Vigabatrin-induced modification of Ki-67 expression in gingival epithelium: immunohistochemical study of a short series

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Objective: To study the expression and role in vigabatrin (VGB)-induced gingival enlargement of Ki-67 antigen and p27^{KIP1}, p21^{WAF1}, and p53, proteins that activate or inhibit cell-cycle progression.

Materials and methods: Six patients treated with VGB for partial epileptic seizures refractory to classic anticonvulsant treatment were studied. Gingival biopsies were taken from four of these patients for immunohistochemical studies; 10 control biopsies from individuals with healthy gingiva and 10 from patients with periodontal disease were also evaluated.

Results: Four of the six patients presented some degree of gingival enlargement (mild or moderate). Nuclear expression of Ki-67 was elevated (mean of 894 positive cells/mm² in VGB-induced gingival enlargement vs. 391 cells/mm² in controls with healthy gingiva and 425 cells/mm² in controls with periodontal disease) (p < 0.01, analysis of variance: ANOVA), and nuclear expression of cyclin-dependent kinase (cdk) inhibitors p27^{KIP1} and p21^{WAF1} was reduced. The patients with gingival enlargement presented inflammatory infiltrate in lamina propria, mainly composed of T lymphocytes (CD3⁺) and plasma cells (CD38⁺), which was even more intense than in the biopsies of patients with periodontal disease.

Conclusion: The overexpression of antigen Ki-67 and slight underexpression of cdk-inhibitors p27^{KIP1} and p21^{WAF1} suggest that VGB induced an increase in cell proliferation and contributed, together with concomitant periodontal disease, to the gingival enlargement.

Vigabatrin (VGB: 1,4-aminohex-5enoic acid, gamma-vinyl gamma-aminobutyric acid) is an antiepileptic drug used in patients who prove refractory to conventional therapy. It acts by increasing the concentration of gammaaminobutyric acid (GABA) through the selective and permanent inhibition of GABA-aminotransferase (1).

The side-effect of VGB with greatest clinical repercussion is retinal dys-function, requiring the regular oph-

thalmic follow up of these patients (2). Only two cases of VGB-induced gingival enlargement have been reported

(1, 3). Both patients were young adults

under 30 years old and the onset of

within

gingival enlargement was

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6 months of the start of VGB treatment (doses of 1.5-2.5 g/day). In both cases, the gingival enlargement was moderate and distributed in both upper and lower arches. In the case published by Katz et al. (1), the gingival enlargement recurred within 1 year after a gingivectomy, and in the patient reported by our group, the gingival enlargement increased slowly and progressively during the third year of follow-up (3). In the present cases, we prefer to use the term gingival enlargement instead of gingival overgrowth for the different clinical presentations and morphological features.

A statistically significant increase in Ki-67 expression was recently demonstrated in the gingival epithelial cells and fibroblasts of the lamina propria of patients with gingival overgrowth induced by nifedipine, phenytoin or cyclosporin A (4, 5). Saito *et al.* found p53 expression in isolated cells of the suprabasal layer of the gingival epithelium in seven out of 11 patients with nifedipine-induced gingival enlargement (4). However, the proliferative effect of VGB on gingival epithelial cells has yet to be determined.

VGB-induced gingival enlargement may be caused in part by an increase or dysregulation of cell proliferation. The present study used immunohistochemical methods to investigate the expression and possible role in VGB-induced gingival enlargement of Ki-67 antigen and p27^{KIP1}, p21^{WAF1} and p53, proteins that stimulate or inhibit cell-cycle progression. We present a short series of six adult patients treated with VGB for partial epileptic seizures refractory to classic anticonvulsant treatment.

Materials and methods

Six patients treated with VGB for partial epileptic seizures refractory to classic anticonvulsant treatment and who received no gingival overgrowthinducing drugs (hydantoin-derived drugs, immunosuppressants, or calcium channel blockers) were recruited from the Department of Neurology of the Virgen de las Nieves University Hospital. These patients were referred to our Department of Periodontology without previous oral evaluation. The gingival enlargement was assessed in a double-blinded fashion by two periodontists according to the clinical criteria proposed by Inglés (6), and the patient's oral health status was assessed by radiographic study and by determination of the plaque index of O'Leary (7), the bleeding index of Ainamo and Bay (8), and pocket depths.

In the patients with gingival enlargement (n = 4), biopsies were taken from an affected lateral sector of the papillary gingiva for histological and immunohistochemical study. Informed consent was obtained in all cases. The specimens were fixed in 4% buffered formalin for 24 h, embedded in paraffin, and stained with hematoxylin-eosin. Immunohistochemistry techniques were used to study the expression of different cyclin-dependent kinase (cdk) inhibitors and of the proliferation markers Ki-67 and p53. In parallel, the immunophenotype of the inflammatory infiltrate of the lamina propria was studied using antibodagainst leukocyte common ies antigen (CD45, clone 1.22/4.14), T lymphocytes (CD3, clone PS1), B lymphocytes (CD20, clone L26), monocyte/macrophage cells (CD68, clone KP1), and plasma cells (CD38, clone 38CO3) (all from Master Diagnóstica, Granada, Spain).

Sections were dewaxed, hydrated, and heat-treated in 0.01 M citrate buffer for antigenic unmasking. The rest of the procedure was carried out using an automatic immunostainer (Labvision, Fremont CA, USA). The incubation time with the primary antibodies was 60 min, and the streptavidin-biotin-peroxidase method (Master Diagnóstica) was used as visualization system. A millimeter scale in the evepiece of a microscope BH2 (Olympus, Tokyo, Japan) with $40 \times$ objective was used to count the positive nuclei per mm² for Ki-67 (clone MIB1; DakoCytomation, Freiburg Germany), p27^{KIP1} (clone DCS-72.f6; Master Diagnóstica), p21^{WAF1} (clone DCS-60.2; Master Diagnóstica), and p53 (clone DO7; Master Diagnóstica) antibodies in the free labial and sulcular gingival epithelium, and the inflammatory cells per mm² in the

lamina propria. As controls, biopsy specimens of the same area (lateral sectors) obtained from 10 dental patients without gingival enlargement or periodontal disease (healthy controls) and from 10 dental patients without gingival enlargement but with periodontal disease (400 × magnification in 10 fields) were similarly studied. The results were expressed as number of positive cells per mm². The morphological and immunohistochemistry study was done in a double-blinded fashion by two pathologists. A morphometric study on histological sections was performed, as previously described (9), to quantify the area and thickness of gingival epithelium in mm^2 , using a computer application of digital image analysis designed by our group with the Visilog 3.6 programme (Noesis, Velize, France).

The Kolmogorov–Smirnov test was used to test the normality of the variables. After the descriptive analysis, one-way analysis of variance (ANOVA) and Student's *t*-test analyses were performed to determine the statistical significance. Samples were stratified into controls and treated patients. The confidence interval was 95% (p < 0.05). The statistical analysis was performed using the SPSS-Windows 10.0 program (SPSS Inc, Chicago, IL, USA).

Results

Clinical dental study

The patients in the study, three males and three females with a mean age of 39.5 years (range, 14–61 years), received treatment with VGB (mean length of treatment, 6.25 years; range, 2.5–8 years) at a mean dose of 2.33 g/day (range 2–3 g/day). The oral examination revealed some degree of gingival enlargement in four of the six patients.

Two of these four patients presented generalized gingival growth of the free and attached gingiva in the anterior and posterior regions of both arches, more marked in the lower arch in one patient. This growth was more pronounced on labial than lingual surfaces and absent in edentulous spaces in both cases. Both patients reported that the gingival enlargement had commenced after 3-5 months of VGB treatment and had progressed slowly. Their gingival enlargement was evaluated as grade 2 according to the index of Inglés et al. (6). The two patients presented generalized horizontal bone loss in both arches, assessed with orthopantomography and by the mobility of teeth or by at least three probes of 6–7 mm in the upper arch at sites where no gingival growth was observed. The other two patients with gingival enlargement presented mild growth, evaluated as grade 1 (Inglés index), with involvement of papillary and free gingiva but no periodontal pockets. The remaining two patients did not present gingival enlargement. The mean plaque and bleeding scores for the overall series of six patients were 51.16% (range 12-75) and 27.5% (range 3-45), respectively.

The 10 dental patients without gingival enlargement or periodontal disease who provided control biopsies had a mean age of 35 ± 2.8 years, mean plaque index of $24\% \pm 2.4$, and bleeding index of $10\% \pm 0.3$, and were evaluated as grade 0 according to the Inglés index. (No overgrowth, firm adaptation of the attached gingiva to the underlying alveolar bone. There is no or slight stippling, as well as no or only slightly granular appearance. A knife-edged papilla is present toward the occlusal surface. There is no increase in density or size of the gingiva.) The 10 dental patients without gingival enlargement but with periodontal disease had a mean age of 47 ± 1.4 years, plaque index of $65\% \pm 4.5$, and bleeding index of $70\% \pm 6.8$, and the average Inglés index was grade 0 with slight stippling, and slightly granular appearance, but with 2.84 \pm 0.94 mm of pocket depth.

Morphological and immunohistochemical study

In the microscopic study, the biopsy specimens with gingival enlargement showed an epithelium with papillomatous lining and elongated rete ridges with divided ends. There were numerous cells in mitosis. In all the specimens, the lamina propria presented a dense extracellular matrix and numerous dilated vessels surrounded by a moderate or intense chronic inflammatory infiltrate.

In the immunohistochemical study, the nuclear expression of Ki-67 was significantly (p < 0.01, ANOVA) higher in the VGB-induced gingival enlargement than in the gingival epithelia of controls (894 \pm 101.6 positive cells/ mm^2 in VGB-induced gingival enlargement group vs. 391 ± 10.1 positive cells/mm² in healthy controls and 425 ± 12.0 positive cells/mm² in controls with periodontal disease) (Table 1, Fig. 1). The expression of nuclei was distributed positive throughout the basal and suprabasal layers, and the labial surface and sulcular epithelium showed a similar number of proliferating cells. No positivity for Ki-67 was observed in the fibroblasts of the lamina propria in any of the biopsy specimens.

We observed a slight increase in epithelial area $(0.385 \pm 0.04 \text{ vs.})$

Table 1. Mean of positive epithelial cells per mm² for Ki-67, p21^{WAF1}, p27^{KIP1}, and p53 in gingival biopsies of VGB-induced gingival enlargement

Antibody	Healthy controls (without periodontal disease) ^a $(n - 10)$	Controls with periodontal disease $(n - 10)^{b}$	Patients treated with VGB $(n - 4)$
Ki-67	391 ± 10.1	425 ± 12.0	(n = 1) 894 ± 101.6*
P27 ^{KIP1} P21 ^{WAF1} P53	$\begin{array}{rrrr} 1558 \ \pm \ 78.1 \\ 122 \ \pm \ 6.7 \\ 23 \ \pm \ 13.6 \end{array}$	$\begin{array}{rrrr} 1503 \ \pm \ 80.4 \\ 101 \ \pm \ 8.4 \\ 18 \ \pm \ 12.9 \end{array}$	$\begin{array}{rrrr} 1029 \ \pm \ 180.5 \\ 78 \ \pm \ 53.8 \\ 101 \ \pm \ 61.8 \end{array}$

Values are expressed as mean/mm² \pm standard deviation of 10 fields of high magnification. ^aMean of 10 healthy control biopsies \pm standard deviation.

^bMean of 10 control biopsies with moderate periodontal disease \pm standard deviation. *p < 0.01, ANOVA test.

 $0.349 \pm 0.02 \text{ mm}^2$) and thickness $(1.16 \pm 0.05 \text{ vs.} 1.0 \pm 0.07 \text{ mm})$ in gingival enlargement biopsies of VGBtreated patients vs. controls. This result indirectly supports an increase in the number of epithelial cells. There was an intense expression of p27^{KIP1}, mainly in the parabasal layer and malpighian layer of the mucosal epithelium. Almost all the fibroblasts and most of the inflammatory infiltrate of the lamina propria were positive for p27KIP1. The number of positive epithelial cells per mm² was very large but always below the number counted in the gingival mucosa of the controls $(1029 \pm 180 \text{ epithelial positive nuclei})$ per mm² in VGB-treated patients vs. 1558 ± 78.1 and 1503 ± 80.4 in healthy controls and controls with respectively) periodontal disease, (Table 1). The expression of p21^{WAF1} was similar among the three groups and was not very intense, with a mean density of 78 positive cells per mm² in epithelium of VGB-treated patients and 122 in epithelium of healthy controls (Table 1). A greater number of p53-positive cells were found in VGBtreated patients vs. either control group, although there was a wide variability among the biopsy specimens.

The VGB-treated patients showed an intense inflammatory infiltrate (CD45⁺) in the lamina propria, significantly more intense (p < 0.01 ANO-VA) than in either control group, with a major predominance of plasma cells $(CD38^+)$ and T-lymphocytes $(CD3^+)$ but few macrophages (CD68⁺) or B-lymphocytes (CD20⁺), which were all always at higher levels than in either control group. Table 2 lists the number of leukocyte subsets per mm² in VGBtreated patients and in the controls with or without periodontal disease. The inflammatory infiltrate was mainly localized in deep areas of the specimen, underlying the papillary sulcular epithelium.

Discussion

Gingival enlargement develops in half of all patients who are treated with hydantoins for epileptic seizures (10). It is an uncommon side-effect in



Fig. 1. Ki-67 expression in gingival mucosa: (A) expression in control, nuclear immunostaining in basal layer (brown deposit); (B) expression in VGB-induced gingival enlargement, major increase in nuclear immunostaining, related to increased proliferation (streptavidin– biotin–peroxidase method, $\times 200$). (C) Intraoral photograph of patient treated with VGB with moderate gingival enlargement.

patients treated with other anticonvulsant drugs, such as phenobarbital (11) or sodium valproate (12), and its histopathologic characteristics are different from those of hydantoin-induced gingival enlargement (13). Only two cases of VGB-induced gingival enlargement have been published (1, 3). In the present study of six VGBtreated patients, however, two presented moderate gingival enlargement and two mild gingival enlargement. The incidence of this side-effect may be underestimated, because clinical attention is focused on retinal changes, the main side-effect of VGB, and patients are unlikely to be referred to a dental specialist for oral evaluation because the gingival enlargement is not usually severe.

Gingival overgrowth induced by hydantoins, cyclosporin A, or calcium channel blockers is characterized by a localized growth of the interdental papillae and free gingiva that progressively covers the dental crown (10, 14, 15). The clinical manifestation was different in the present cases. The growth was diffuse, regular and generalized in both free and attached gingiva, with no tendency to cover the dental crowns.

The regulation and expression of proteins involved in the cell cycle may be altered when gingival enlargement is produced. Ki-67 antigen expression has been observed to be present in the nuclei of proliferating cells located in G₁, S, G₂ and M phases of the cell cycle and absent in quiescent cells $(G_0 \text{ phase})$ (16). In the present patients, the density of Ki-67-positive cells per mm² was significantly higher than in the controls. This same effect has been reported for gingival overgrowthinducing drugs such as nifedipine, phenytoin or cyclosporin A (4, 5). However, the proliferative effect of VGB on gingival epithelial cells has yet to be determined. There is no published evidence that the other drugs administered to the present patients, carbamazepine, levetiracetam, and clobazam, have an effect on cell proliferation. With respect to the effect on inflammatory infiltrate, only carbamazepine has been reported to cause immunosuppression (13).

Progression through the cell cycle is governed by cdks, whose activity is inhibited by cdk inhibitors such as p27KIP1 and p21WAF1. p27KIP1 is a member of the CIP/KIP family of cdk inhibitory proteins that negatively regulate cell proliferation. p27KIP1 acts in G₁ to inhibit cdks and preferentially inhibits S-phase kinases, thereby halting cell cycle progression. p27KIP1 blocks the cell cycle by binding to the cyclin E-Cdk2 complex (17). Many normal tissues, such as epithelium or lymphocytes, have nuclear levels of p27 that can be immunohistochemically detected (18). The number of epithelial cells that expressed p27KIP1 was lower

Table 2. Quantitation of leukocyte subpopulations in gingival lamina propria of patients with VGB-induced gingival enlargement

Leukocyte subsets	Healthy controls ^a (n = 10)	Controls with periodontal disease $(n = 10)^{b}$	Patients treated with VGB $(n = 4)$
CD45 (leukocyte common antigen) CD3 (T lymphocytes) CD20 (B lymphocytes) CD38 (plasma cells) CD68 (monocyte/macrophage cells)	$\begin{array}{rrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrr$	$\begin{array}{rrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrrr$	$\begin{array}{r} 4569.0 \pm 649* \\ 1163.1 \pm 1144 \\ 548.3 \pm 525 \\ 2619.8 \pm 1571\dagger \\ 198.0 \pm 96 \end{array}$

Values are expressed as mean/mm² \pm standard deviation of 10 fields measured.

^aMean of 10 healthy control biopsies \pm standard deviation.

^bMean of 10 control biopsies with moderate periodontal disease \pm standard deviation. *p < 0.01, ANOVA test.

†Healthy controls vs. patients treated with VGB and healthy controls vs. controls with periodontitis (p < 0.001, Student's *t*-test).

in the gingival enlargement of our VGB-treated patients than in the epithelium of the healthy or periodontal disease controls. This reduced expression, also reported in endocrine organs with hyperplasia (19), may be related to the greater epithelium proliferation in our patient and may be partly responsible for the development of the gingival enlargement.

p21^{WAF1} could inhibit cell-cycle progression by binding to cyclin E– cdk2, cyclin A–cdk2 or cyclin D–cdk4 complexes, thereby inactivating their kinase activities (20). Few p21^{WAF1}positive epithelial cells were found in any of the biopsies of the VGB-treated patients, supporting the hypothesis of a reduced inhibition of proliferation.

Wild-type p53 protein arrests cells in the G1 phase of the cell cycle and allows time for repair of the damaged DNA before entry into S (21). In normal tissues without impaired DNA, the halflife of wild-type p53 protein is too short to permit immunohistochemical detection. Nevertheless, antigen-retrieval techniques have resulted in the increased detection of wild-type p53 protein in normal tissues and it is possible to immunohistochemically detect some p53-positive cells. DO7 clone appears to be a more robust antibody for the detection of wild-type p53 (22). Similar to our findings, Saito et al. found p53 expression in isolated cells of the suprabasal layer of the gingival epithelium in patients with nifedipine-induced gingival enlargement (4).

It has recently been proposed that the immune response may be altered in drug-induced gingival enlargement (23). In our patients, the proportion of inflammatory cells per mm² (CD45⁺, $CD38^+$ or $CD3^+$) was far higher than the mean reported in normal gingival biopsies or in gingival overgrowth induced by cyclosporin A or nifedipine (14), although the low expression of Ki-67 (< 5%) and high expression of $p27^{KIP1}$ (> 80%) in the inflammatory infiltrate indicate scant local proliferation. This increase in inflammatory cells undoubtedly contributed to enlarge the gingiva and may even be implicated in the pathogenic mechanism of the gingival enlargement, as reported for other drugs. The fact that the gingival enlargement persisted 6 months after the withdrawal of VGB in one patient suggests that the inflammatory infiltrate of periodontal disease may participate in the development of this gingival enlargement, in an idiosyncratic response to the drug.

In conclusion, the overexpression of antigen Ki-67 and slight underexpression of the cdk-inhibitors p27^{KIP1} and p21^{WAF1} suggest that VGB induced an increase in epithelial cell proliferation and contributed to the gingival enlargement, together with the concomitant periodontal disease, by a mechanism that has yet to be elucidated.

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