An Involvement of Granulocyte Medullasin in Phenytoin-Induced Gingival Overgrowth in Rats

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Received December 27, 2001 Accepted March 22, 2002

ABSTRACT—To investigate the relationship between histological changes and distributions of medullasin, a neutrophil elastase-like serine proteinase, in phenytoin-induced gingival overgrowth, we established a rat model of gingival overgrowth. Thirty-two, 20-day-old male Fischer 344 rats were fed a diet containing phenytoin and sacrificed at 1, 2, 4 and 8 weeks. Control rats (n = 40) were fed the same diet, but without the drug and killed at the same weeks as experimental rats (n = 32) and 0 week (n = 8). The mandible specimens were resected and sectioned bucco-lingually between the first and second molars. A marked inflammatory-cell infiltration and elongated rete pegs were seen in the phenytoin-treated group. The extent of the overgrowth assessed by computer image analysis and the density of medullasin-positive cells by immunohistochemistry in the approximal gingiva showed a significant increase in the phenytoin-treated group compared to the control group. A marked infiltration of the positive cells in experimental rats was observed as early as 2 weeks when gingival overgrowth was not fully established. Medullasin-positive cells were mostly neutrophils and partly macrophage-like cells. These findings suggest that medullasin may be involved in mainly host defense and secondarily collagen metabolism in the phenytoin-induced rat model of gingival overgrowth.

Keywords: Medullasin, Serine proteinase, Phenytoin, Gingival overgrowth, Immunohistochemistry

Phenytoin is an anti-epileptic drug known to cause gingival overgrowth (1), the precise mechanism of which remains unclear (2 – 5). Gingival overgrowth may be due to increased proliferation and synthesis of fibroblasts (6), leading to an accumulation of connective tissue components such as collagens. Various lysosomal proteinases may contribute to this protein turnover and/or be involved in abnormal collagen metabolism in medication-induced gingival overgrowth (3, 4, 7). Medullasin (EC 3.4.21.37) is a neutrophil elastase-like serine proteinase with a molecular mass of 31,800 and optimum pH of 8.5, and it is mainly found in granulocytes and erythroblasts (8, 9). This enzyme is readily secreted into extracellular spaces at sites of inflammation and is involved in normal host defense mechanisms including the development of inflammation (10), the induction of DNA and RNA synthesis in human lymphocytes and the activation of monocytes/macrophages (11). Medullasin may be involved in periodontal diseases, since it degrades a wide variety of important molecules in gingival connective tissue including collagens and proteoglycan. We have previously demonstrated both immunohistochemically and immunohistochemically the involvement of this protein in periodontal breakdown (12 – 14). Moreover, we examined the distribution of medullasin in tissue overgrowth and reported its involvement in nifedipine-induced human gingival overgrowth (15). More recently, we clarified a possible role of medullasin in nifedipine-induced rat gingival overgrowth, following the observations of the establishment of gingival overgrowth at 8 weeks and the host-defense role of this enzyme throughout the study (16). Besides, a marked infiltration of inflammatory cells including medullasin-positive cells was found at as early as 2 weeks of treatment, suggesting the impor-

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tance of the inflammatory response in the establishment of gingival overgrowth. However, this finding was rather different from a previous study by another group where there was very little infiltration of inflammatory cells (17). Similarly, the association of the inflammatory response with the establishment of phenytoin-induced rat gingival overgrowth remains obscure (18). Therefore, we investigated a possible role of medullasin in the establishment and maintenance of phenytoin-induced gingival overgrowth and then discussed a necessity of the inflammatory response to cause the overgrowth.

MATERIALS AND METHODS

Animals
Seventy-two specific pathogen-free 20-day-old male Fischer 344 rats were obtained from a commercial source and housed individually in stainless-steel cages. They were kept in a temperature-controlled room set at 24°C. The animals were divided at random into two equal groups, experimental (n = 32) and control (n = 32 plus 0-week rats (n = 8)). Experimental rats were fed a diet of CE-2 (Clea Japan, Inc., Osaka) supplemented with phenytoin; control rats had the same diet without phenytoin. During the course of the experiments, the body weight and food consumption of each rat were measured once weekly. Gingival overgrowth was induced according to the method of Morisaki et al. (18) with a slight modification. Briefly, 5 days after weanling rats were fed an ordinary diet, experimental rats were given phenytoin at a dose of 1 mg/g CE-2 for the first week and then increased up to 2 mg/g CE-2 throughout the 8 weeks of the study. Control rats were only fed CE-2. The phenytoin-treated rats were of similar weight and activity to their phenytoin-untreated controls throughout the 8-week study period. Animals were sacrificed at weeks 0, 1, 2, 4 and 8 after the commencement of phenytoin administration. Animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Materials
Phenytoin was purchased from Wako Ind. (Osaka) and the avidin-biotin-peroxidase complex (ABC) purchased from Seikagaku Corp. (Tokyo). All other chemicals were reagent-grade from various commercial sources. The antiserum against medullasin purified from human bone marrow cells was raised in rabbits as described by Aoki (8).

Immunohistochemistry
At death, the mandible was immediately resected and washed with phosphate-buffered saline (PBS), pH 7.4, for 30 min. It was then fixed in periodate-lysine-paraformaldehyde fixative for 12 h, followed by demineralization in 10% EDTA for 28 days. After a thorough washing with PBS, the specimens were embedded in paraffin wax, sectioned at 4-μm thickness in the buccolingual direction between the first and second molars (Fig. 1) and then stained to show medullasin activity by the ABC technique as described by the manufacturer. Non-specific protein binding was blocked with 10% normal goat serum for 30 min and the sections immersed in the primary antibody solution (rabbit antihuman medullasin polyclonal antibody, 1:800) at 4°C overnight. Antibody was diluted and sections were washed in PBS containing 0.5% milk protein and 0.05% Triton X-100. The sections were then incubated at room temperature with biotinylated secondary antibody (anti-rabbit immunoglobulin) for 10 min. After a further wash, they were deperoxidized to remove endogenous peroxidase with 3% hydrogen peroxide in methanol for 15 min, followed by the addition of ABC. After another wash in the same buffer, the reaction was developed by incubation for 2–8 min in Karnovsky solution (3,3′-diaminobenzidine tetrahydrochloride in 100 ml of 0.05 M Tris buffer, pH 7.4, containing 0.3% hydrogen peroxide and distilled water). The reactions were counterstained with methyl green, rinsed with PBS, dehydrated with fresh ethanol, and mounted. Normal rabbit serum was substituted for primary antibody on each section as a non-immune control. One section from each sample was stained with hematoxylin and eosin to identify and measure the degree of gingival overgrowth.

Morphometric analysis
To evaluate gingival overgrowth in each gingival biopsy, black-and-white microphotographs were taken with a camera (BX50; Olympus, Tokyo) at ×13.2 magnification.
The photographs were digitized in a scanner (GT4000; Epson, Tokyo) to 300-dots/inch images in PICT format. Image files were analyzed with the NIH image software (ver. 1.55) on a Macintosh computer (Power Book G3). The components assessed for gingival overgrowth are depicted in Fig. 2A. The area used for the assessment of gingival overgrowth was defined as the area between the straight line connecting the mucogingival junctions of both sides and the free gingival margin.

Quantitative evaluation of enzyme-positive cells
The number of medullasin-positive cells was counted with a photomicroscope (Optiphot-2; Nikon, Tokyo) equipped with an ocular grid at ×200 magnification. As shown in Fig. 2B, immunoreactive cells were counted in each 0.0625-mm² area in the buccal and lingual pocket epithelium and connective tissue adjacent to the pocket epithelium, including the midportion of approximal gingiva. The results were expressed as the mean percentage of positive cells per total cells in the 0.0625-mm² area for each biopsy. All evaluations and measurements were made by one well-trained investigator, counting three times the positive cells at the same location.

Statistical analyses
Data are expressed as the mean ± S.D. Differences in the area of defined gingival tissues and the changes in the mean percentages of medullasin-positive cells during the experiment were evaluated statistically with the Mann-Whitney U test. A P-value <0.05 denoted a statistically significant difference.

RESULTS

Evidence of gingival overgrowth
It is apparent that gingival overgrowth was induced in experimental rats, especially in approximal gingival tissues. A representative example of the histological changes and gingival overgrowth in 8-week-old rats is shown in Fig. 3A. Inflammatory cells are more frequent in experimental rats than those in the control (Fig. 3B).

Evaluation of gingival overgrowth
The extent of gingival overgrowth was evaluated histomorphometrically. Treatment with phenytoin resulted in a progressive enhancement of the tissues (Table 1). Although the gingival area increased with time in both groups, there was a significant increase between weeks of the treatment, especially in the experimental group. Gingival overgrowth was apparent in experimental rats at 4 weeks and continued to the 8th week. In contrast, there was no significant increase in the tissues of control rats except between 0 week and 1 week.

Immunohistochemistry
Gingival tissues were examined immunohistochemically for the presence and distribution of cells positive for medullasin. There were only a few inflammatory cells and medullasin-positive cells in tissues of the control rats throughout the study (Fig. 4: B, D and F). In experimental rats, the similar pathological change was observed at 1 week. At 2 weeks, however, a number of inflammatory cells including medullasin-positive cells infiltrated the connective tissue and the pocket epithelium (Fig. 4A). At 4 weeks of treatment, there was a dense population of inflammatory cells and medullasin-positive cells, but elongation of oral epithelium was seldom observed (Fig. 4C). At 8 weeks of phenytoin treatment, the oral epithelium was rather elongated, while the pocket epithelium showed proliferation and had become larger (Fig. 4E). Clusters consisting of neutrophils, macrophage-like cells and lymphocytes had formed in the connective tissue beneath the elongated epithelium. The enzyme was expressed in most neutrophils and some macrophage-like cells throughout the study. By contrast, there was a gradual increase in population of the cells in control rats with time, but not so remarkably (Fig. 4: B, D and F). Medullasin-positive cells were predominantly
neutrophils and to a lesser extent macrophage-like.

Quantitative analysis

The mean percentages of medullasin-positive cells were evaluated (Fig. 5). The number of medullasin-positive cells in experimental rats at 1 week was almost the same as that of control rats. However, the number of the cells in experimental group increased significantly between 1 week and 2 weeks, and then increased with time, but not significantly. Also, it was significantly higher than that in the controls at 2, 4 and 8 weeks. In contrast, there was no significant change in the percentage with age in control rats.

DISCUSSION

To date, very little information is available on the role of proteinases in phenytoin-induced gingival overgrowth (3, 19, 20). A series of immunohistochemical studies from our
Fig. 4. Immunohistochemical distribution of medullasin-positive cells in a representative approximal gingival tissue sample at 2 weeks (A, B), 4 weeks (C, D) and 8 weeks (E, F) (A, C, E: experimental; B, D, F: control; original magnification ×66). At 2 weeks, the number of positive cells in experimental rats is higher than in the control (A). Medullasin is present mainly in neutrophils (arrow). Insert: enlargement of a neutrophil showing the staining profile of medullasin (original magnification ×132). In experimental rats, the pathophysiological change at 4 weeks became more distinctive than that at 2 weeks (C). Note lack of change in control rats (D). Although positive cells are mainly neutrophils, some medullasin-positive macrophage-like cells (arrowhead) infiltrated the connective tissue adjacent to the pocket epithelium. Insert: enlargement of a macrophage-like cell showing the staining profile of medullasin (original magnification ×132). At 8 weeks, the positive cells in experimental rats have infiltrated more intensively than those in control rats at 8 weeks and in experimental rats at 4 weeks (E and F). The enzyme is found in most neutrophils in the pocket epithelium and adjacent connective tissue and in some of macrophage-like cells infiltrating in a cluster beneath the elongated epithelium.
lagen fibers in phenytoin-induced overgrown tissues show a regular arrangement unlike nifedipine-induced overgrowth.

The most significant role of medullasin is thought to induce inflammation. Actually, we found an accumulation of inflammatory cells including medullasin-positive cells in the overgrown gingival specimens. On the contrary, there were quite few inflammatory- and medullasin-positive-cells in the control specimens, suggesting the necessity of inflammatory response in the induction of rat gingival overgrowth. We found a marked infiltration of medullasin-positive inflammatory cells at 2 weeks of treatment, probably indicating the involvement of medullasin in the activation of the inflammatory response. However, gingival overgrowth was not yet fully established. At 4 weeks, we found gingival overgrowth in all experimental rats. The proportion of medullasin in the tissues increased up to 4 weeks with established gingival overgrowth, suggesting indirect association of medullasin with gingival overgrowth. The overgrowth almost reached a plateau at 8 weeks (further data not shown), although the increase in the proportion of medullasin-positive cells continued up to the longer treatment period (32 weeks, data not shown). Medullasin is distributed mainly in neutrophils and partly in monocytes/macrophages, indicating its significant role in host defense. Therefore, the gradual increase in medullasin throughout the experiment may suggest that there is a progressive inflammation in overgrown tissues even after the establishment of gingival overgrowth.

Nevertheless, the necessity of inflammation in drug-induced gingival overgrowth is controversial. Previous reports (17, 18) revealed a passive association of inflammation with the establishment of the overgrowth, since there was a slight infiltration of inflammatory cells in both experiments. However, the observed areas in their studies were buccal and lingual (not approximal), different from our areas (approximal). As stated above, gingival overgrowth occurs mostly in approximal areas, since bacterial plaque readily accumulates between teeth and can cause inflammation (16, 24, 25). Considering these, approximal gingival tissues could be more susceptible to gingival overgrowth due to the infiltration of inflammatory cells.

Besides, the gingival overgrowth in this study is more remarkable in lingual approximal areas as compared with buccal ones. The precise reason is still unclear, but the tooth shape in approximal areas may affect the attachment of bacterial plaque onto the tooth surface. Further studies are needed to clarify the reason.

The dosage of phenytoin given to rats in this experiment should be discussed. Although the dose (2 mg/g) was determined following the study of Morisaki et al. (18), it seems to be overdosed to the rats. However, the author stated that the minimum dietary concentration (2 mg/g) of phenytoin to elicit gingival overgrowth from rats conse-
quently leads to their serum level of about 13 – 14 μg/ml, the level of which is approximately equivalent to the therapeutic serum level of phenytoin (10 – 20 μg/ml) in humans. Therefore, it may not be appropriate to regard the dose used in this study as an overdose. Fortunately, the phenytoin-treated rats in our experiment were of similar weight and activity throughout the study to the untreated control rats, probably indicating the absence of such kinds of side effects in the rats. Nevertheless, the appropriate (minimum) dose to cause gingival overgrowth should be reconsidered.

In this study, we could not establish a specific role of medullasin in collagen metabolism of the gingival overgrowth. However, medullasin possibly has a secondary influence on the metabolism by modulating cytokine (26) and growth factors (27) following the activation of macrophages (11). Further in vivo and in vitro studies are in progress in our laboratories.

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