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Participation of Tyrosine Kinase and Phospholipase Cγ in Isradipine-induced Proliferation of Cultured Human Gingival Fibroblasts

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Abstract: Some kinds of drugs such as calcium (Ca^{2+}) channel antagonists, antiepileptics and immunosuppressants cause gingival overgrowth as a side effect, the mechanism of which is still unclear. We have examined the effects of isradipine, one of the dihydropyridine-derivative Ca^{2+} channel antagonists, on cultured human gingival fibroblast Gin-1 cells. In the present study, to elucidate the mechanism by which isradipine causes gingival overgrowth, we examined whether tyrosine kinase (TK) and phopholipase Cγ (PLCγ) are involved in the isradipine-induced proliferation of gingival fibroblasts. Herbimycin A (1 μM) remarkably inhibited the isradipime (10 μM)-induced proliferation. Both U73122 (5 μM), a PLCγ inhibitor, and xestospongin C (5 μM), an antagonist of a receptor of inositol 1,4,5-trisphosphate in Ca^{2+} stores, significantly reduced the $[Ca^{2+}]i$ raised by isradipine (10 μM). Thus, the findings obtained here indicate that TK and PLCγ are closely involved in the isradipine-induced $[Ca^{2+}]i$ rise to elicit gingival overgrowth.

Key words: isradipine; tyrosine kinase; phopholipase Cγ; gingival overgrowth

Introduction

Some kinds of drugs such as calcium (Ca^{2+}) channel antagonists (nifedipine, etc.), antiepileptics (phenytoin, etc.) and immunosuppressants (cyclosporin A, etc.) cause gingival overgrowth as a side effect (Varnfield and Botha 2000). Various kinds of mechanisms have been proposed to explain the gingival overgrowth caused by such drugs (Metcalfe et al. 1986; Brown et al. 1991; Kataoka et al. 2001; Spolidorio et al. 2002; Johnson et al. 2000), ever since phenytoin-induced gingival hyperplasia was first reported in 1939 (Kimball 1939). Kataoka et al. (2001) recently described that the decrease in collagen degradation due to lower phagocytosis is closely associated with the increase in type I collagen accumulation in nifedipine-treated rat gingiva.

We have been examining the effects of isradipine, a dihydropyridine-derivative Ca^{2+} channel antagonist, on cultured human gingival fibroblasts. From the results obtained earlier that isradipine enhanced the proliferation, the release of bFGF, and the production of type I collagen (Hattori et al. 2004), we considered that the possibility that isradipine might also cause gingival overgrowth. Furthermore, we had previously observed unexpectedly that isradipine raised the $[Ca^{2+}]i$ in gingival fibroblasts (Hattori and Wang 2005). Thus, we supposed that Ca^{2+} might act as an essential second messenger to exert its proliferative effect. In the present study, in order to elucidate the mechanism by which isradipine causes gingival overgrowth, we examined whether or not tyrosine kinase (TK) and phopholipase Cγ (PL Cγ) are involved in the isradipine-induced proliferation of gingival fibroblasts. The findings obtained indicate that both TK and PLCγ take part in the $[Ca^{2+}]i$ elevation and the proliferation.

Materials and Methods

Normal human gingival fibroblast Gin-1 cells obtained from Dainippon Pharmaceutic Co. Ltd. (Japan) were used in these experiments. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum.

Isradipine-induced fibroblast proliferation in the presence and absence of herbimycin A was examined by using the reagent water-soluble tetrazolium-1 (WST-1), which is supplied in a commercially available assay kit. The cells were cultured in 96-well microculture plates. Control wells contained only isradipine, whereas herbimycin A along with the isradipine was added to the test wells. Both isradipine and herbimycin A were dissolved in dimethyl sulfoxide (DMSO) as a solvent, and the final concentration of DMSO in each well did not exceed 1 %. The number of cells per well was 1×10^4 at the starting point. The cell proliferation of fibroblasts with or without herbimycin A was examined over a time course of 10 days. Briefly, at selected times during the culture period, WST-1 was added to each well, and the plate was then incubated at 37 °C for 2 hours before the measurement.

The cells were kept in a solution consisting of 135 mM-NaCl, 5 mM-KCl, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 10 mM glucose, and 20 mM-HEPES-NaOH (pH 7.4) and were loaded with the dye, 5μM fura-2 acetoxymethyl ester (fura-2/AM), during a 45-min incubation at 37 °C. The excitation was provided by light from a Xenon lamp that was passed through a 340- or 360-nm filter. The wavelength of emission for analysis was

500 nm. Changes in the fluorescence intensity of fura-2 in the cells were recorded with a video-imaging analysis system.

Tissue culture reagents were purchased from Gibco (USA). Isradipine was a generous gift from Novartis Pharma (Switzerland). Herbimycin A was obtained from Sigma (USA). U73122 and xestospolin C came from Calbiochem (Germany). Fura-2/AM was from Dojindo Laboratories (Japan). All other chemicals were from Nacalai Tesque (Japan).

Each value of the data represents the mean value ± the standard error of the mean and the number of observations (N). Statistical analyses of the data were performed by using Student's simple t-test in the case of counting cell number and by the 2-sided paired t-test in the case of measuring $[Ca^{2+}]i$. Differences between mean values were considered significant if the probability of error (p) was less than 0.05.

The detailed methods for cell counting and $[Ca^{2+}]i$ measuring were described before (Hattori and Maehashi 1999; Hattori and Wang 2004).

Results

Fig. 1 shows the effects of herbimycin A, a TK inhibitor, on the isradipine-induced proliferation of Gin-1 cells. Herbimycin A (1 μM) remarkably inhibited the isradipine (10 μM)-induced proliferation. Especially, there was significant difference between the control group (isradipine alone) and test one (isradipine plus herbimycin A) beginning 5 days after the start of the cultures.

Fig. 2 illustrates the influence of U73122, a PLCγ inhibitor, on the isradipine-induced rise in $[Ca^{2+}]i$. This $[Ca^{2+}]i$ rise slowly appeared about 30 sec after isradipine application. U73122 (5 μM) significantly reduced the $[Ca^{2+}]i$ raised by isradipine (10 μM).

Finally, the effect of xestospogin C, an antagonist of a receptor of inositol 1,4,5-trisphosphate (IP_3) in Ca^{2+} stores, on the isradipine-induced $[Ca^{2+}]i$ rise was examined. As seen in Fig. 3, xestospongin C (5 μM) significantly reduced the isradipine (10 μM)-induced $[Ca^{2+}]i$ rise.

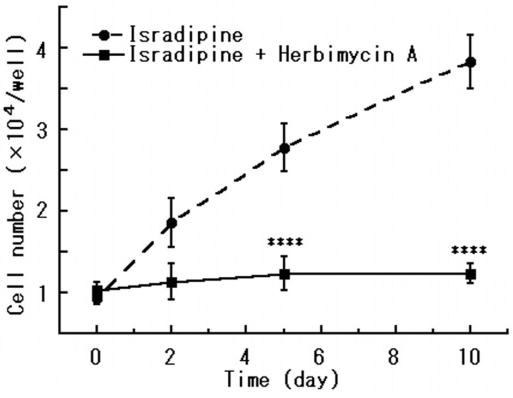

Fig. 1. Effect of herbimycin A on the isradipine-induced proliferation of Gin-1 cells. Herbimycin A (1μM) remarkably inhibited the isradipine (10 μM)-induced proliferation. ****p < 0.001, N = 8 for each datum point.

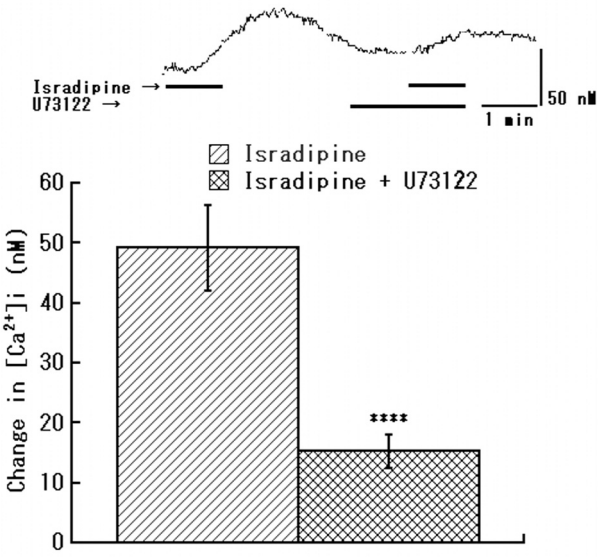

Fig. 2. Influence of U73122 on the $[Ca^{2+}]i$ raised by isradipine (10 μM). The trace above the graph shows the time course of the inhibiting effect of U73122. Peak points of the $[Ca^{2+}]i$ were adopted as data. U73122 significantly reduced the $[Ca^{2+}]i$. ****p < 0.001, N = 16.

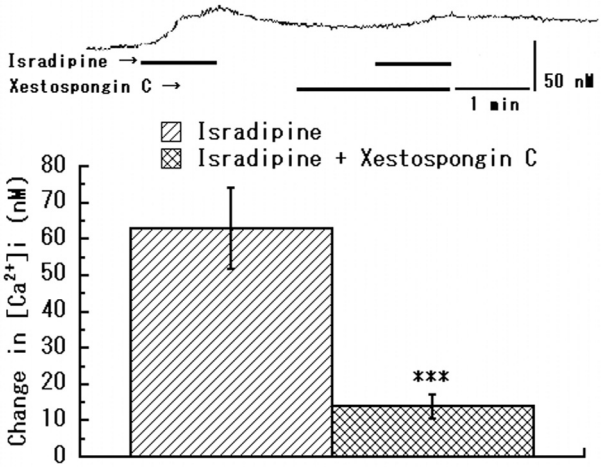

Fig. 3. Effect of xestospongin C on the rise in $[Ca^{2+}]i$ elicited by isradipine (10 μM). Xestospongin C (2 μM) significantly reduced the $[Ca^{2+}]i$. ***p < 0.005, N = 14.

Discussion

A number of Ca^{2+} channel blockers cause gingival overgrowth, for example, nifedipine, diltiazem, oxodipine, verapamil, nitrendipine, and felodipine (Varnfield and Botha 2000). With regard to the mechanism, numerous reports have been published. For example, Brown et al. (1991) described that inflammation from bacterial plaque is involved in the pathogenesis of drug-induced gingival hyperplasia. Spolindorio et al. (2002) observed that gingival overgrowth was caused by nifedipine and that the fibroblast and collagen density increased in parallel with the severity of the overgrowth. Brunius and Modéer (1989) claimed that phenytoin influences the cellular calcium metabolism

in fibroblasts, which action may contribute to the pathogenesis of gingival overgrowth. Moreover, Modéer et al. (1991) reported a relationship between phenytoin-induced increase in the $[Ca^{2+}]i$ in gingival fibroblasts and the clinical development of gingival overgrowth. The latter 2 reports appear to be reasonable, for one of the early events immediately induced by mitogens is an increase in cytosolic Ca^{2+} (Munaron 2002) and because the Ca^{2+} ionophore A23187 stimulates DNA synthesis in invertebrate and mammalian oocytes (Metcalfe et al. 1986).

We observed the isradipine-induced proliferation of cultured human gingival fibroblasts (Hattori et al. 2004) and recently found that isradipine raised the $[Ca^{2+}]i$ (Hattori and Wang 2005). From these facts, we supposed that cytosolic Ca^{2+} plays a key role in the proliferation of gingival fibroblasts. The result that herbimycin A inhibited the proliferation (Fig. 1) indicates that TK is closely associated with the proliferation. From the result that U73122 reduced the isradipine-induced $[Ca^{2+}]i$ rise (Fig. 2), we showed that PLCγ is related to the $[Ca^{2+}]i$ rise. It is unquestionable that IP_3 stimulates IP_3 receptors in the endoplasmic reticulum to cause the release of Ca^{2+}; because xestosporin C, an IP_3 receptor antgonist, decreased the isradipine-induced rise in $[Ca^{2+}]i$ (Fig. 3). Munaron et al. (2004) recently claimed that Ca^{2+} influx is a key signal in the control of cell proliferation, but they did not make mention of the Ca^{2+} release from Ca^{2+} stores.

In Fig. 4, we propose the following mechanism to be reasonable for isradipine-induced gingival overgrowth: First of all, isradipine elevates the $[Ca^{2+}]i$ by stimulating both nonselective cation channels (Hattori 2003) and Ca^{2+} release from intracellular stores (i.e., endoplasmic reticulum; Hattori and Wang 2005). Cytosolic Ca^{2+} elicits exocytosis (Becherer et al. 2003), which releases the growth factor bFGF (Hattori et al. 2004; Hattori and Wang 2005). This factor then, combines with TK-coupled receptors in autocrine and/or paracrine fashion to activate TK (Bansal et al. 2003), which in turn activates PLCγ (Haendeler et al. 2003). The action of this phospholipase raises the concentration of IP_3 by cleaving diacylglycerol from phosphatidylinositol 4,5-bisphosphate to generate IP_3 (Rameh et al. 1988). IP_3 stimulates IP_3 receptors in endoplasmic reticulum to cause the release of Ca^{2+}. This released Ca^{2+} then accelerates bFGF exocytosis through a positive feedback. In a different way, TK activates Ras proteins (Smith et al. 1986). The activated form of Ras proteins is associated with the activation of MAP kinase (Moodie et al. 1993), whose action promotes the transcription of c-fos genes (Gille et al. 1995) involved in cellular proliferation (Calaf and Hei 2004). Moreover, TK enhances collagen synthesis (Amemiya et al. 1999). Finally, gingival overgrowth is completed as a result of excessive fibroblast proliferation (Hattori et al. 2004) and type I collagen production (Hattori et al. 2004).

The findings obtained here led us to the conclusion that TK and PLCγ are closely involved in the isradipine-induced elevation of $[Ca^{2+}]i$ leading to gingival overgrowth.

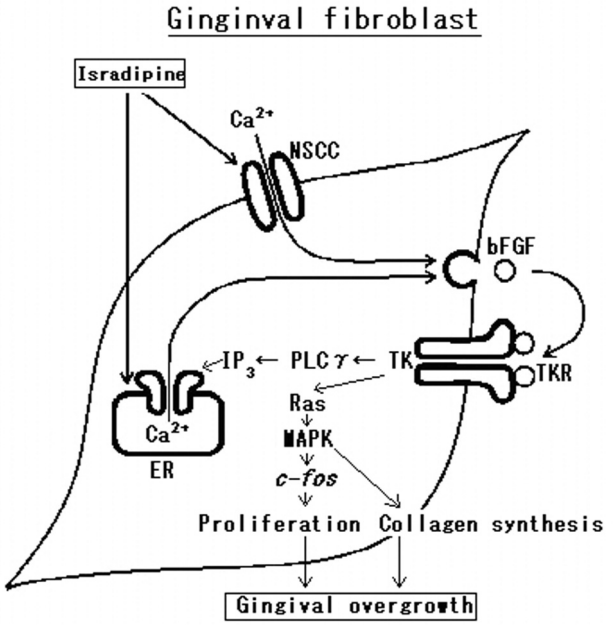

Fig. 4. Mechanism of isradipine-induced gingival overgrowth. NSCC: nonselective cation channel; bFGF: basic fibroblast growth factor; TKR: tyrosine kinase-coupled receptor; TK: tyrosine kinase; PLCγ: phospholipase Cγ; IP_3: inositol 1,4,5-trisphosphate; ER: endoplasmic reticulum; MAPK: MAP kinase. At first, isradipine raises the $[Ca^{2+}]i$ in gingival fibroblasts. This action finally becomes an indispensable trigger of gingival overgrowth.

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