INTRODUCTION

High mobility group protein box 1 (HMGB1) is a protein present in the nucleus of all cells, and it plays a key role in maintenance of nuclear homeostasis. HMGB1 is organized into three different regions including two DNA binding domains called box A and B, and an acidic C-terminal tail. Besides box A and B, two nuclear localization signals are present in the protein structure at amino acids 28–44 and 179–185. The protein binds to the minor groove of linear DNA, and bends the double helix up to 90° or more, thereby altering chromatin architecture and allowing recruitment of transcription-regulating factors [1]. Numerous lines of evidence point to HMGB1 as a key regulator of enhanceosome organization, transcription factor recruitment, and overall activation of the basal transcriptional machinery [1,2].
Surprisingly, a large body of experimental evidence demonstrates that HMGB1 is also endowed with extracellular signaling functions on various cell types, including those of the immune system [3-5]. The protein has been recently included into the “alarmin” family, a term identifying a group of endogenous factors, also known as “endokines,” which once released in the extracellular space interact with membrane receptors on immune cells to activate the inflammatory response [6]. Of note, HMGB1 can be released from the nucleus to the extracellular milieu in response to different stimuli. While necrotic cells passively release the protein following nuclear and plasma membrane rupture, cells undergoing apoptosis retain HMGB1 into the nucleus strongly associated with condensed chromatin. Besides passive release during necrosis, HMGB1 can be actively secreted by several cell types [7]. Among these, activated immune cells constitute a major source of extracellular HMGB1 in inflamed tissues [8].

Once released, HMGB1 induces proliferation, cell migration, and survival, as well as inflammation. HMGB1 behaves as a prototypical cytokine once extracellularly released and plays an important role in development and maintenance of the inflammatory response. However, it is worth noting that HMGB1 release occurs considerably later than secretion of other classical proinflammatory mediators (tumor necrosis factor-α, interleukin-1β), indicating that the protein is a delayed mediator of inflammation [9].

In light of its remarkable pathogenetic relevance, great effort has been focused on counteracting the proinflammatory effects of HMGB1. Although different strategies have been proposed at the preclinical level, almost none of them have reached clinical application [3]. A notable exception is glycyrrhizin (GLT), an alkaloid extracted from Glycyrrhiza Glabra used in Japan as a remedy to treat different inflammatory disorders including hepatitis. Recent findings demonstrate that GLT binds to a specific pocket of HMGB1, thereby precluding the protein’s ability to bind to its cognate receptors and to exert proinflammatory and chemotactic effects [10]. Different formulations containing GLT have therefore been proposed as therapeutic tools for treatment of disorders whose pathogenesis is postulated to be prompted and/or sustained by HMGB1.

As for the potential pathogenetic role of HMGB1 in the respiratory tract, very recent studies have shown increased levels of the protein in patients affected by asthma [11]. However, it is not known whether HMGB1 plays a role in inflammatory processes of the upper respiratory tract such as those during rhinitis or nasal polyps. In the present study, we therefore evaluated the HMGB1 levels in nasal fluids of patients with different forms of rhinitis. Given that HMGB1 plays a key role in promoting survival of eosinophils, leukocytes with key pathogenetic roles in allergic and nonallergic immune disorders of the nasal mucosa, we also checked whether GLT affects the survival of different human leukocytes including eosinophils.

**MATERIALS AND METHODS**

**Subjects and experimental design**

This study included children with allergic rhinitis (AR) recruited by family paediatricians of Messina. The nasal HMGB1 concentration was measured in the laboratory of Unit of Genetics and Paediatric Immunology of the Policlinico of Messina. All participating subjects were of Italian origin.

The details of the study were explained to the parents/guardians of the children who were participating in the study, and written informed consent was obtained from each parent/guardian. A physician performed the physical examination of each child. Demographic data and other clinical characteristics of the study subjects were collected using the questionnaires. AR was diagnosed according to the presence of typical nasal symptoms (nasal itching, sneezing, watery rhinorrhea, and nasal obstruction) and sensitization to pollen allergen, documented by a positive skin prick test (SPT). Inclusion criteria were: age of between 6 and 16 years, a diagnosis of AR, sensitization to Parietaria pollen, and the presence of nasal symptoms.

Exclusion criteria were: perennial AR, any respiratory tract infection within 1 month before the start of the study, other nasal disease (including structural abnormalities, nasal polyposis and clinically relevant septum deviation), asthma comorbidity (excluded by history and physical examination), and any treatment in the previous month capable of interfering with the results. The study was carried out from March 2013 to May 2013, the time during which the subjects were naturally exposed to pollens. The study was conducted on 170 AR subjects (87 males and 83 females; median age, 10.3 ± 3.4 years). All children presented with: nasal obstruction, nasal itching, sneezing, and watery rhinorrhea, an increased level of total serum IgE and positive results for the Parietaria pollen SPT.

In accordance with the study protocol, patients were divided into three groups; one group received 1 puff of saline (29 males and 28 females), one group received 1 puff of Budesonide (32 males and 25 females) and one group received 1 puff of Narivent (DMG, Rome, Italy) (30 males and 26 females) into each nostril 2 times a day for 1 week. HMGB1 levels in nasal fluids were evaluated before and after treatment.

**Nasal fluid collection procedure**

All subjects underwent nasal lavage as described by Salpietro et al. [12]. Briefly, healthy controls and patients affected by different types of rhinitis or nasal polyps as well as AR patients before and after 1 week of treatment with saline, Narivent or Budesonide were placed in a sitting position with the head slightly tilted. Nasal lavage was performed with a pipette, by injecting 1.5 mL of saline solution (0.9% NaCl at 37°C) into the nostril of the patient, after closing the possible escape route represented by the other nostril with a patch. The recovered liquid was collected after a few seconds with the same pipette. Sampling was performed for...
both nostrils. The fluid was stored at -20°C until HMGB1 measurement. The recovery volume was on average more than 90% after nasal lavage.

**HMGB1 measurement**

HMGB1 concentration was measured in the nasal fluid collected from each patient (as described above) by performing an enzyme-linked immunosorbent assay (ELISA) using an HMGB1 ELISA Kit II developed by Shino-Test Corporation (IBL International GmbH, Hamburg, Germany) following the manufacturer’s instructions. Briefly, samples were diluted 1:5 with sample diluent and added to microtiter plates, which were then incubated for 24 hours at 37°C. After washing, 100 µL/well of antihuman HMGB1 peroxidase-conjugated monoclonal antibody was added and the plates were incubated at room temperature for 2 hours. After extensive washing, tetramethyl benzidine dihydrochloride was added to each well. The enzyme reaction was allowed to proceed for 30 minutes at room temperature. The chromogenic substrate reaction was stopped by adding the stop solution, and the absorbance of each well was determined on a microplate reader at 450 nm. The HMGB1 concentrations were calculated with reference to a standard curve, with a detection limit of 0.2 ng/mL.

**Quantitation of GLT and glycyrrhetinic acid**

Plasma samples were collected from patients treated with acute (1 hour after one puff per nostril) or chronic (after 1 week of treatment with 1 puff per nostril twice a day) Narivent administration or from healthy subjects 1 hour after consumption of a licorice candy (containing 20.5 ± 3 mg of GLT). Samples were quantitated by means of liquid chromatography-mass spectrometry. Briefly, specimens were extracted with methanol and injected in a high performance liquid chromatography apparatus consisting of a reversed-phase C18 (ODS) column with a mobile phase of methanol 1%, formic acid (75:25, vol/vol). Mass spectrometry determination was performed using negative electrospray ionization (negative ESI) in the selected ion monitoring mode. GLT was monitored at the m/z 821.1 channel, glycyrrhetinic acid (GA) at the m/z 469.2 channel, and internal standard (linuron) at the m/z 248.9 channel. The calibration curve was linear over the range from 0.05 to 10 µg/mL with a correlation coefficient above 0.99.

**Leukocyte preparation and cell death evaluation**

Blood samples from healthy donors were stratified on a Ficoll layer. After centrifugation at 1,500 rpm for 30 minutes at room temperature, peripheral blood mononuclear cells were collected at the interface with Ficoll and used for preparation of monocytes and lymphocytes as previously described [5]. Cellular pellet containing red blood cells and granulocytes was used for preparation of eosinophils (isolation kit 130-092010) and neutrophils (CD16 microbead 130-045-701) by means of magnetic separation procedure using Miltenyi kits following the manufacturer’s instructions. Cells were cultured as previously described [5]. Cell death was assessed by Propidium iodide labeling and cytofluorometric analysis as previously described [13].

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**RESULTS**

Levels of HMGB1 in nasal fluids of patients with rhinitis and polyps

We first investigated whether the levels of HMGB1 were altered in nasal fluids of patients affected by different forms of rhinitis. To this end, we collected nasal fluids from healthy subjects and patients affected by AR, non-AR with eosinophilia syndrome (NARES) or nasal polyps (12 subjects per group), and evaluated their HMGB1 content by means of a commercial ELISA kit (see MATERIALS AND METHODS SECTION for further detail). As shown in Fig. 1A, we found significant increases in the HMGB1 content in all the three groups of rhinitis patients. When HMGB1 levels were compared with those in patients pooled into a single group, a 3-fold increase in HMGB1 levels was observed (Fig. 1B). The present data showing that the level of proinflammatory cytokine HMGB1 is increased in nasal fluids of patients with different forms of rhinitis underscore the therapeutic potential of HMGB1-inactivating strategies in treatment of inflammatory disorders of the nasal mucosa. The nasal formulation Narivent is a GLT-containing medical device used to treat inflammation of the nasal mucosa [14]. We therefore compared the efficacy of topical treatments with saline, Narivent, or Budesonide in reducing the HMGB1 content in nasal fluids of patients with AR (see MATERIALS AND METHODS SECTION for patient details). As shown in Fig. 1C, we found that, at variance with saline, Narivent and Budesonide equally reduced the HMGB1 content in nasal fluids after 1 week of treatment. Reportedly, GLT is known to inhibit 11p-hydroxysteroid dehydrogenase 2 and to promote arterial hypertension via cortisol accumulation [15]. However, it is not known whether nasal formulations containing GLT can increase cortisol levels because of systemic absorption. To address this question, we first evaluated the plasma levels of GLT and of its principal metabolite GA in healthy subjects. We found that both acute (i.e., 30 minutes after two puffs/nostril, 4.99 ± 0.35 mg GLT/puff) or prolonged administration (i.e., two puffs/nostril twice a day per one week) resulted in GLT and GA concentrations in plasma in the order of the method detection limit (0.2 ng/mL). Notably, ingestion of a candy containing a standard amount of GLT (20.88 ± 3.8 mg) resulted in plasma levels (30 minutes after ingestion) that were 2 folds higher than those found in patients receiving GLT for one week (Fig. 1D). In keeping with the very low plasma concentrations of GLT and GA, in subjects receiving Narivent, plasma cortisol levels were not different from those in controls (Fig. 1E). Taken together, these findings indicate that nasal treatment with GLT leads to poor systemic absorption that is not sufficient to affect the plasma cortisol levels and blood pressure, at least in the time window analysed. Data also
indicate that ingestion of a single licorice candy is enough to cause significantly higher plasma GLT/GA concentrations than those in patients treated with nasal GLT formulation for an entire week.

Release of HMGB1 by different human leukocytes and effects of GLT

The identification of increased HMGB1 concentrations in nasal fluids of rhinitis patients clearly underscores the pathogenetic relevance of this proinflammatory agent in immune disorders of the nasal mucosa. It is well known that the latter are frequently associated with eosinophilic infiltrates [16,17]. Given the ability of HMGB1 to activate and promote survival of eosinophils [18], as well as the high levels of the protein in nasal fluids of patients with AR and NARES (Fig. 1A) (in which the eosinophilic infiltrate is well represented and is of pathogenetic relevance), we next planned to evaluate the ability of different leukocytes to release HMGB1. Interestingly, we found that among human macrophages and granulocytes isolated from peripheral blood of healthy subjects, eosinophils were able to release a higher amount of HMGB1 within 12 hours of incubation (Fig. 1C). Of note, monocytes, lymphocytes, and neutrophils released an amount of HMGB1 (Fig. 2A) that was almost half of that released by eosinophils. To further investigate the ability of the protein to affect half-life of leukocytes in culture, we took advantage of the ability of GLT to bind and inactive HMGB1. We therefore exposed cultured leukocyte populations to GLT and evaluated their survival. GLT was used at a concentration of 1%, 5 folds lower than that in the anti-inflammatory nasal preparation Narivent. As shown in Fig. 2B, we found that GLT selectively killed eosinophils and had no effect on the other types of leukocytes.

**DISCUSSION**

To the best of our knowledge, this is the first evidence to suggest that the proinflammatory protein HMGB1 is increased in human nasal fluids during inflammatory disorders of the nasal mucosa in addition to AR [12]. Notably, NARES patients showed the high-
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est HMGB1 levels, which was in line with the key proinflammatory role of HMGB1 in eosinophils [18]. As for polyps, we speculate that the increased levels of HMGB1 were due to the well-known inflammatory response that accompanies polyp development. Also, in keeping with the different degrees of the inflammatory component in different polyps, we found that HMGB1 levels in nasal fluids from patients with polyps showed highest variability (Fig. 1A).

It is worth noting that the present study, by showing that the level of the proinflammatory cytokine HMGB1 is increased in nasal fluids of patients with different forms of rhinitis, underscores the therapeutic potential of HMGB1-inactivating strategies in treatment of inflammatory disorders of the nasal mucosa. Several compounds have been proposed as potential remedies that are able to counteract the proinflammatory role of HMGB1. These are, for instance, the HMGB1 receptor antagonist HMGB1-BoxA, the anti-HMGB1 antibodies, or the HMGB1-scavenging compounds thrombomodulin, polymyxin B, and GLT. To the best of our knowledge, GLT is the sole compound used to inhibit HMGB1 signalling in the clinic due to its anti-inflammatory properties (see http://www.minophagen.co.jp). In this respect, the nasal formulation Narivent is a recently developed GLT-containing medical device used to treat inflammation of the nasal mucosa [15]. Therefore, on one hand, the use of GLT-containing formulations may help unravel the pathogenetic role of HMGB1 in rhinitis-related disorders, and on the other hand, it may represent innovative tools for the treatment of upper airway inflammation.

We originally reported that among human leukocytes, eosinophils are the major source of extracellular HMGB1. Of note, we reasoned that the presence of HMGB1 in leukocyte culture media is not due to cell death-dependent passive release because during the early incubation times we performed evaluation of entrance of propidium Iodide (a prototypical marker of cell death) which showed no signs of cell demise (data not shown). Evidence that eosinophils show higher HMGB1 release among white blood cells is in keeping with the survival-promoting effect of HMGB1 on eosinophils [18]. Also, the finding that GLT selectively induced eosinophil death indicates that GLT is not cytotoxic per se. It also suggests that eosinophils are the only leukocyte subtype that needs HMGB1 for survival. To the best of our knowledge, this is the first evidence suggesting that signalling from extracellular HMGB1 is selectively required by eosinophils to avoid cell death. Why HMGB1 signalling is dispensable for survival of the other leukocyte population is currently not known. In this regard, it will be interesting to decipher the mechanisms underpinning the survival-promoting effect of HMGB1 in eosinophils and to investigate whether/why these mechanisms do not operate in other leukocytes. As a final note, we reasoned that the reduced HMGB1 levels in nasal fluids of patients treated with Narivent can be attributed to the ability of GLT to selectively kill eosinophils, which are a major source of extracellular HMGB1. Overall, this study improves our understanding of the effects of HMGB1 on human leukocytes, and corroborates the therapeutic potential and safety profile of GLT in topical treatment of rhinitis.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.
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