiltrate, atrophy or destruction of acini, and fibrosis (1, 11).

Our purpose was to study the most relevant histopathological features for oral cGVHD diagnosis and the frequency and immunophenotypical distribution of inflammatory cells in the oral mucosa and LSG of patients with oral cGVHD.

Material and methods

Patients

The files of the BMT Unit of the State University of Campinas were searched for patients who had undergone biopsy of the lip salivary gland (LSG) and oral mucosa (OM) in the late BMT period. Twenty-five patients with diagnosis of oral cGVHD (seven females and nine males, median age 35 (14–54) years) were included in this study. The selected biopsy specimens were those obtained by the date on which the disease was clinically diagnosed. The hematologic diseases treated with BMT, sex and age of patients at day 0 is shown in Table 1. Sixteen A-BMT patients that did not develop cGVHD in any organ composed the control group.

Histologic study of LSG and OM

There were 22 biopsy specimens with both OM and LSG, and three with only LSG. In the control group, 14 biopsy specimens had both OM and LSG, and two had only LSG.

The sections were blindly and independently evaluated by two observers (ABS and MLC). In LSG specimens, the following aspects were evaluated: degree of interstitial lymphocytic infiltration, presence of periductal lymphocytic infiltration, degree of atrophy or destruction of acini and/or ductal epithelium, ductal dilatation, and fibrosis. Lymphocytic infiltration, destruction of the ductal and acini epithelium and fibrosis were graded, according to the severity of involvement, as: absent; slight; moderate or severe. In OM specimens, epithelial changes, such as basal cell degeneration, apoptotic bodies, and cleavage under epithelium, exocytosis, and lymphocytic infiltration of the connective tissue were evaluated. Histologic alterations were graded as absent; slight; moderate or severe.

Table 1 Clinical data

Patient	*Age/Sex*	*Blood disease*	*cGVHD day of diagnosis post-BMT*
1	41/M	MDS	281
2	38/M	AML	100
3	35/M	CML	547
4	20/M	AA	226
5	46/M	MM	167
6	29/F	CML	230
7	42/F	CML	124
8	26/M	PNH	263
9	44/M	CML	100
10	38/F	CML	100
11	43/F	CML	220
12	43/F	CML	582
13	25/M	CML	100
14	24/M	CML	241
15	54/M	CML	210
16	41/M	CML	201
17	24/F	CML	158
18	30/F	CML	224
19	20/M	AML	179
20	44/M	CML	249
21	14/F	ALL	346
22	41/M	CML	100
23	43/M	CML	1006
24	35/M	AML	248
25	42/M	AML	350

MDS, myelo-displastic syndrome; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; AA, aplastic anemia; MM, multiple myeloma; PNH, paroxysmal nocturnal hemoglobinuria; ALL, acute lymphocytic leukemia.

Table 2 Immunomarkers

Antibody	*CD*	*Source*	*Spec/Refer.*
UCHL-1	45Ro	Dako[a]	T, B, M, G (12,13)
OPD4	–	Dako[a]	CD4 (14) M (15)
C8/144	8	Dako[a]	C/S T (16,17)
P6 M1	68	Dako[a]	M (18)
L 26	20	Dako[a]	B (19) T* (20)

[a]Dako Comp., Carpenteria, CA, USA.
Spec/Refer., specificity/references; T, T subsets; B, B lymphocytes; M, monocyte-derived cells; G, granulocytes; C/S T, cytotoxic/suppressor T cells; T*, some normal peripheral blood T cells.

Immunohistochemical study of LSG and OM

Sections of 3 μm were prepared and mounted on silanized glass slides, deparaffinized, and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The antigen retrieval procedure was then performed by placing the sections in a glass container with citric acid (pH = 6.0) that was placed into a microwave oven (Panasonic®, Manaus, Brazil). The sections were then treated by two cycles of 12 min at a high level (1380 W) and incubated for 12 h with primary monoclonal antibodies (Table 2).

Subsequently the sections were incubated with a secondary antibody for 30 min. After this step, the revelation of specimens was performed through diaminobenzidine and counterstaining with Harris hematoxylin. Negative controls were performed on sections of the same specimens that were similarly processed, except that no primary antibodies were used.

All immunostained inflammatory cells and all negative cells were blindly and separately recorded in four different areas within the epithelium and underlying connective tissue through a Carl Zeiss KS400 microscope in high (400×) magnification. In the LSG, the most affected secretory units were chosen. All stained cells, and all negative cells were also recorded in ten different areas. They were analyzed according to the number of positive and negative cells per square unit as much as to the percentage of positive cells.

Statistical analysis

Comparison among proportions was studied through the chi-square method. The averages of dependent and independent variables were compared through Student's *t*-test, with significance level of 0.05.

Results

Histopathologic findings

The most common histopathological features in the OM of cGVHD patients were a moderate lymphohistiocytic infiltration, basal cell degeneration and exocytosis

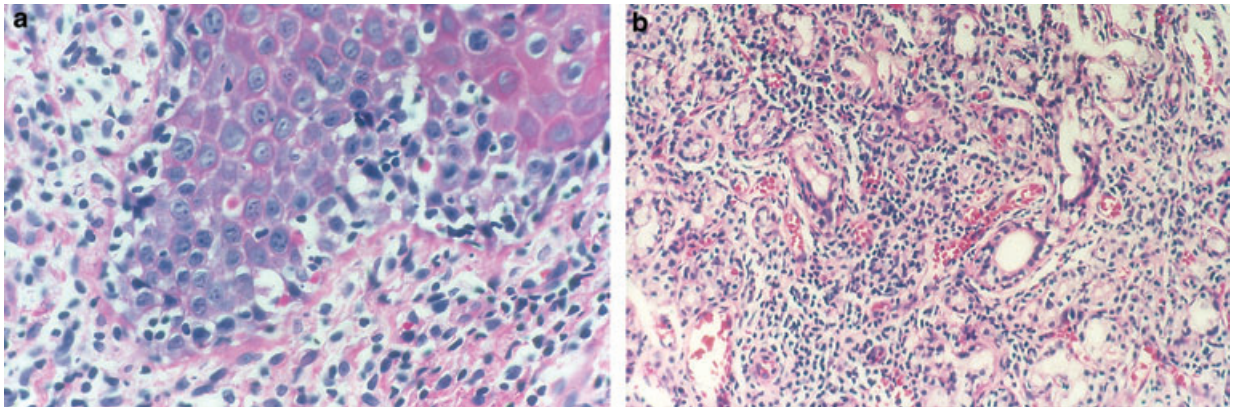

Figure 1 Oral cGVHD in (a) lip mucosa: apoptotic bodies, lymphocytic infiltration, and exocytosis; (b) minor salivary gland: severe lymphocytic infiltration and acinic loss (H & E, original magnification (a) ×400; (b) ×200).

(Fig. 1a). Apoptotic bodies were observed in all cases. Partial cleavage between the epithelial and connective tissues was present in 32% of the cases and total separation in 5% of the cases.

In LSG, 60% of the cases displayed moderate lymphohistiocytic infiltrate. The degree of acini destruction was rather variable: 16% of the cases did not present any sign of atrophy, and the remaining cases had slight (28%), moderate (52%) or severe (4%) atrophy (Fig. 1b). Interstitial fibrosis was slight in most patients. There were no differences in ductal dilatation criteria between patients that had cGVHD and the control group. Several plasma cells were observed in both the OM and the salivary glands.

Immunohistochemical findings

The number of positive cells was different ($P < 0.01$) between the groups for CD68, CD45, and CD8, but not for CD4 and CD20 in the epithelium (Table 3). The same results were found in the connective tissue and LSG, except for the CD4-stained cells that were statistically different between groups ($P < 0.05$). For all markers larger numbers of cells were observed in lip mucosa and glands of cGVHD patients, relatively to control group.

The total number of cells was significantly ($P < 0.001$) smaller within the epithelium, compared to subepithelial tissue for CD68, CD45, CD4, and CD8, but not for CD20. When the number of positive cells in the connective tissue was compared to those in LSG, significant differences ($P < 0.05$) were observed just for CD8 and CD20 cells that were more numerous within lip connective tissue. There were no statistical differences between lip connective tissue and minor salivary glands for CD68, CD45, and CD4-stained cells counting. However, CD8 and CD20-stained cells were more numerous in subepithelial lip connective tissue.

In labial mucosa, CD45 (T) lymphocytes predominated, followed by CD8 (cytotoxic T) cells, CD68 (macrophages), and CD4 cells (Fig. 2a–c). The CD20 (B lymphocytes) cells were seldom seen. In LSG, T (CD45+) cells predominated, followed by macrophages (CD68+), cytotoxic (CD8+) T lymphocytes, and helper (CD4+) T lymphocytes (Fig. 2d–f).

Table 3 Number of immunostained cells in control – and cGVHD group

CD	*Tissue*	*Control group median value (range)*	*cGVHD group median value (range)*
CD68	Oral epithelium+	0.90 (1.31)	4.15 (3.28)**
	Connective tissue+	5.09 (4.98)	13.79 (6.69)**
	Minor salivary gland+	4.06 (4.50)	16.98 (9.80)*
CD45	Oral epithelium+	2.37 (2.83)	7.17 (5.97)**
	Connective tissue+	5.36 (4.44)	24.3 (10.43)**
	Minor salivary gland+	3.27 (3.70)	24.79 (16.23)*
CD4	Oral epithelium	0.03 (0.11)	0.29 (0.78)
	Connective tissue+	1.05 (2.19)	5.33 (5.72)***
	Minor salivary gland+	0.57 (0.80)	5.68 (4.91)*
CD8	Oral epithelium+	1.67 (1.88)	6.07 (5.59)**
	Connective tissue+	2.36 (2.77)	15.7 (12.7)**
	Minor salivary gland+	0.31 (0.48)	11.13 (10.36)*
CD20	Oral epithelium	0.00	0.00
	Connective tissue	0.00	0.10 (0.3)
	Minor salivary gland	0.00	0.90 (2.4)

Median value, +: statistically significant differences by *t*-test.
*$P < 0.001$; **$P < 0.01$; ***$P < 0.05$.

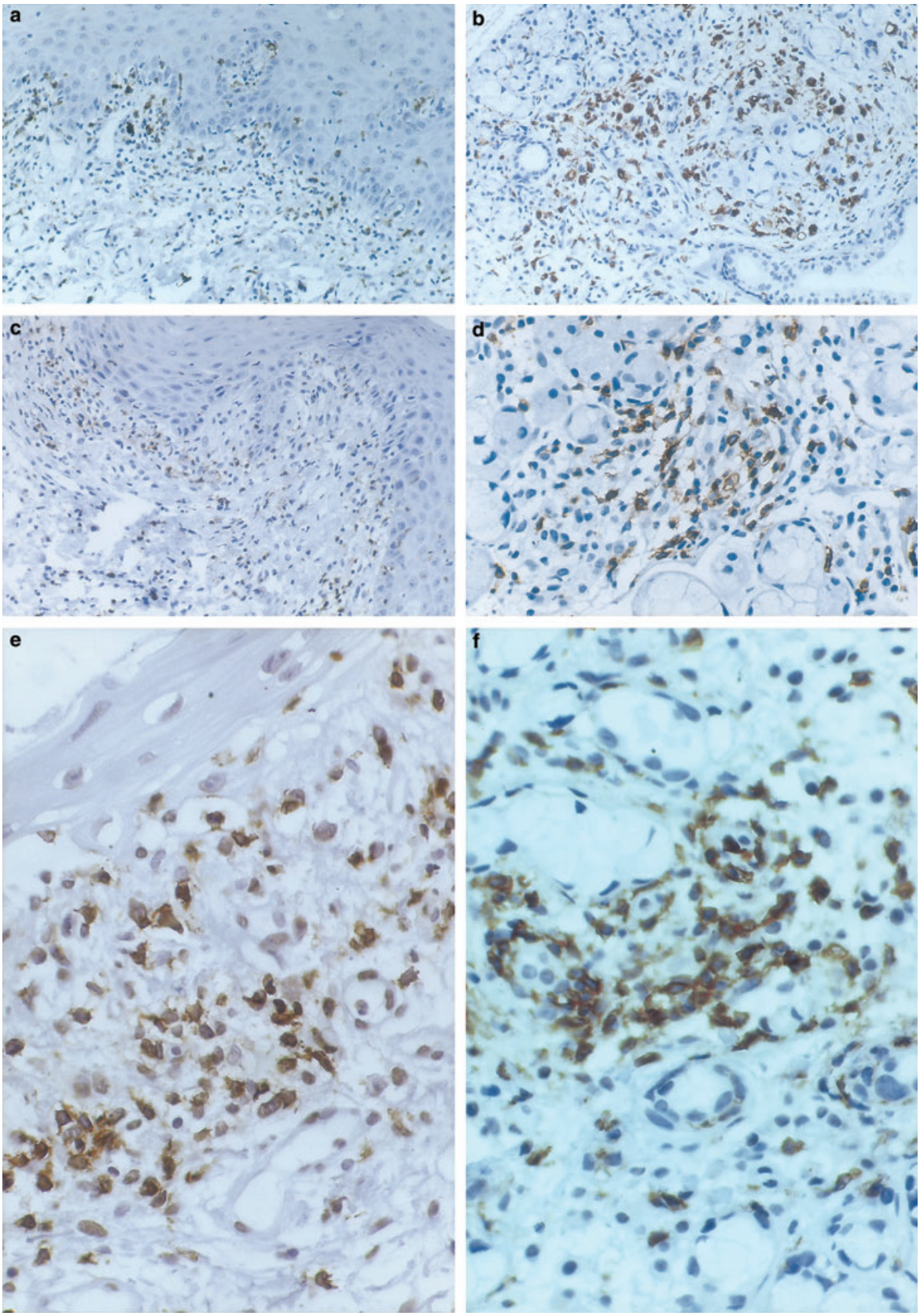

Figure 2 Oral cGVHD in lip mucosa: (a) CD68-positive cells; (c) CD8-positive cytotoxic T lymphocytes; (e) CD45-positive T lymphocytes. Oral cGVHD in minor salivary gland: (b) CD68-positive cells; (d) CD8-positive cytotoxic T lymphocytes; (f) CD45-positive T lymphocytes (original magnification (a), (b) and (c) ×200; (d), (e), and (f) ×400).

Discussion

Chronic GVHD remains a substantial problem after allogeneic BMT. Earlier and more precise diagnosis is important both for predicting the outcome of the disease and determining the optimum treatment at an early stage. Skin and liver manifestations can be confused with other disorders. Oral involvement has been described as one of the first signs or symptoms of the disease. Furthermore, OM and salivary gland alterations have been reported to reflect the status of cGVHD better than other affected organs (5, 21). In that way, it is important to establish the most important histological criteria for diagnosis. The most frequent findings in our study were: basal cells hydropic degeneration, presence of apoptotic bodies, lymphocytic infiltration, and focal or total cleavage between the epithelium and connective tissue. Nakamura et al. (5) reported the presence of hydropic degeneration in 4 of 11 cGVHD patients. In our study, it was seen in 16 of 25 cases (73%) and differences are probably due to the developmental phase of disease.

Moderate lymphocytic infiltrate, variable degrees of acinic atrophy, and slight interstitial fibrosis represented the most common histopathological picture in LSG of chronic GVHD patients. Most authors reported that ductal dilatation is one of the features for the diagnosis of cGVHDc (5, 7, 21, 22). However, in our study there was no difference in ductal dilatation between the patients that had cGVHD and the control group. It is possible that ductal dilatation can be due to the conditioning regimen rather than to cGVHD.

The immunologic mechanisms underlying GVHD are poorly understood. The complex pathophysiology of GVHD fundamentally depends on interactions between antigen-presenting cells of the recipient and T cells of the donor (23). The contribution of cytokines to the inflammation and tissue damage that characterize GVHD, including IL-2, IL-6, IL10, IFN-γ, TNF-α, and IGF has been pointed out (23, 24). Recent studies suggest that small variations in the genes that encode these proteins, called single-nucleotide polymorphisms, determine the relative levels of cytokines under conditions of stress and may therefore predict outcomes after hematopoietic stem-cell transplantation (23). However, an imbalance between effector and suppressor immune functions, self- and non-self-discriminating T lymphocytes can also occur. Host factors such as microbial status or integrity of specific tissues have also been claimed to be involved in the pathogenesis of cGVHD (25).

Salivary glands are susceptible target organs in cGVHD because of their high expression of histocompatibility antigens or their accessibility to pathogenic lymphocytes. In our study, we subdivided the OM in epithelial and connective tissue in order to compare those two tissues with each other and with LSG, relatively to the number of immunostaining cells. The results revealed that LSG are more frequently affected by cGVHD than the OM, as 100% of cGVHD patients presented histologic alterations in labial glands and only 77% in lip mucosa. Our results are in agreement with other authors that compared the significance of mucosa vs. LSG in the histologic diagnosis of cGVHD (5, 21). Therefore, it is important that the LSG be examined to establish the diagnosis and determine the grade of cGVHD.

There are some studies about the T-cell infiltrate in cGVHD of the OM, but results are incongruent. In the OM we observed a pre-dominance of CD8-positive cells in cGVHD patients. Nakamura et al. found a slight pre-dominance of CD8 positive T cells. Conversely, Mattsson et al. (26) and Hasseus et al. (2) reported dominance of CD4 positive T cells. B cells are reported to be virtually absent in oral cGVHD (5), as was observed in our work, although many plasma cells were seen. The reasons for such diversity in T-helper/cytotoxic cells ratio are still not clear, but could be due to diversity of immunohistochemical analysis techniques used, to the scarce number of cases studied by Hasseus and Mattsson (2, 26), and/or to the degree of cGVHD involvement. It was shown that CD4 and CD8 donor T cells induce GVHD in different ways (27). The CD4-mediated GVHD progresses rapidly, whereas CD8-mediated GVHD develops slowly (27, 28). The CD4 T cells are primarily responsible for the induction of acute GVHD, since depletion of CD4 T cells, but not CD8 T cells, from donor inoculum markedly inhibits GVHD mortality (27, 29).

Mattsson et al. (26) reported the macrophage frequency in the inflammatory infiltrate to be less than 1% in oral cGVHD. In our study, those cells constitute a significant part of the infiltrate, suggesting a more important participation in the etiopathogenesis of the disease.

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