Proteomic Profiling and Differential Messenger RNA Expression Correlate HSP27 and Serpin Family B Member 1 to Apical Periodontitis Outcomes

Franco Cavalla, DDS, MS,[*†‡] *Claudia Biguetti, DDS, MS,*[†‡§] *Sameer Jain, BDS, MS,*[§] *Cleverick Johnson, DDS, MS,*[¶] *Ariadne Letra, DDS, MS, PhD,*[‡§‖] *Gustavo Pompermaier Garlet, DDS, MS, PhD,*[†] *and Renato Menezes Silva, DDS, MS, PhD*[‡§]

Abstract

Introduction: Understanding protein expression profiles of apical periodontitis may contribute to the discovery of novel diagnostic or therapeutic molecular targets. **Methods:** Periapical tissue samples (n = 5) of patients with lesions characterized as nonhealing were submitted for proteomic analysis. Two differentially expressed proteins (heat shock protein 27 [HSP27] and serpin family B member 1 [SERPINB1]) were selected for characterization, localization by immunofluorescence, and association with known biomarkers of acute inflammatory response in human apical periodontitis (n = 110) and healthy periodontal ligaments (n = 26). Apical periodontitis samples were categorized as stable/inactive (n = 70) or progressive/active (n = 40) based on the ratio of expression of receptor activator of nuclear factor kappa-B ligand (*RANKL*)/osteoprotegerin (*OPG*). Next, the expression of *HSP27*, *SERPINB1*, C-X-C motif Chemokine Receptor 1 (*CXCR1*), matrix metalloproteinase 8 (*MMP8*), myeloperoxidase (*MPO*), and cathepsin G (*CTSG*) messenger RNA was evaluated using real-time polymerase chain reaction. Data analysis was performed using the Shapiro-Wilk test, analysis of variance, and the Pearson test. P values <.05 were considered statistically significant. **Results:** Proteomic analysis revealed 48 proteins as differentially expressed in apical periodontitis compared with a healthy periodontium, with 30 of these proteins found to be expressed in all 4 lesions. The expression of HSP27 and SERPINB1 was ~2-fold higher in apical periodontitis. Next, an increased expression of HSP27 was detected in epithelial cells, whereas SERPINB1 expression was noted in neutrophils and epithelial cells. *HSP27* and *SERPINB1* transcripts were highly expressed in stable/inactive lesions (P < .05). Significant negative correlations were found between the expression of *HSP27* and *SERPINB1* with biomarkers of acute inflammation including *CXCR1*, *MPO*, and *CTSG*. **Conclusions:** Our data suggest HSP27 and SERPINB1 as potential regulators of the inflammatory response in apical periodontitis. Additional functional studies should be performed to further characterize the role of these molecules during the development/progression of apical periodontitis. *(J Endod 2017;43:1486–1493)*

Key Words

Apical periodontitis, gene expression, heat shock protein 27, protein expression, SERPINB1

Significance

Understanding protein expression profiles of periapical lesions may contribute to the discovery of novel diagnostic or therapeutic molecular targets.

Apical periodontitis is the inflammatory destruction of periradicular tissues caused by persistent microbial infection within the root canal system. Factors contributing to persistent apical periodontitis include inadequate aseptic control, poor access cavity design, missed canals, inadequate instrumentation, and defective temporary or permanent restorations (1). However, it has been recently proposed that an imbalance in the expression of genes involved in healing and genetic predisposition can also contribute to persistent apical periodontitis (2).

Chronic inflammation is the hallmark of apical periodontitis and drives the pathological changes that lead to tissue destruction in the periapical environment as well as the progression of the lesion. Even after conventional endodontic treatment, the persistence of infectious stimuli from residual microorganisms or their antigens can perpetuate the inflammatory process, precluding the return to homeostasis, regression, and healing (3).

From a clinical perspective, the ability to distinguish between a progressive lesion and a stable lesion that is undergoing regression and healing is critical to determine the need of additional treatment interventions, including periapical surgery. Currently, the decision of additional treatment is based on previous treatment history along with clin-

From the *Department of Conservative Dentistry, School of Dentistry, University of Chile, Santiago, Chile; †Department of Biological Sciences, School of Dentistry of Bauru, University of São Paulo, Bauru, São Paulo, Brazil; ‡Center for Craniofacial Research, University of Texas School of Dentistry at Houston, Houston, Texas; and §Department of Endodontics, School of Dentistry at Houston, ¶Department of General Practice and Dental Public Health, and ‖Department of Diagnostic and Biomedical Sciences, University of Texas Health Science Center at Houston, School of Dentistry at Houston, Houston, Texas.

Address requests for reprints to Dr Renato Menezes Silva, Department of Endodontics, University of Texas School of Dentistry at Houston, 7500 Cambridge Street, Suite 6411, Houston, TX, 77054. E-mail address: renato.m.silva@uth.tmc.edu

0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2017.03.014

ical and radiologic signs and symptoms evaluated during follow-up visits. Additional diagnostic tools such as the use of a molecular signature of apical periodontitis would provide clinical benefits in terms of determining when complex interventions are needed (4).

Proteomic analysis of apical periodontitis tissues represents a powerful and unbiased approach to identify key molecules that are differentially expressed and produced within the lesions (5). These molecules might be key components of the biological pathways involved in lesion resolution or progression.

In the present study, we performed proteomic analysis of apical periodontitis and healthy periodontal tissue samples using 2-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry. Selected differentially expressed proteins were further investigated using quantitative real-time polymerase chain reaction (PCR) and immunofluorescence. We also verified the association of selected protein targets with the expression of known biomarkers of acute inflammatory response.

Material and Methods

Sample Population

This study was approved by the Institutional Review Boards at the University of Texas Health Science Center at Houston, Houston, TX, and the University of São Paulo (Faculdade de Odontologia de Bauru Universidade de São Paulo), Bauru, São Paulo, Brazil. Patients were recruited from the endodontics and oral surgery clinics at these institutions. The subjects were patients with apical periodontitis (N = 114, 19–61 years of age) presenting with periapical rarefaction characterized radiographically by the disappearance of the periodontal ligament space and discontinuity of the lamina dura. All patients were referred for endodontic surgery after conventional root canal treatment had failed. Patients with medical conditions requiring the use of systemic modifiers of bone metabolism or other assisted drug therapy (ie, systemic antibiotics, anti-inflammatory drugs, and hormone therapy) 6 months before initiation of the study, patients with preexisting conditions such as periodontal disease, and pregnant or lactating women were excluded from the study. At the time of surgery, periapical biopsies were collected, and each specimen was divided into 2 fragments of similar size and stored in 10% formalin or rinsed in phosphate-buffered saline (PBS) and immediately frozen at −80°C. Samples stored in 10% formalin were submitted to routine histologic processing, serial sectioning for hematoxylin-eosin staining, and histopathological analysis. All experimental samples were histologically diagnosed as apical granulomas with or without epithelium (72%) and radicular cysts (28%). The clinical diagnosis included previously endodontically treated teeth (100%) with asymptomatic apical periodontitis (43%), symptomatic apical periodontitis (19%), and chronic apical abscess (38%).

Healthy periodontal ligament tissues were used as control tissues and were collected from individuals referred for the extraction of premolars for orthodontic reasons (n = 27, aged 19–24 years). In total, 114 human apical periodontitis samples and 27 healthy periodontal ligaments were collected postoperatively and used in this study.

2D-DIGE

Five tissue samples were submitted to 2D-DIGE. Of these, 4 periapical tissue samples were from cases with a histopathological diagnosis of a periapical granuloma and a clinical diagnosis of a previously endodontically treated tooth with a chronic apical abscess (n = 2) and a previously endodontically treated tooth with asymptomatic apical periodontitis (n = 2). One healthy periodontal ligament tissue obtained from a premolar extracted for orthodontic purposes was used as the control specimen.

Samples were incubated in 2-dimensional cell lysis buffer (30 mmol/L Tris-HCl (ThermoFisher, Waltham, MA), pH = 8.8, containing 7 mol/L urea, 2 mol/L thiourea, and 4% CHAPS [ThermoFisher]), sonicated at 4°C, and kept in a shaker for 30 minutes at room temperature. Samples were then centrifuged at 25,000g for 30 minutes at 4°C, and the supernatants were collected. Protein concentration was measured using the Bradford method (6). Each sample was diluted in 2-dimensional cell lysis buffer to a protein concentration of 8 mg/mL. We incubated 30 ng protein concentrate from each sample with 1 μL size/charge-matched CyDye (GE Healthcare Life Sciences, Marlborough, MA) at 0.2 nmol/μL for 30 minutes in ice inside a dark chamber. We added 1 μL lysine 10 mmol/L to the mixture and incubated for additional 15 minutes. Samples were labeled, mixed with buffer, and actively rehydrated as described elsewhere (7). The conditions for isoelectric focusing were as follows: 300 V for 2 hours, 500 V for 2 hours, 1000 V for 2 hours, and 8000 V for 8 hours. We equilibrated the focused strips, rinsed them in running buffer, and performed the second-dimension electrophoresis in a 12% agarose sodium dodecyl sulfate gel at 10 mA for approximately 30 minutes.

Image scans were performed immediately using Typhoon TRIO (GE Healthcare Life Sciences). Scanned images were analyzed using Image QuantTL software (GE Healthcare Life Sciences) and subjected to in-gel and cross-gel analysis using DeCyder software version 6.5 (GE Healthcare Life Sciences). Changes in protein expression between lesions and control tissue and between individual lesions were obtained from cross-gel DeCyder software analysis. Protein spots of interest were picked up by an Ettan Spot Picker (GE Healthcare Life Sciences) based on the in-gel analysis and spot-picking design using DeCyder software. The protein spots were washed and digested in gel with Trypsin Gold–modified porcine trypsin protease (Promega, Madison, WI). The digested tryptic peptides were further desalted by Zip-tip C18 (Millipore, Billerica, MA); peptides were eluted with 0.5 μL matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid, and 25 mmol/L ammonium bicarbonate) and spotted on a matrix-assisted laser desorption ionization-time of flight mass spectrometry.

Mass Spectrometry and Database Search

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry and time-of-flight/time-of-flight tandem mass spectrometry were performed on a 5800 mass spectrometer (AB Sciex, Framingham, MA). Matrix-assisted laser desorption ionization–time-of-flight mass spectra were acquired in the reflectron positive ion mode (8). Time-of-flight/time-of-flight tandem mass spectrometry fragmentation spectra were acquired for each sample, averaging 2000 laser shots per fragmentation spectrum on each of the 5 to 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Both the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer (version 3.5; Matrix Science, Boston, MA) equipped with the MASCOT search engine (Matrix Science) to search the database of the National Center for Biotechnology Information nonredundant (9) or the Swiss Protein database (10). Searches were performed without constraining the protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with 1 missed cleavage allowed in the search parameters. Only candidates with either a protein score confidence interval or an ion confidence interval greater than 95% were considered.

Microscopic Analysis

Heat shock protein 27 (HSP27) and serpin family B member 1 (SERPINB1) were selected as targets for additional investigation based

on their ~2-fold overexpression in apical periodontitis compared with healthy tissue and considering their putative regulatory role in inflammation.

Formalin-fixed paraffin-embedded tissue samples of nonepithelialized apical granulomas, epithelialized apical granulomas, and apical cysts were obtained from our in-house tissue collection and sectioned at 6 μm for standard hematoxylin-eosin staining and immunolocalization of HSP27 and SERPINB1. Formalin-fixed paraffin-embedded sections were cleared in xylene and rehydrated in gradated alcohol baths. Antigen retrieval was performed in 10 mmol/L sodium citrate buffer (pH = 6) at 96°C for 5 minutes. Sections were then permeabilized with 0.5% Triton X-100 (ThermoFisher) in PBS, blocked with 1% bovine serum albumin in 10% goat serum, and incubated with either rabbit polyclonal antihuman HSP27 (cat no. AB5579; Abcam, Cambridge, UK) or rabbit monoclonal antihuman SERPINB1 (cat no. AB181084, Abcam) antibody overnight at 4°C. Sections were washed in PBS and incubated with Alexa 488 goat antirabbit secondary antibody (ThermoFisher), counterstained with 4',6-diamidino-2-phenylindole, and mounted. Images were acquired using an Eclipse Ni-U upright fluorescence microscope (Nikon, Tokyo, Japan) equipped with a Zyla 5.5 sCMOS camera (Andor Technologies, Belfast, UK).

Quantitative Real-time PCR

We measured the messenger RNA (mRNA) levels of *HSP27*, *SERPINB1*, receptor activator of nuclear factor kappa-B ligand (*RANKL*), osteoprotegerin (*OPG*), C-X-C motif chemokine receptor 1 (*CXCR1*), matrix metalloproteinase 8 (*MMP8*), myeloperoxidase (*MPO*), and cathepsin G (*CTSG*) in 110 periapical granulomas and 26 healthy periodontal ligament tissue samples as previously described (11). The total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). RNA quality was analyzed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and complementary DNA synthesis was achieved using 3 μg RNA/sample as a template in a reverse transcription reaction (QuantiTectRT Kit, Qiagen).

Samples were categorized as either progressive/active (n = 40) or stable/inactive (n = 70) based on the ratio of *RANKL/OPG* expression as previously described (12). Next, mRNA levels of *HSP27*, *SERPINB1*, *CXCR1*, *MMP8*, *MPO*, and *CTSG* were evaluated using on-demand TaqMan gene expression assays (Invitrogen, Carlsbad, CA) in a Viia7 Real Time PCR instrument (LifeTechnologies, Carlsbad, CA). The reaction conditions were as follows: 40 cycles at 95°C for 10 minutes, 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes. Results are depicted as the expression levels of target genes relative to the expression of housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase [*GAPDH*] and β-actin) in each sample using the $2^{-\Delta\Delta Ct}$ method (13).

Statistical Analysis

The Shapiro-Wilk test was used to test the distribution of the data, whereas analysis of variance was used to test the differences in the relative expression of the investigated genes between lesions and control tissues and between progressive and stable lesions. We also used the Pearson correlation to analyze the association between selected variables. All statistical analyses were performed in Stata/IC 14.1 (Stata Corp, College Station, TX) and GraphPad Prism 7.0 (GraphPad, La Jolla, CA). Depending on the analysis, fold changes greater than or around 2 or P values <.05 were considered statistically significant.

Results

2D-DIGE

The complete data obtained from the database search including the top-ranking protein name, accession number, molecular weight, isoelectric point, peptide count, and Mascot scores are provided as Supplementary Material.

We found 48 differentially expressed proteins between the control tissue and the 4 apical periodontitis samples (Supplemental Table S1 is available online at www.jendodon.com). Of these, 30 were present in all 4 lesions; 19 of 30 (63.3%) proteins were down-regulated, whereas 11 of 30 (36.6%) were up-regulated (Table 1). These peptides were further categorized based on the 12 putative functional pathways in which they belong as follows: 15 (50%) were cytoskeleton proteins (actin cytoplasmic 2, annexin A1, gelsolin, involucrin, keratin type I cytoskeletal 13, keratin type I cytoskeletal 14, keratin type I cytoskeletal 16, keratin type II cytoskeletal 1, keratin type II cytoskeletal 2 oral, keratin type II cytoskeletal 3, keratin type II cytoskeletal 4, keratin type II cytoskeletal 5, keratin type II cytoskeletal 6A, tropomyosin alpha-4 chain, and vimentin), 3 (10%) were blood plasma proteins (apolipoprotein A-I, serotransferrin, and serum albumin), 2 (6.6%) were antioxidant defense system enzymes (superoxide dismutase mitochondrial and thioredoxin), and 2 (6.6%) were cell cycle regulating proteins (14-3-3 protein sigma and protein S100-A9); the remaining 8 peptides included a Ca^{+2} signaling protein (calreticulin), a protein biosynthesis molecule (elongation factor 2), a metabolic enzyme (alpha-enolase), a transport protein (fatty acid–binding protein, epidermal), a serine protease inhibitor family member (SERPINB1), a stress response protein (HSP27), a coagulation cascade protein (protein-glutamine gamma-glutamyltransferase E), and an immune response protein (immunoglobulin gamma-1 chain C region).

HSP27 and SERPINB1 expression was up-regulated in apical periodontitis tissues by 3.79-fold and 1.93-fold, respectively, when compared with the control. Because of their previously reported role in inflammation and apical periodontitis (14–16), these proteins were selected for further characterization and association analysis with known acute inflammatory response markers (*CXCR1*, *MMP8*, *MPO*, and *CTSG*) (17–20).

Microscopic Analysis

Microscopic analysis of the hematoxylin-eosin–stained apical periodontitis sections revealed the presence of granulomatous tissue with a dense fibrous capsule, fibroblasts, and a large area filled with chronic and acute inflammatory infiltrate. This infiltrate was composed by lymphocytes intermixed with neutrophils, macrophages, and plasma cells (Fig. 1*A–L*). Three different patterns of apical periodontitis were noted: nonepithelialized (Fig. 1*A–D*) and epithelialized (Fig. 1*E–H*) granulomas and apical cysts (Fig. 1*I–L*). Highly epithelialized granulomas contained granulation tissue infiltrated by scattered and nonorganized epithelium cords. An apical cyst exhibited a typical cavity-lining epithelium (ie, a hyperplasic, nonkeratinized, and stratified epithelium surrounded by a thick granulation tissue) (21).

Immunofluorescence assays revealed the expression of HSP27 and SERPINB1 in apical periodontitis (Fig. 2). HSP27 expression was not detected in nonepithelialized apical granulomas (Fig. 2*A–D*); however, an increased expression was noted in the epithelia of epithelialized granulomas and apical cysts (Fig. 2*E–L*, respectively). In the case of epithelialized granulomas, the expression of HSP27 was confined to the hyperplasic nonkeratinized epithelial cords infiltrating the granulomatous tissue (Fig. 2*E–H*), with epithelial cells showing a homogenous cytoplasmic immune staining in all layers. Apical cysts showed a similar pattern of immune staining, with a strong cytoplasmic signal in all layers of the epithelium lining the cystic cavity (Fig. 2*K* and *L*).

Similarly, SERPINB1 was expressed by the epithelial cells of epithelialized granulomas and apical cysts (Fig. 2*F'* and *J'*, respectively). The staining pattern was cytoplasmic and homogeneous in all layers

TABLE 1. Pathways Regulated in Apical Periodontitis Compared with Control Samples

Pathway	*N* (%)	*N* (%) up-regulated	*N* (%) down-regulated
Antioxidant defense system	2 (6.6)	1 (50)	1 (50)
Blood plasma protein	3 (10)	3 (100)	0
Ca+2 signaling	1 (3.3)	1 (100)	0
Cell cycle regulator	2 (6.6)	0	2 (100)
Coagulation cascade	1 (3.3)	0	1 (100)
Cytoskeleton	15 (50)	3 (18.75)	12 (81.25)
Immune response	1 (3.3)	1 (100)	0
Metabolic enzyme	1 (3.3)	0	1 (100)
Protein biosynthesis	1 (3.3)	0	1 (100)
Serpin family member	1 (3.3)	1 (100)	0
Stress response	1 (3.3)	1 (100)	0
Transport protein	1 (3.3)	0	1 (100)
	Total	11 (36.6)	19 (63.3)

of the hyperplasic epithelia. In the epithelialized granuloma, strong staining was evident in the scattered islets and cords of epithelia (Fig. 2*F'* and *G'*). In the apical cyst, the immune staining followed the same pattern, and the signal was evident in all layers of the epithelia lining the cystic cavity (Fig. 2*I'–L'*).

SERPINB1 was also expressed by the infiltrating polymorph nuclear neutrophils (PMNs) in the nonepithelialized granuloma, epithelialized granuloma, and apical cyst (Fig. 2*D'*, *H'*, and *L'*). The staining was very specific to PMNs, and no other infiltrating cell showed positive staining. In the nonepithelialized granuloma, the PMNs were clustered in defined sections of the lesion surrounded by other infiltrating leukocytes and granulomatous tissue (Fig. 2*A'–D'*). In the epithelialized granuloma, the infiltrating PMNs were located preferentially in the central part of the lesion (Fig. 2*G'* and *H'*). The apical cyst also showed clustered infiltration of PMNs in the central portion of the lesion (Fig. 2*I'*). Some PMNs transmigrated through the epithelia into the cystic cavity (Fig. 2, *arrow* in *L'*).

Expression Analyses

Increased levels of *HSP27* and *SERPINB1* were detected by quantitative real-time PCR in inactive lesions when compared with the control

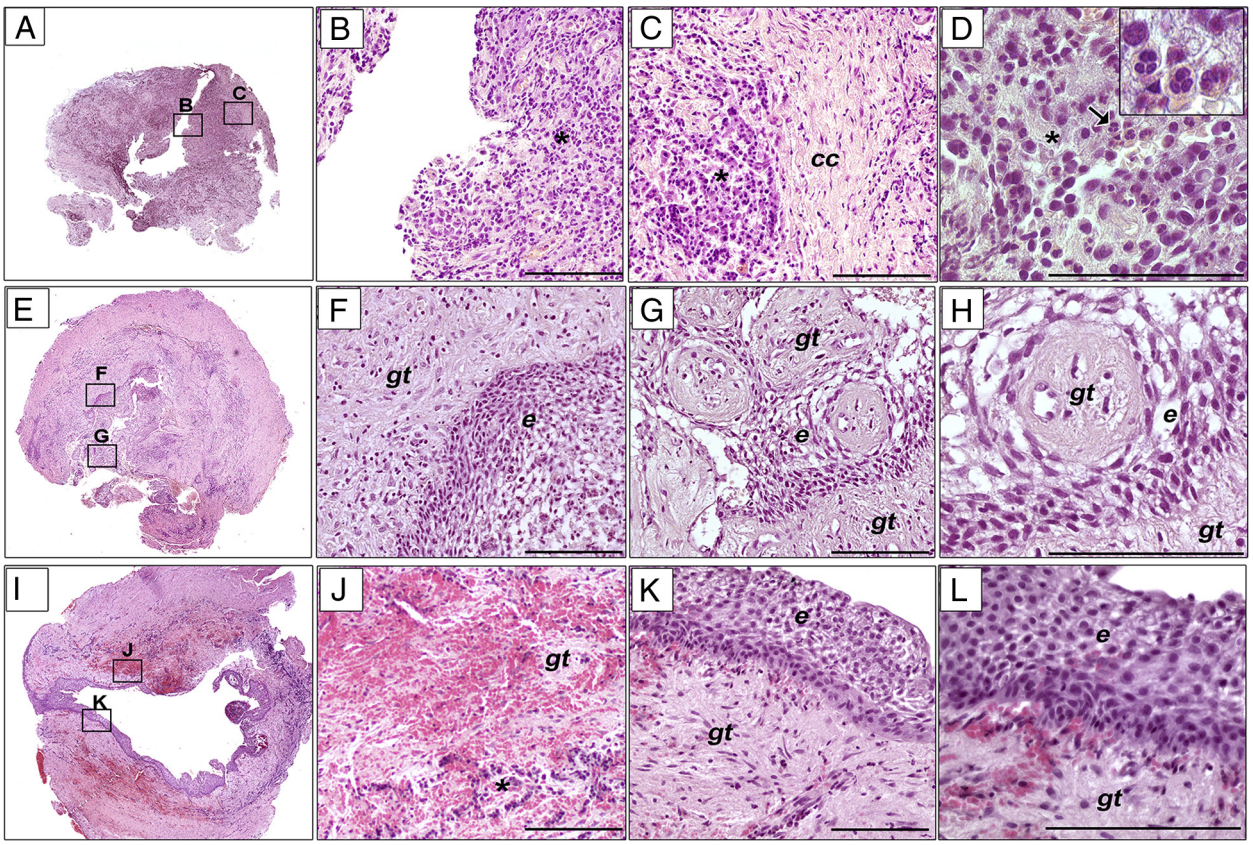

Figure 1. Histophatological characterization of inflammatory apical periodontitis. (*A–L*) A mix of inflammatory cells (*) including neutrophils (*arrows*) were predominant in all lesions. (*A–D*) A nonepithelialized granuloma with a clear decrease of inflammatory infiltrate into the thick collagenous capsule (cc). (*E–H*) A highly epithelialized granuloma containing granulation tissue (gt) enclosed by nonkeratinized epithelium (e) cords. (*I–L*) An apical cyst lined by a hyperplasic, nonkeratinized, stratified epithelium and a granulation tissue (gt) surrounding the lesion. Hematoxylin-eosin stain. Scale bar = 250 μm.

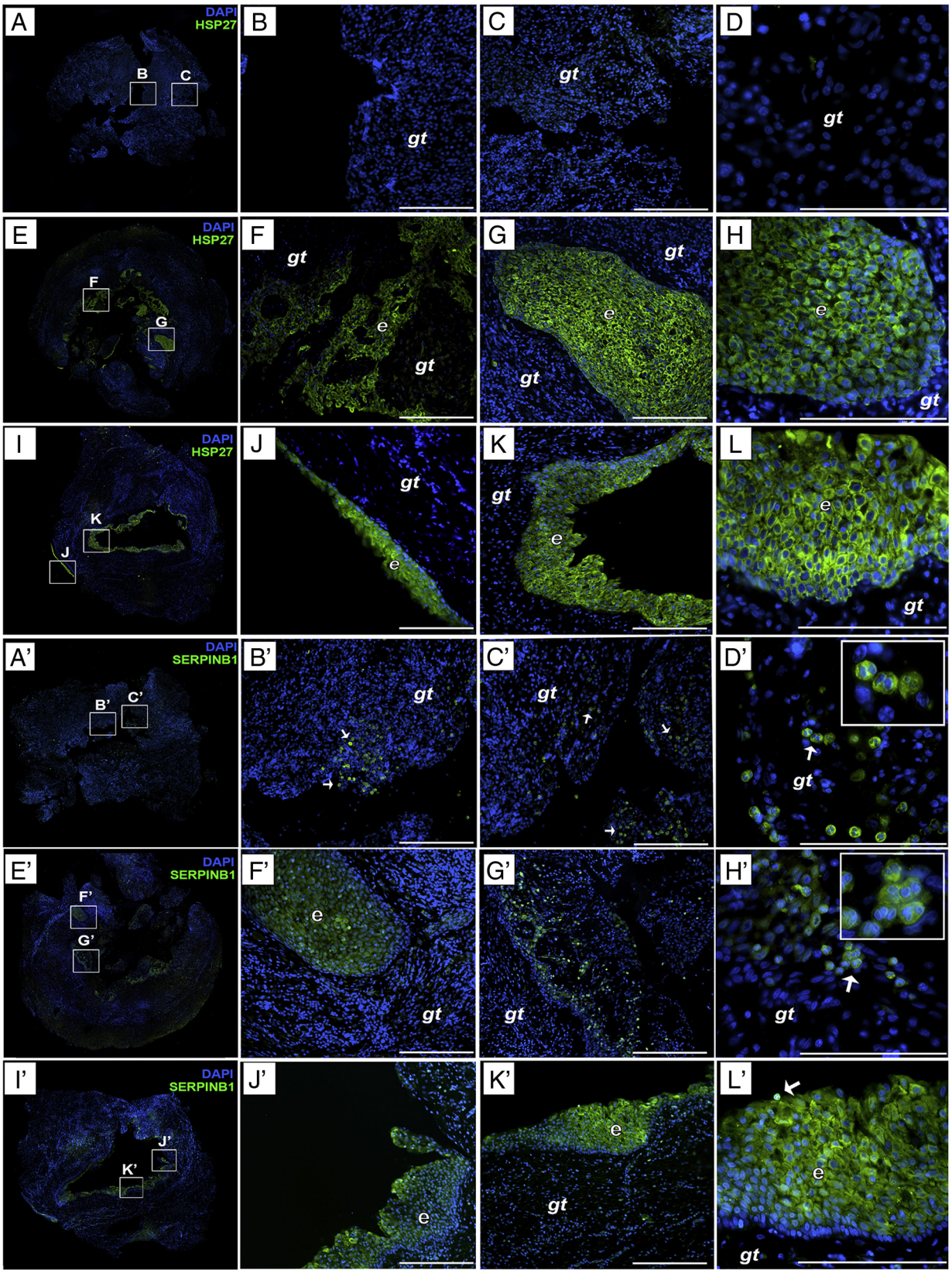

Figure 2. The expression of HSP27 and SERPINB1 in inflammatory apical periodontitis (*A–D* and *A'–D'*: nonepithelialized granuloma, *E–H* and *E'–H'*: epithelialized granuloma, and *I–L* and *I'–L'*: apical cyst). (*A–I*) For HSP27, there was a high expression of HSP27 in the cytoplasm of epithelial cells (e). (*A'–I'*) For SERPINB1, there was a high expression in the cytoplasm of neutrophils (*arrows*) and the epithelial cells (e). All cells present in the granulation tissue (gt) and epithelium (e) were identified using DAPI nuclear staining (blue). Scale bar = 250 μm.

tissue ($P < .05$) (Fig. 3*A*). A significantly higher expression of *HSP27* and *SERPINB1* was also detected in inactive lesions in comparison with active lesions ($P < .05$) (Fig. 3*B*). Moreover, a significant positive correlation was noted between *HSP27* and *SERPINB1* expression levels in apical periodontitis ($r^2 = 0.12$, $P < .001$) (Fig. 3*C*).

We then tested the potential association between *HSP27* expression levels with the expression of known acute inflammation biomarkers and detected significant negative correlations with *CXCR1* ($r^2 = 0.12$, $P < .001$), *MPO* ($r^2 = 0.06$, $P = .007$), and *CTSG* ($r^2 = 0.08$, $P = .002$) (Fig. 3*D*). Likewise, *SERPINB1* showed significant negative correlations with *CXCR1* ($r^2 = 0.09$, $P = .001$), *MMP8* ($r^2 = 0.12$, $P < .001$), and *MPO* ($r^2 = 0.15$, $P < .001$) and a significant moderate (−) correlation with *CTSG* ($r^2 = 0.52$, $P < .001$) (Fig. 3*E*).

Discussion

In this study, we used proteomic analysis as an unbiased approach to identify differentially expressed proteins in apical periodontitis in comparison with healthy control tissue. Overall, of the 48 proteins identified as differentially expressed, 30 were found to be expressed in all 4 lesions. Of these, 19 of 30 (63.3%) were down-regulated, whereas 11 of 30 (36.6%) were up-regulated. Most of these peptides belonged to biological pathways with functions related to the cytoskeleton, antioxidant defense system, and cell cycle, whereas a few peptides have suggested functions in calcium signaling, biosynthesis and metabolism, protein transport, inhibition of proteases, stress response, immune response, and coagulation cascade.

Interestingly, most of the differentially expressed proteins in apical periodontitis appeared down-regulated, and these were mainly related to cytoskeleton assembly and cell-cell interaction pathways. Although speculative, we may argue that the down-regulation of cytoskeleton proteins might reflect the tissue collapse and disorganization resultant of the chronic inflammatory process affecting the apical tissue during the progression of apical periodontitis. Recently, chronic inflammation has been linked to cytoskeletal changes, and direct links between cytoskeletal proteins and proinflammatory signaling are beginning to emerge in the literature (22).

The up-regulated proteins included an antioxidant enzyme, 3 blood plasma proteins, a calcium signaling protein, 2 cytoskeleton proteins, an immune response protein, a serpin family member, and a stress response protein. We focused our analysis on 2 of the significantly up-regulated proteins, HSP27 and SERPINB1, because of their putative roles as key

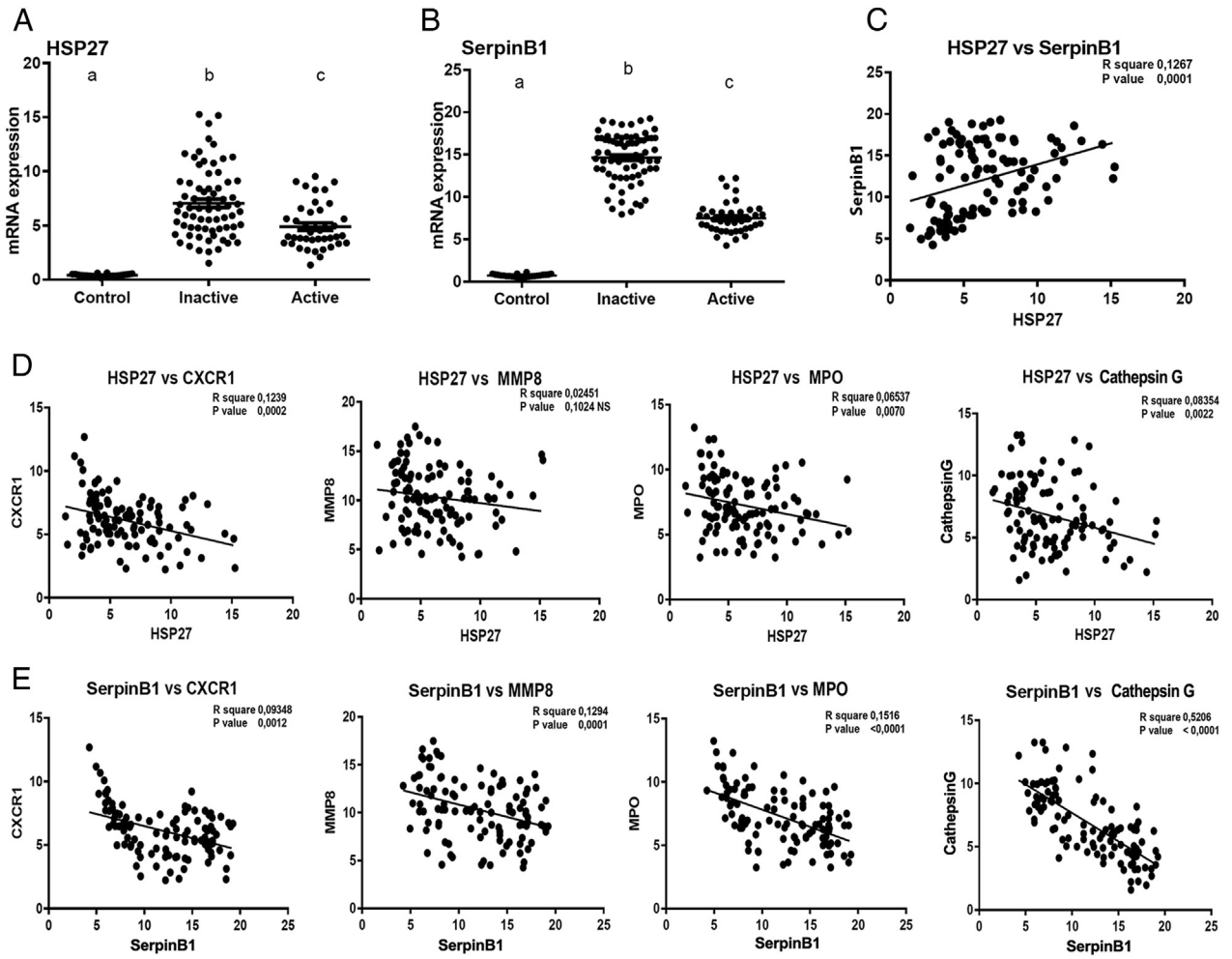

Figure 3. The expression of HSP27 and SERPINB1 in apical periodontitis and correlation analysis with additional immune inflammatory markers. (*A*) The relative expression of HSP27 in healthy apical tissues (controls) and active and inactive apical periodontitis. (*B*) The relative expression of SERPINB1 in healthy apical tissues (controls) and active and inactive apical periodontitis. In *A* and *B*, different letters represent statistically significant differences ($P < .05$). Pearson correlation analysis between (*C*) the relative expression of *HSP27* and *SERPINB1* in apical periodontitis; between (*D*) *HSP27* and *CXCR1*, *MMP8*, *MPO*, and *CTSG*, and between (*E*) *SERPINB1* and *CXCR1*, *MMP8*, *MPO*, and *CTSG*. $n = 110$ apical granulomas and $n = 26$ healthy periapical tissues.

regulators of tissue metabolism and their tissue protective roles during various chronic inflammatory conditions and animal models (23, 24). HSP27 was 3.79-fold up-regulated in apical periodontitis. This protein belongs to the heat shock protein gene family and has an important role in the inhibition of apoptosis in thermal and chemical stress, protecting the cells from injury in hostile environments (25, 26). In contrast, *HSP27*-deficient mice display impaired wound healing (27). Additional evidence suggests that HSP27 can modulate neutrophil chemotaxis and activity (28, 29), which could account for the local immune regulation of apical periodontitis. The results of our immunofluorescence assays revealed the expression of HSP27 predominantly in the epithelial cells of both epithelialized granulomas and apical cysts. These results corroborate previous reports in which HSP27 expression was found in odontogenic epithelial cells, dental lamina, and the enamel organ of tooth germs but not in fibroblasts or other stromal cells (30). Furthermore, the observed overexpression of the HSP27 protein in periapical granulomas is in line with our previously published findings in which HSP27 mRNA was significantly up-regulated in periapical granulomas and in lipopolysaccharide-stimulated macrophages (15). HSP27 expression was significantly higher in inactive lesions, implicating a potential role for this molecule in regulating lesion progression (15). Nevertheless, additional mechanistic studies are needed to determine a functional role for HSP27 in the pathogenesis of apical periodontitis.

SERPINB1 expression was also higher (~2-fold) in apical periodontitis. SERPINB1 is a potent inhibitor of neutrophil serine proteases (elastase and cathepsin G) and commonly up-regulated in ulcerative colitis (31). This protein also plays a crucial role in tissue protection against neutrophil-induced damage, acting as an apoptosis inhibitor in a caspase-independent pathway mediated by cathepsin G (32). We have previously shown the overexpression of another serpin family member (*SERPINE1*) in inactive apical periodontitis (2); the current findings of SERPINB1 up-regulation in apical periodontitis reinforce the evidence for a role of this gene family in the pathogenesis of the condition. Our results showed that SERPINB1 is exclusively expressed in the epithelium and in infiltrating PMNs present in the granulation tissue. We may speculate that whether in the epithelium or in the granulation tissue, SERPINB1 is serving a protective role, inhibiting the cathepsin G–mediated apoptosis of neutrophils and epithelial cells and thereby limiting the damage derived from the diffuse and uncontrolled secretion of neutrophil elastase and cathepsin G. The results of our quantitative PCR support this hypothesis because *SERPINB1* appears significantly more up-regulated in stable lesions than in progressive lesions. Interestingly, the negative correlation between *CTSG* and *SERPINB1* in apical periodontitis suggests the involvement of this protein in neutrophil-mediated inflammation. Recent evidence suggests that neutrophils can exert a regulatory role during the acute phase of the immune response, partially because of the release of neutrophil extracellular traps (NETs). These NETs contain DNA and up to 20 different inflammatory mediators and effector molecules, such as cathepsin G and elastase. The up-regulation of SERPINB1 might regulate and reduce the destructive potential of NET release, inhibiting the direct degradation of the tissue and helping to stabilize the lesion (33).

Interestingly, the results of our correlation analyses suggest dynamic and interactive roles for *SERPINB1* and *HSP27* in the regulation of apical periodontitis through their modulation of the expression of additional biomarkers of acute inflammation. Of note, significant negative correlations were identified for both *SERPINB1* and *HSP27* with *CXCR1*, *MMP8*, *MPO*, and *CTSG*. We observed a strong negative correlation was observed between the expression of *HSP27* and *CXCR1*, the receptor for interleukin 8 (CXCL8) and the main mediator of neutrophil infiltration in periapical inflammation (17). Additionally, significant negative correlations were observed between *HSP27* with *MPO* and *CTSG*, suggesting that its expression is correlated with decreased neutrophil infiltration and activity. Likewise, significant negative correlations were observed between the expression of *SERPINB1* with all the selected biomarkers of acute inflammation. The strongest negative correlation was noted for *CTSG* (r^2 = 0.52), which possibly suggests a direct regulatory link between the expression of *SERPINB1* and the inhibition of the expression of cathepsin G, which is 1 of the main proteolytic effector molecules released by neutrophils and responsible for direct tissue degradation and proinflammatory signaling (34, 35).

In summary, using an unbiased proteomic analysis, we identified HSP27 and SERPINB1 as differentially expressed proteins in apical periodontitis and confirmed their predominant epithelial localization. We also observed interesting correlations between the expression of these proteins with other inflammatory molecules that may provide insights into the functional role of HSP27 and SERPINB1 as regulators of apical periodontitis.

Acknowledgments

The authors thank the study participants.

Supported by the AAE Foundation and IFEA Jean-Marie Laurichesse Research Grant Award.

The authors deny any conflicts of interest related to this study.

Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (http://dx.doi.org/10.1016/j.joen.2017.03.014).

References

1. Nair PN. On the causes of persistent apical periodontitis: a review. Int Endod J 2006;39:249–81.
2. Garlet GP, Horwat R, Ray HL Jr, et al. Expression analysis of wound healing genes in human periapical granulomas of progressive and stable nature. J Endod 2012;38:185–90.
3. Cavalla F, Araujo-Pires AC, Biguetti CC, Garlet GP. Cytokine networks regulating inflammation and immune defense in the oral cavity. Curr Oral Health Rep 2014;1:104–13.
4. Weissman J, Johnson JD, Anderson M, et al. Association between the presence of apical periodontitis and clinical symptoms in endodontic patients using cone-beam computed tomography and periapical radiographs. J Endod 2015;41:1824–9.
5. Arentz G, Weiland F, Oehler MK, Hoffmann P. State of the art of 2D DIGE. Proteomics Clin Appl 2015;9:277–88.
6. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
7. Friedman DB, Lilley KS. Optimizing the difference gel electrophoresis (DIGE) technology. Methods Mol Biol 2008;428:93–124.
8. Cantel S, Brunel L, Ohara K, et al. An innovative strategy for sulfopeptides analysis using MALDI-TOF MS reflectron positive ion mode. Proteomics 2012;12:2247–57.
9. Pruitt KD, Tatusova T, Maglott DR. NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res 2005;33(Database issue):D501–4.
10. Bairoch A, Apweiler R. The SWISS-PROT protein sequence data bank and its supplement TrEMBL in 1999. Nucleic Acids Res 1999;27:49–54.
11. Araujo-Pires AC, Francisconi CF, Biguetti CC, et al. Simultaneous analysis of T helper subsets (Th1, Th2, Th9, Th17, Th22, Tfh, Tr1 and Tregs) markers expression in periapical lesions reveals multiple cytokine clusters accountable for lesions activity and inactivity status. J Appl Oral Sci 2014;22:336–46.
12. Menezes R, Garlet TP, Letra A, et al. Differential patterns of receptor activator of nuclear factor kappa B ligand/osteoprotegerin expression in human periapical granulomas: possible association with progressive or stable nature of the lesions. J Endod 2008;34:932–8.
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–8.
14. Batulan Z, Pulakazhi Venu VK, Li Y, et al. Extracellular release and signaling by heat shock protein 27: role in modifying vascular inflammation. Front Immunol 2016;7:285.

15. Goodman SC, Letra A, Dorn S, et al. Expression of heat shock proteins in periapical granulomas. J Endod 2014;40:830–6.
16. Loison F, Xu Y, Luo HR. Proteinase 3 and Serpin B1: a novel pathway in the regulation of caspase-3 activation, neutrophil spontaneous apoptosis, and inflammation. Inflamm Cell Signal 2014;1:e462.
17. Silva TA, Garlet GP, Lara VS, et al. Differential expression of chemokines and chemokine receptors in inflammatory periapical diseases. Oral Microbiol Immunol 2005; 20:310–6.
18. Ruest LB, Ranjbaran H, Tong EJ, et al. Activation of receptor activator of nuclear factor-kappaB ligand and matrix metalloproteinase production in periodontal fibroblasts by endothelin signaling. J Periodontol 2016;87:e1–8.
19. Rathnayake N, Gustafsson A, Norhammar A, et al. Salivary matrix metalloproteinase-8 and -9 and myeloperoxidase in relation to coronary heart and periodontal diseases: a subgroup report from the PAROKRANK Study (Periodontitis and Its Relation to Coronary Artery Disease). PLoS One 2015;10:e0126370.
20. White PC, Chicca IJ, Cooper PR, et al. Neutrophil extracellular traps in periodontitis: a web of intrigue. J Dent Res 2016;95:26–34.
21. Schulz M, von Arx T, Altermatt HJ, Bosshardt D. Histology of periapical lesions obtained during apical surgery. J Endod 2009;35:634–42.
22. Kopecki Z, Ludwig RJ, Cowin AJ. Cytoskeletal regulation of inflammation and its impact on skin blistering disease epidermolysis bullosa acquisita. Int J Mol Sci 2016;17:1116.
23. Dai HJ, Li DW, Wang YX, et al. Induction of heat shock protein 27 by bicyclol attenuates d-galactosamine/lipopolysaccharide-induced liver injury. Eur J Pharmacol 2016;791:482–90.
24. Gong D, Farley K, White M, et al. Critical role of serpinB1 in regulating inflammatory responses in pulmonary influenza infection. J Infect Dis 2011;204:592–600.
25. Strauch A, Haslbeck M. The function of small heat-shock proteins and their implication in proteostasis. Essays Biochem 2016;60:163–72.
26. Haslbeck M, Vierling E. A first line of stress defense: small heat shock proteins and their function in protein homeostasis. J Mol Biol 2015;427:1537–48.
27. Crowe J, Aubareda A, McNamee K, et al. Heat shock protein B1-deficient mice display impaired wound healing. PLoS One 2013;8:e77383.
28. Lam PY, Harvie EA, Huttenlocher A. Heat shock modulates neutrophil motility in zebrafish. PLoS One 2013;8:e84436.
29. Jog NR, Jala VR, Ward RA, et al. Heat shock protein 27 regulates neutrophil chemotaxis and exocytosis through two independent mechanisms. J Immunol 2007;178: 2421–8.
30. Kumamoto H, Suzuki T, Ooya K. Immunohistochemical analysis of inducible nitric oxide synthase (iNOS) and heat shock proteins (HSPs) in ameloblastomas. J Oral Pathol Med 2002;31:605–11.
31. Uchiyama K, Naito Y, Takagi T, et al. Serpin B1 protects colonic epithelial cell via blockage of neutrophil elastase activity and its expression is enhanced in patients with ulcerative colitis. Am J Physiol Gastrointest Liver Physiol 2012;302:G1163–70.
32. Baumann M, Pham CT, Benarafa C. SerpinB1 is critical for neutrophil survival through cell-autonomous inhibition of cathepsin G. Blood 2013;121:3900–7. S1–S6.
33. Yang H, Biermann MH, Brauner JM, et al. New insights into neutrophil extracellular traps: mechanisms of formation and role in inflammation. Front Immunol 2016;7:302.
34. Henry CM, Sullivan GP, Clancy DM, et al. Neutrophil-derived proteases escalate inflammation through activation of IL-36 family cytokines. Cell Rep 2016;14: 708–22.
35. Eipper S, Steiner R, Lesner A, et al. Lactoferrin is an allosteric enhancer of the proteolytic activity of cathepsin G. PLoS One 2016;11:e0151509.