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AQP1 expression in human gingiva and its correlation with periodontal and peri-implant tissue alterations

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ABSTRACT

Aquaporins (AQPs) are a family of hydrophobic integral membrane proteins that function as transmembrane channels and play an important role in tissue homeostasis. Aquaporin-1 (AQP1), in particular, has been reported to be involved in several biological processes including inflammation, angiogenesis, wound healing and others. Periodontitis and peri-implantitis can be defined as inflammatory processes that affect the tissues surrounding a tooth or an osseointegrated implant, respectively. To date, there are limited data about the involvement of AQPs in these diseases. The aim of this study was to evaluate the possible link between the histomorphological alterations and the expression of AQP1 in healthy, pathological and healed periodontal and peri-implant gingival tissues. The results obtained showed that changes in organization of collagen fibers were observed in periodontitis and peri-implantitis, together with an increase in the percentage of area occupied by inflammatory cell infiltration and an increase of AQP1 immunostaining, which was located in the endothelial cells of the vessels within the *lamina propria.* Moreover, in healed periodontal and peri-implant mucosa a restoration of histomorphological alterations was observed together with a concomitant decrease of AQP1 immunostaining. These data suggested a possible link between the degree of inflammatory state and the presence of AQP1, where the latter could be involved in the chain of inflammatory reactions triggered at periodontal and peri-implant levels.

Introduction

In recent years, increasing oral implantology research has resulted in a rapid development of this discipline with the osseointegrated implant widely regarded as the best replacement for the lost tooth. Soft tissues surrounding healthy and osseointegrated dental implants share macroscopic anatomical–functional characteristics with the gingival tissue around the teeth, although at a microscopic level there are considerable differences in the histological composition and vascularization (Berglundh et al., 1992). Moreover, implants can incur complications and/or failures due to the same phenomena which, in some cases, lead to the loss of the dental elements, such as occlusal trauma or bacterial colonization (Mombelli, 1997).

Periodontitis and peri-implantitis can be defined as inflammatory processes that affect the tissues surrounding, respectively, a tooth or an osseointegrated implant, and that are responsible for progressive bone resorption over time, which can lead to the loss of the tooth or implant (Page and Schroeder, 1976; Mombelli and Lang, 1998). Conventional tools for the diagnosis of these pathologies including circumferential probing to verify the amount of bone loss, analysis of the mobility of the tooth/implant and radiological examination (Ericsson and Lindhe, 1993), can provide information about the degree of tissue destruction and periodontal and peri-implant disease status, but cannot predict the risk of the progression of these pathologies.

Research has increasingly involved the study and understanding of the molecular and biological mechanisms that are activated during inflammation of the gingival tissues. The involvement of metalloproteinases (MMPs), responsible for the remodeling and degradation of extracellular matrix components, and heat shock

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proteins (HSPs), a group of polypeptides whose expression is increased when cells have to deal with infectious and inflammatory conditions, has been extensively studied in periodontal and peri-implant diseases. Our previous data supported the crucial role of the extracellular matrix in maintaining tissue homeostasis, showing that in peri-implantitis a clearly enhanced immunolabelling of HSP25 and HSP60 was observed in gingival epithelium, indicating a signal of local altered homeostasis (Borsani et al., 2005, 2007).

Aquaporins (AQPs) play an important role in tissue homeostasis. AQPs are a family of hydrophobic integral membrane proteins that function as transmembrane channels through which water crosses the biological membranes of different cell types (King and Agre, 1996). Thirteen mammalian AQPs (AQP0–12) have been identified to date (King and Yasui, 2002; Verkman, 2002) and, although they all share structural similarities, their expression is tissue specific. Different AQP isoforms have been found in various mammalian tissues including the nervous system, kidney, eye, gastrointestinal tract, skin and respiratory tract (Lehmann et al., 2004; Liu and Wintour, 2005; Buffoli, 2010). This family has been highly conserved throughout evolution and is subdivided into two main groups based on structural and functional differences: (1) *Aquaporins* (AQP0, 1, 2, 4, 5, 6 and 8) which pass water through the single pore; (2) *Aquaglyceroporins* (AQP3, 7, 9 and 10) which are permeable to water as well as to neutral solutes, including glycerol and urea (Wang et al., 2003; Verkman, 2009; Loreto et al., 2012). In addition, a third group of AQPs was discovered by Ishibashi (2006) that is called *Superaquaporins* or *Subcellular Aquaporins*. This latter group originally included only AQP11 and AQP12, but the subfamily was later renamed as "*Unorthodox AQPs*" and mammalian AQP6 and AQP8 were also included (Benga, 2009). AQPs have also been proposed to transport other small molecules and gases, including carbon dioxide, ammonia, nitric oxide and hydrogen peroxide (Herrera and Garvin, 2011; Verkman, 2011).

Several studies described AQP involvement in various physiological functions and pathological conditions in different organs (Verkman, 2011). Mice and humans lacking AQP1, which is expressed in the kidney proximal tubule, show defective fluid absorption (Preston et al., 1994; Ma et al., 1998). Mice lacking AQP5, which is expressed in salivary and airway submucosal glands, show defective secretion of saliva and airway mucus (Song et al., 2002; Shen et al., 2011). AQP-facilitated fluid secretion has also been found in the ocular ciliary epithelium, which produces aqueous fluid (Verkman et al., 2008) and in the brain choroid plexus, which produces cerebrospinal fluid (Bonomini and Rezzani, 2010).

AQPs are reported to be also expressed in various tissue of the gastrointestinal tract and orofacial tissues (Ma and Verkman, 1999; Hatakeyama et al., 2001; Wang et al., 2003; Felszeghy et al., 2004; Nandasena et al., 2007; Laforenza, 2012; Loreto et al., 2012; Yoshii et al., 2012), however, only very limited data on AQP expression in gingival tissues have been reported in the literature (Tancharoen et al., 2008).

Angiogenesis, together with inflammatory infiltrate, may be important in the evolution of inflammatory and healing processes involving gingival tissues (Cornelini et al., 2003; Kasprzak et al., 2012). Among the different AQP isoforms, AQP1 has been found to play a fundamental role as a water channel in cell migration, which is central to diverse biological phenomena including inflammation, angiogenesis, wound healing, tumor spreading and organ regeneration (Saadoun et al., 2005; Benga, 2012).

Considering the lack of data regarding the relationship between AQP1 and tissues surrounding teeth and implants, our study aimed to provide further information, evaluating, both qualitatively and quantitatively, the presence and the location of this isoform in the gingival tissues surrounding the teeth and implants in physiological, pathological and healing conditions.

Materials and methods

Patients and collection of the samples

Eighteen healthy and non-smoking patients (six men and twelve women, aged 16–67 years) were selected for this study. The study was approved by the Ethical Committee of "Spedali Civili" Hospital of Brescia and conducted in accordance with the Helsinki Declaration. Informed consent was obtained from all patients.

Patients were classified into six groups ($n = 3$ per group):

(Group 1) Subjects with natural teeth (healthy periodontal tissue): Probing Pocket Depth (PPD) ≤ 3 mm, Bleeding on Probing (BOP) negative (−), no tooth mobility or degree 1 mobility;
(Group 2) Subjects with periodontitis: PPD ≥ 4 mm, BOP positive (+), tooth mobility (degree 2 or 3), possible involvement of the furcation;
(Group 3) Subjects with periodontitis after periodontal therapy (including causal, additional and support therapy): 1 year of maintenance;
(Group 4) Subjects with healthy peri-implant tissue in an osseointegrated and functionalized implant of at least 1 year: PPD ≤ 3 mm, BOP−, no implant mobility, no suppuration and peri-implant marginal bone height preservation;
(Group 5) Subjects with peri-implantitis: PPD ≥ 4 mm, BOP+ and/or suppuration, presence of marginal bone loss in radiographic images, with the possible development of crater-shaped bone defects;
(Group 6) Subjects with peri-implantitis after cumulative interceptive-supportive therapy (CIST protocol): 1 year of maintenance.

Gingival biopsies (2 mm × 2 mm) from the attached tissue around natural teeth and implants were taken by scalpel from the palatal/lingual margin during external gingivectomy surgical procedures. Gingival biopsies of healthy periodontal tissue were obtained from teeth that had to be extracted for orthodontic reasons. The collection of the samples was carried out in all cases after topical anesthesia with 2% mepivacaine and epinephrine 1:100,000. Tissue sample were fixed in 10% buffered formalin (pH 7.0–7.2) for 24 h, conventionally dehydrated and embedded in paraffin wax according to standard procedure. Serial sections (7 μm thick) were cut using a microtome (Microm HM 325, Thermo Scientific, Walldorf, Germany) and collected on poly-L-lysine coated glass slides.

Histomorphological analysis

Sections were deparaffinized in xylene, rehydrated in descending concentrations of ethanol, water and stained with: Hematoxylin–Eosin staining for the evaluation of the inflamed area; Sirius Red staining for assessing connective tissue.

Hematoxylin–Eosin staining

The sections were processed according to the standard protocol. In order to quantify the percentage of inflamed area, a digitally fixed image (standardized arbitrary area) for each section (5 serial sections/subject) was evaluated using an optical light microscope (Olympus BX50, Olympus, Hamburg, Germany) at a final magnification of 100× in a blind study by two investigators unaware of the group assignment. Quantitative analysis of the percentage of inflamed area was performed using a digital color video camera equipped with an image analysis program (Image Pro-Plus, Milan, Italy).

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Sirius Red staining

For Sirius Red staining, the sections were incubated in 1% acid phosphomonolybdic aqueous solution for 5 min and then in 0.1% (w/v) Sirius Red (Direct Red 80, 365548, Sigma–Aldrich, Milan, Italy) in saturated picric acid solution for 60 min, according to Puchler's method (Sweat et al., 1964; Allon et al., 2006) All sections were then analyzed using a light microscope (Olympus BX50, Olympus, Hamburg, Germany) equipped with U-POT polarizer (Olympus, Hamburg, Germany) to analyze the organization of collagen fibers, according to Rodella et al. (2006): under polarized light microscopy, the dense collagen fibers were stained orange-red, whereas the thin collagen fibers appeared green. A qualitative analysis was performed in a blind study by two investigators unaware of the group assignment.

AQP1 immunohistochemistry

AQP1 immunohistochemistry was performed manually and the assay was complete simultaneously for all six groups. Serial sections were deparaffinized in xylene, rehydrated in descending concentrations of ethanol, water and phosphate buffered saline (PBS) and heat treated in 0.05 M citrate buffer solution pH 6.0 (Bio-Optica, Milan, Italy) for antigen unmasking at 98 °C for 20 min and cooled at room temperature (RT) for another 20 min. Endogenous peroxidase activity was blocked by incubation with a solution of 3% hydrogen peroxide for 10 min. Sections were then incubated with normal goat serum (Vector Labs., Burlingame, CA, USA) for 60 min, and serially treated overnight (ON) with rabbit polyclonal antibody against AQP1 (Alpha Diagnostic International, San Antonio, TX, USA) diluted 1:200 in PBS containing 3% normal goat serum and 0.1% Triton X-100. After incubation in the primary antibody, the sections were sequentially incubated with biotinylated anti-rabbit immunoglobulins and avidin–biotin peroxidase complex (Vector Labs, Burlingame, CA, USA). The product of the reaction was visualized using hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich, St. Louis, MO, USA) as chromogen. All sections were counterstained with Hematoxylin, dehydrated and mounted. Control reactions were performed by omitting the primary antibody.

Quantitative analysis of AQP1 immunostaining

The immunohistochemical data were evaluated quantitatively by two blinded investigators, using an optical microscope (Olympus BX50, Olympus, Hamburg, Germany). Digital images (200×, final magnification) of sections were analyzed using a digital color video camera equipped with an image analyzer (Image Pro-Plus, Milan, Italy) and the degree of immunostaining was calculated as integrated optical density (IOD) in 5 fields (standardized arbitrary fields) for each subject (5 serial sections/subject).

Double immunofluorescence analysis: AQP1 and CD141

The sections were deparaffinized in xylene, rehydrated in descending concentrations of ethanol, water and phosphate buffered saline (PBS) and heat treated in 0.05 M citrate buffer solution pH 6.0 (Bio-Optica, Milan, Italy) for antigen unmasking at 98 °C for 20 min and cooled to RT for another 20 min. Endogenous peroxidase activity was blocked by incubation with a solution of 3% hydrogen peroxide for 10 min. The sections were incubated in 5% bovine serum albumin (BSA) diluted in PBS containing 0.1% Triton X-100 for 30 min and then incubated ON in rabbit polyclonal primary antibody directed against AQP1 (1:200, Alpha Diagnostic International, San Antonio, TX, USA) mixed with mouse monoclonal primary antibody CD141 Thrombomodulin (1:25, Neomarkers, Fremont, CA, USA), an integral membrane protein expressed on the surface of the endothelial cells. After incubation in the primary antibody, the sections were sequentially incubated with appropriate fluorescent secondary antibodies diluted 1:200 in PBS (anti-rabbit, Alexa-Fluor 555, red fluorescent dye and anti-mouse Alexa-Fluor 588, green fluorescent dye, Invitrogen, Carlsbad, CA, USA). The co-localization was evaluated on digitally images acquired with laser scanning confocal microscopy (LSM 510, Zeiss, Oberkochen, Germany).

Statistical analysis

For statistical comparison among the experimental groups, the data were analyzed by ANOVA test corrected by Bonferroni ($P < 0.05$ considered statistically significant). The results obtained for all analyses are presented as means ± SE.

Results

Histomorphometric analysis: Hematoxylin–Eosin staining

The histomorphometric analysis of the Hematoxylin–Eosin stained sections revealed a significant increase ($P < 0.05$) in the percentage of inflamed area in the *lamina propria* of the samples from subjects with periodontitis and peri-implantitis compared to healthy periodontal and peri-implant tissues (group 2 *vs* group 1: 10.04 ± 0.75 *vs* 3.25 ± 0.41; group 5 *vs* group 4: 10.99 ± 0.64 *vs* 2.27 ± 0.43). The percentage of inflamed area was restored in healed periodontal and peri-implant tissues after appropriate therapy (group 3: 2.16 ± 0.27; group 6: 3.21 ± 0.3). See Fig. 1.

Histomorphological analysis: Sirius Red staining

Under polarized light microscopy, the dense collagen fibers were stained orange-red and the thin collagen fibers appeared green (Rodella et al., 2006). The staining was present in the *lamina propria* of all groups, whereas the epithelium was negative. In the healthy tissues (groups 1 and 4), the collagen fibers were well organized showing mainly green fibers, which were constitutively present (Fig. 2A and D). In the samples from subjects with periodontitis and peri-implantitis (groups 2 and 5), the collagen fibers appeared disorganized without any directionality and an increase in orange-red fibers, which characterized the fibrotic process, was observed (Fig. 2B and E). In healed periodontal and peri-implant mucosa (groups 3 and 6), a partially restored pattern was observed and mainly collagen fibers appearing green were present (Fig. 2C and F), similarly to the healthy tissues.

Qualitative and quantitative evaluation of AQP1 immunostaining

Immunohistochemical staining showed that the AQP1 immunostaining positivity was restricted to the endothelial cells of blood vessels within the *lamina propria* of gingival tissue for all groups (Fig. 3). The quantitative analysis allowed us to calculate and compare the values of integrated optical density (IOD) of AQP1 immunostaining among the different groups. The data obtained showed that AQP1 immunostaining increased significantly ($P < 0.05$) in the samples from subjects with periodontitis and peri-implantitis respect to healthy periodontal and peri-implant tissues, respectively: group 2 *vs* group 1: 33.09 ± 0.51 *vs* 22.32 ± 0.57; group 5 *vs* group 4: 38.06 ± 0.84 *vs* 30.7 ± 0.71. In healed periodontal mucosa the AQP1 immunostaining decreased significantly ($P < 0.05$) compared to the samples from subjects with periodontitis (group 3 *vs* group 2: 27.95 ± 0.97 *vs* 33.09 ± 0.51), but it was also significantly ($P < 0.05$) higher compared to the healthy group: group 3 *vs* group 1: 27.95 ± 0.97 *vs* 22.32 ± 0.57. Similar

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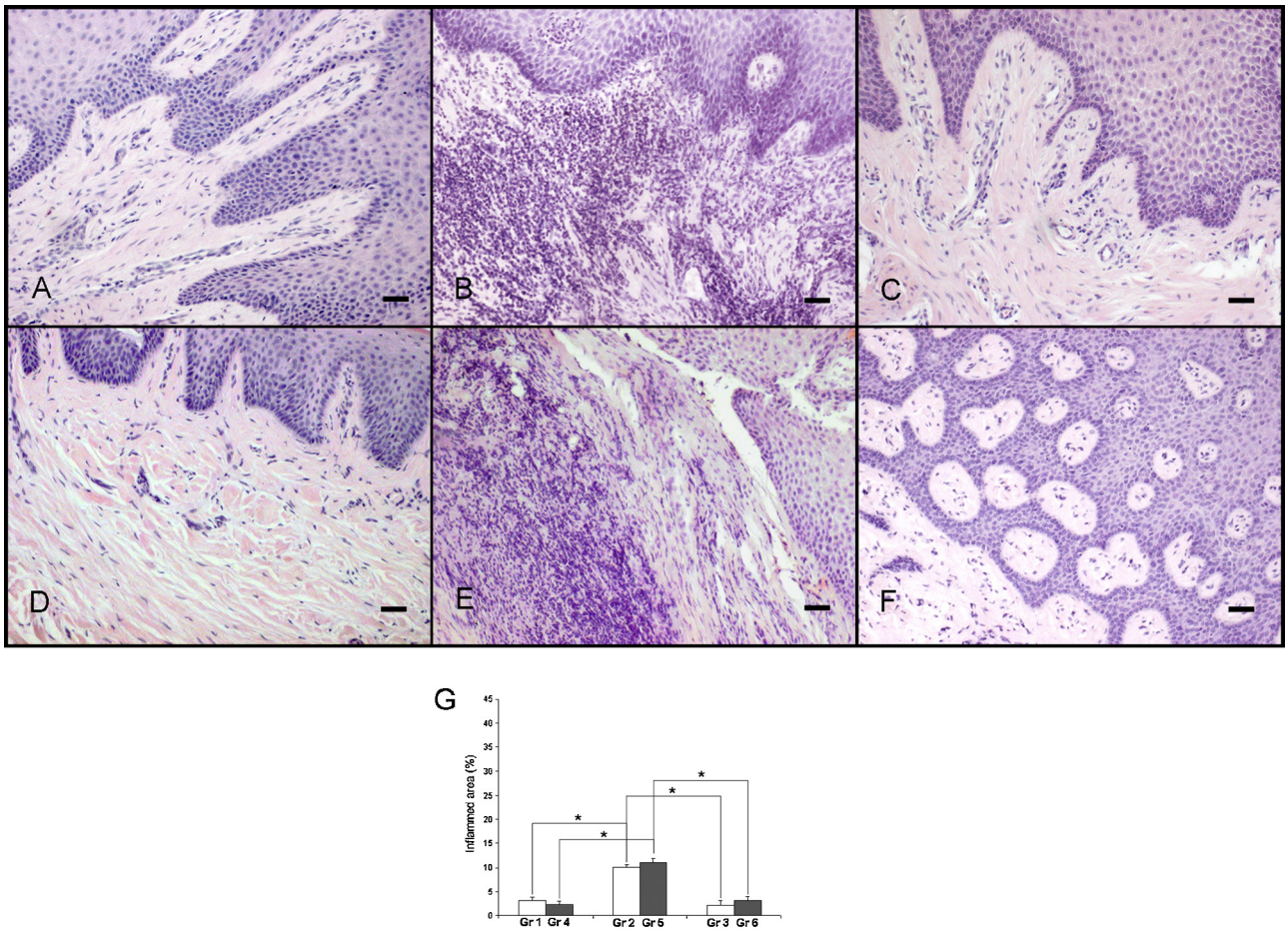

Fig. 1. Hematoxylin–Eosin staining. (A) Healthy periodontal tissue, Group 1. (B) Periodontitis, group 2. (C) Healed periodontal tissue, group 3. (D) Healthy peri-implant mucosa, group 4. (E) Peri-implantitis, group 5. (F) Healed peri-implant tissue, group 6. (G) Quantitative analysis of inflamed area (%), $^{*}P < 0.05$. Scale bar = 40 μm.

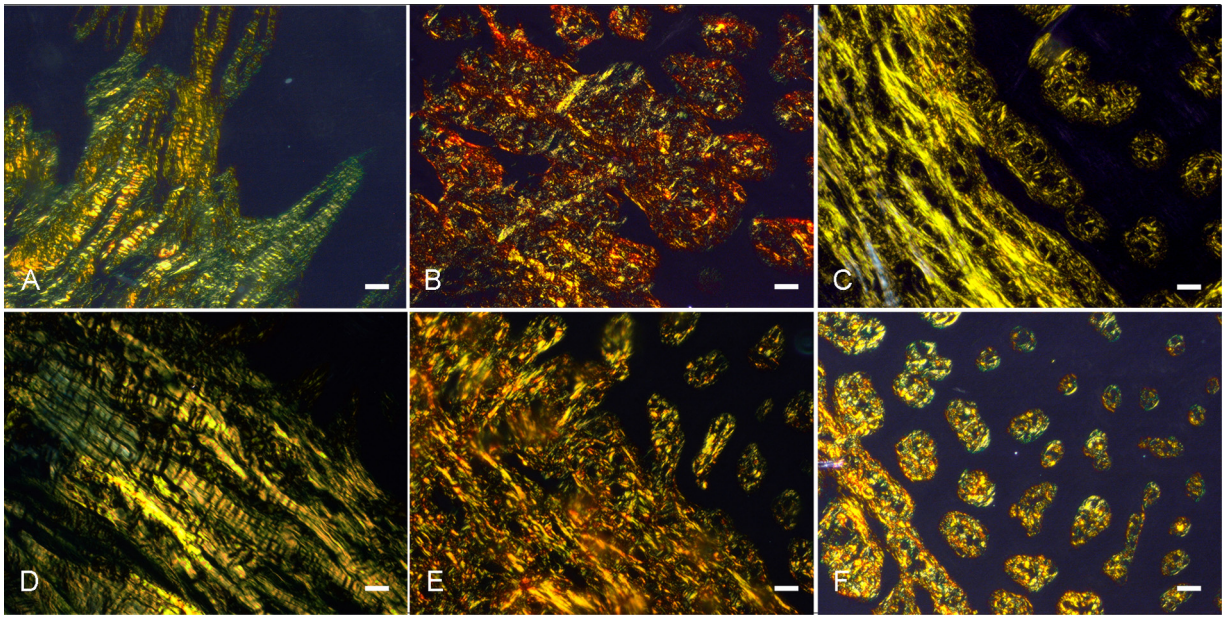

Fig. 2. Sirius Red staining. (A) Healthy periodontal tissue, Group 1. (B) Periodontitis, Group 2. (C) Healed periodontal tissue, Group 3. (D) Healthy peri-implant mucosa, Group 4. (E) Peri-implantitis, Group 5. (F) Healed peri-implant tissue, Group 6. Scale bar = 40 μm.

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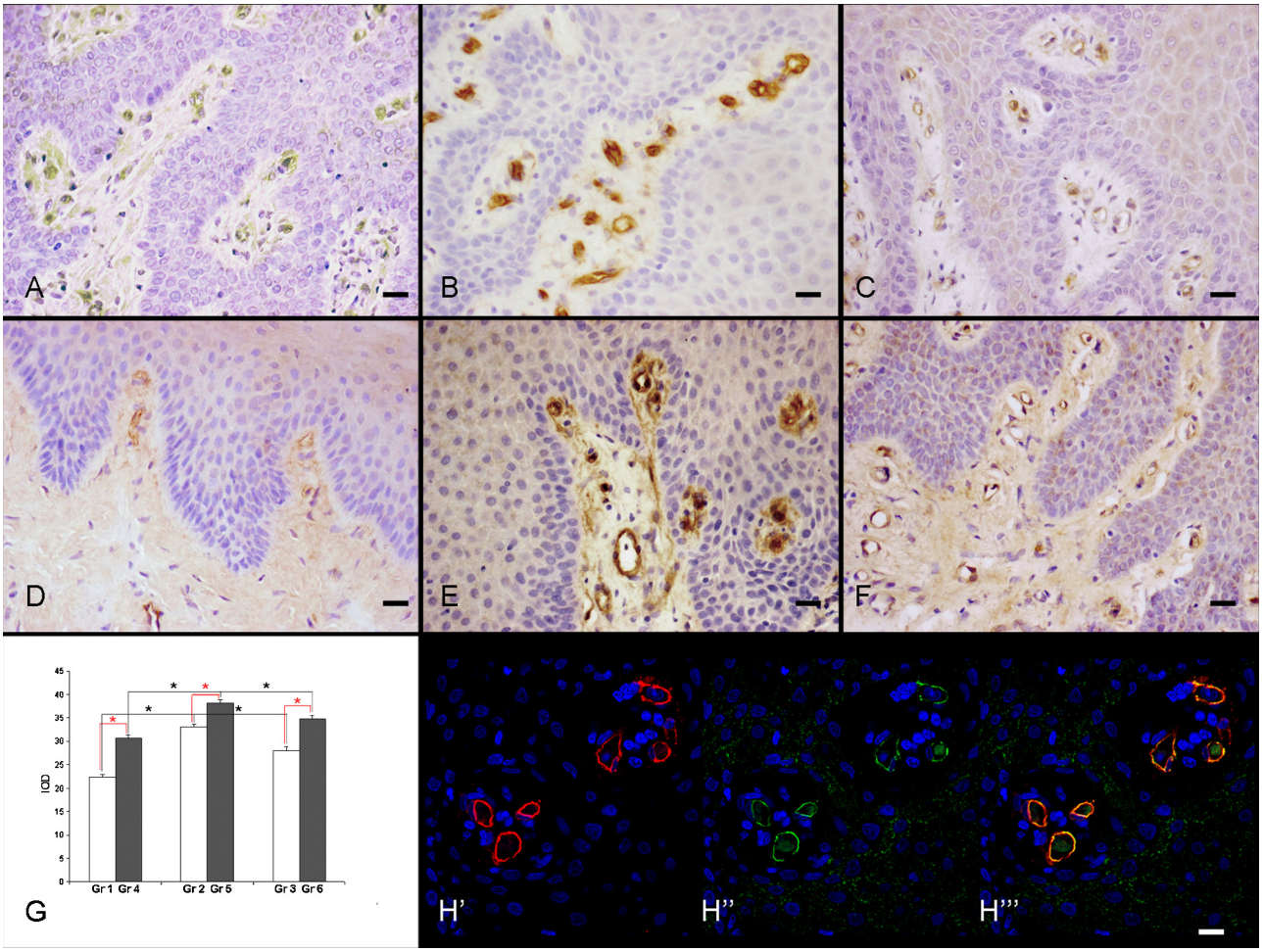

Fig. 3. AQP1 immunohistochemistry. (A) Healthy periodontal tissue, Group 1. (B) Periodontitis, Group 2. (C) Healed periodontal tissue, Group 3. (D) Healthy peri-implant mucosa, Group 4. (E) Peri-implantitis, Group 5. (F) Healed peri-implant tissue, Group 6. (G) Quantitative analysis of AQP1 immunostaining (IOD) $^{*}P < 0.05$. (H) Co-localization of AQP1 (red color, H′) and CD141 (green color, H″) immunofluorescence; overlapping (H‴). Scale bars: A–F = 20 μm; H = 15 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

results were observed in peri-implant tissues: in fact, we observed that AQP1 immunostaining was significantly lower ($P < 0.05$) in the healed peri-implant tissue compared to the samples from subjects with peri-implantitis: group 6 *vs* group 5: 34.74 ± 0.68 *vs* 38.06 ± 0.84; nevertheless, it was significantly higher ($P < 0.05$) compared to the healthy group: group 6 *vs* group 4: 34.74 ± 0.68 *vs* 30.7 ± 0.71 (Fig. 3A–F). Moreover, comparing the periodontal and the peri-implant tissues, AQP1 immunostaining was significantly lower ($P < 0.05$) in the former then the latter. The quantitative data of AQP1 immunostaining are summarized in Fig. 3G.

Double immunofluorescence analysis confirmed the AQP1 localization in blood vessels, showing a co-localization of AQP1 with CD141 (Fig. 3H).

Discussion

Whereas several studies in dentistry focused on the bone surrounding teeth and implants (Tehemar et al., 2003; Molly et al., 2004; Novaes et al., 2004; Papalexiou et al., 2004; Spiechowicz et al., 2004), relatively few studies have investigated the soft tissues, which are the first to be altered in cases of pathology and are often critical for teeth and implant integrity (Berglundh et al., 1992; Nanci and Bosshardt, 2006).

In response to a bacterial challenge, gingival tissue can elicit cytokines and lead to dramatic changes in surrounding periodontal tissue homeostasis (Okada and Murakami, 1998). Among the different molecules involved in tissue inflammation, AQPs have been reported to play a role in the progression and resolution of the inflammatory status. In fact, some studies conducted in mice lacking AQP1 demonstrated the role of this protein in peritoneal membrane inflammation, showing a decrease of vascular proliferation and leukocyte recruitment in this animal model (Nishino and Devuyst, 2008). In addition, previous reports showed that Aqp1−/− mice showed decreased angiogenesis and a significant attenuation of the inflammatory infiltrate. In addition, it is reported that AQPs play a fundamental role in cell migration and, consequently, in the different several biological phenomena, such as angiogenesis, wound healing, tumor spread and organ regeneration (Saadoun et al., 2005).

At least eight AQPs are reported to be expressed in various tissue of the gastrointestinal tract, including AQP1, 3, 4, 5, 7, 8, 9, 10 and 11 (Ma and Verkman, 1999; Hatakeyama et al., 2001; Laforenza, 2012). In particular, AQP transcripts are present in the mucosa of the human gastric pyloric antrum and body, in the mucosa of human duodenum and ascending colon, where they play a crucial role in maintaining body water homeostasis and ensure digestive and absorptive functions (Laforenza, 2012). Moreover, AQP expression profiles seem to vary in different human orofacial tissues, such as fetal teeth, masseter muscle, infrahyoid muscle, Meckel's cartilage, gingiva, submandibular gland and temporomandibular joint

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disk (Wang et al., 2003; Loreto et al., 2012). The presence of AQPs in human and mouse tooth germs (Felszeghy et al., 2004) and in rat periodontal tissue was previously reported (Nandasena et al., 2007; Yoshii et al., 2012); in addition, the expression of AQP3, AQP7 and AQP10 in human gingiva by RT-PCR analysis was reported (Wang et al., 2003). Although it is conceivable that these channel proteins maintain oral tissue homeostasis, very limited data on AQP expression in gingiva have been reported in the literature. In particular, a study (Tancharoen et al., 2008) showed that AQP3 expression was located circumferentially on the epithelial cell boundaries in the basal and suprabasal gingival layers and it was up-regulated in periodontitis lesions, suggesting a possible role for AQP3 in the regulation of gingival epithelial cell migration and in periodontal pocket formation.

Our histomorphological data showed that changes in organization of collagen fibers were observed in periodontitis and peri-implantitis, together with an increase in the percentage of area occupied by inflammatory cell infiltration. During gingival and peri-implant mucosa inflammation, in fact, the cell infiltrate occupies an increasing proportion of the connective tissue and might cause a collagen degradation or a fibrotic reaction (Borsani et al., 2005) by stimulative effects of inflammatory mediators of the connective tissue (Page and Schroeder, 1976; Younes et al., 2009). These alterations could be due to changes in the tissue homeostasis that could be related to both an increase in inflammatory cell infiltration and extracellular matrix remodeling (Baniţă et al., 2008). So, considering that among the different AQP isoforms, AQP1 has been reported to be involved in several biological processes including inflammation, angiogenesis, wound healing, tumor spread and organ regeneration (Saadoun et al., 2005; Nishino and Devuyst, 2008; Benga, 2012), the aim of this study was to evaluate the possible alteration in AQP1 expression in healthy, pathological and healed periodontal and peri-implant tissues.

Our results showed that AQP1 immunostaining was co-localized with CD141 and was located in the endothelial cells of the vessels within the *lamina propria*, where it is closely associated with the vascular permeability and may be involved in the interstitial and vascular fluid transfer in the surrounding mucosal tissue (Nielsen et al., 1993; Verkman, 2002; Saadoun et al., 2005; Tancharoen et al., 2008).

AQP1 immunostaining increased significantly with periodontitis and peri-implantitis and was partially restored after appropriate therapy in both diseases; in fact, even if there was a significant decrease of AQP1 immunostaining compared to the subjects with periodontitis and peri-implantitis, significantly higher values were found for healed periodontal and peri-implant tissues respect the healthy groups. These results were overlapped with the percentage of inflamed area calculated in the same groups, suggesting a possible link between the degree of inflammatory state and the presence of AQP1, where the latter could be involved in the chain of inflammatory reactions triggered at periodontal and peri-implant levels. These data are in agreement with other previous studies that showed a correlation between AQP1 expression and inflammation in different human and animal models, such as in the peritoneal tissue of peritoneal dialysis patients and in inflammatory sublaryngeal edema in rat (Oh et al., 2011; Liu et al., 2012).

In addition, considering that some evidences suggested a role of AQP1 for mediating the hydration changes accompanying the deposition of extracellular matrix (Shanahan et al., 1999), the concomitant increase of AQP1 expression, the changes in collagen fiber organization and, in particular, the increase in orange-red fibers which characterized the fibrotic process, during periodontotis and peri-implantitis may be linked; these relationships could be also supported by the decrease in AQP1 expression corresponding to a concomitant decrease of the red color fibers in healed periodontal and peri-implant tissues. Moreover, some evidence has supported the possible relationship between the expression of AQP1 and the synthesis of extracellular matrix in atherosclerotic plaques (Shanahan et al., 1999).

In conclusion, our results support a correlation between AQP1 expression and the histomorphological alterations observed in periodontal and peri-implant soft tissues. These preliminary data will be supported by increasing the sample size of the study, with the purpose of being able to define a further, and more sensitive, prognostic index of periodontal and peri-implant disease.

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