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Original

Effects of interleukin-1α on the production and release of basic fibroblast growth factor in cultured nifedipine-reactive gingival fibroblasts

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Abstract: The effect of interleukin-1α (IL-1α) on the production of basic fibroblast growth factor (bFGF) in human gingival fibroblasts originated from a nifedipine-reactive patient was investigated. Ca^{2+}-mobilizing agents, thapsigargin and bradykinin, were also tested to determine whether they affected the production and release of bFGF. The release of bFGF from IL-1α-pretreated cells in relation to the transient increase in intracellular Ca^{2+} ($[Ca^{2+}]i$) and extracellular Ca^{2+} levels was also investigated. IL-1α and thapsigargin yielded significantly higher bFGF production, and also enhanced bFGF mRNA expression. IL-1α-pretreated cells showed significantly greater release of bFGF under the present experimental conditions. Levels of released bFGF were significantly higher in cells pretreated with IL-1α, followed by bradykinin and thapsigargin in the presence of extracellular Ca^{2+}. The transient mobilization of intracellular Ca^{2+} accelerated the release of bFGF in IL-1α-pretreated cells, but not in untreated cells. Thus, IL-1α increases bFGF production in nifedipine-reactive gingival fibroblasts and also influences the release of bFGF in the IL-1α-pretreated cells. (J. Oral Sci. 50, 83-90, 2008)

Keywords: IL-1α; bFGF; nifedipine-reactive human gingival fibroblasts; gingival overgrowth.

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Introduction

In a series of studies on the mechanism of gingival overgrowth caused by calcium channel blockers, we have investigated the effect of nifedipine, one of the dihydropyridine-calcium channel blockers, on cell proliferation, DNA synthesis, and collagen synthesis in human gingival fibroblasts originating from nifedipine-reactive patients (nifedipine responders; NIFr) and nifedipine-non-reactive patients (nifedipine non-responders; NIFn), and found that nifedipine enhanced these activities (1). We have also reported that a mixture of interleukin-1α (IL-1α) and nifedipine (NIF) accelerated human gingival fibroblast cell proliferation, suggesting that IL-1α might play an important role in gingival overgrowth caused by nifedipine (2). IL-1 has been reported to be a potent regulator of fibroblast proliferation (3-7), and is also known to induce the synthesis of basic fibroblast growth factor (bFGF) in human peritoneal mesothelial cells (8), rabbit corneal endothelial cells (9), and human osteoblasts (10). FGF was originally identified by its growth-stimulatory effect on 3T3 fibroblast cells (11). bFGF is a member of the large FGF family consisting of 30 members in humans (12), and is implicated in gingival overgrowth (13). It is involved in various cellular processes such as stimulation of DNA synthesis and cell proliferation, angiogenesis, and tissue repair, as well as differentiation and cell migration. Recently, we reported that the proliferation rate of NIFr cells in the presence of bFGF was significantly higher than that of NIFn cells as a result of active G_1/S transition of NIFr cells, as assessed by increments of cyclin E, pCDK2, and pRB (ser807/811)

protein in the cells (14).

Therefore we considered it worthwhile to investigate whether NIFr cells produced bFGF by IL-1α treatment in relation to gingival overgrowth. The present study was undertaken to clarify the effect of stimulants such as nifedipine, IL-1α, bradykinin, and thapsigargin on bFGF production in NIFr cells. We also investigated the release of accumulated bFGF in NIFr cells.

Materials and Methods

Cells

Cell cultures were prepared using the method reported previously (1,14,15). Briefly, cultures of fibroblast-like cells were established from a gingival specimen taken from a female nifedipine-reactive patient (nifedipine responder, duration of nifedipine medication at 40 mg/day: 2 years 8 months), aged 47 years, during clearance of remaining teeth. The plan to use gingival samples was approved by the Committee on Studies Involving Human Beings of Nihon University School of Dentistry at Matsudo (EC 099-001). The patient gave written informed consent for use of the gingival specimen. Since NIFr cells are significantly more affected than NIFn cells (14), we focused only on this cell line in the present study. Fibroblasts were obtained by trypsinization of primary cell outgrowths. After establishment of the cells, they were maintained in DMEM-10 (Dulbecc's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, 100 U/ml penicillin G, and 0.2 μg/ml amphotericin B), and routinely passaged using 0.25% trypsin and 0.02% EDTA in Dulbecco's phosphate-buffered saline (DPBS). Homogeneity of the fibroblasts was confirmed by flow cytometry (FACS Vantage, Nippon Becton Dickinson Co. Ltd., Tokyo, Japan). DMEM, DPBS, streptomycin, penicillin G, amphotericin B, trypsin, and FCS were purchased from Gibco Laboratories (Carlsbad, CA, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA). The fibroblasts used for experiments proliferated in the logarithmic phase between the 5th and 8th passages.

Determination of bFGF level in extracellular fluid and whole cells

Cells (2.5×10^4 cells/well) in 500 μl of DMEM-10 were allowed to settle in a 24-well multi-well plate for 48 h and then the medium was replaced with DMEM-1 (DMEM supplemented with 1% FCS and the same antimicrobial agents as those of DMEM-10). After the cells had been arrested in G_0/G_1 phase by DMEM-1, semi-confluent cells were treated with 100 pg/ml IL-1α, 5 nM bradykinin or 1 μM thapsigargin in DMEM-1 for 0, 3, 6, 12, 24, 48, and 72 h. Since NIFr cells proliferated in the presence of 10 ng/ml bFGF, and NIFr cells produced bFGF upon treatment with 100 pg/ml IL-1α in DMEM-0.5 (14), we used semi-confluent cells for pretreatment with 100 pg/ml IL-1α in DMEM-1. After the treatment, the extracellular solution was collected and the cells were washed three times with DPBS and then scraped off in 1 ml of DPBS supplemented with 0.01% BSA and 1% Nonidet P-40. The cell suspension was then sonicated for 20 s at 50 V (Ultrasonic Processor, VP-5, Taitec, Saitama, Japan) and kept at 4°C for 1 h. The extracellular fluid and lysed cell suspension were kept at -30°C until use. bFGF level was measured using a bFGF ELISA system (Amersham International plc, UK). The level of bFGF in the extracellular solution served as the extracellular fluid bFGF level and that of the whole cells served as the whole cell bFGF level. The bFGF level in the absence of drugs served as the control. The sum of whole cell and extracellular fluid bFGF levels was regarded as the total bFGF level.

Effect of IL-1α on expression of bFGF mRNA

After cells (1.0×10^6 cells/culture plate) had been arrested by DMEM-1 in a 100-mm culture plate, they were treated for 12 h with 1 μM nifedipine, 100 pg/ml IL-1α, or 1 μM thapsigargin. The total RNA was extracted from cells using a RNeasy Mini Kit (QIAGEN KK, Tokyo, Japan). RT-PCR was carried out using a OneStep RT-PCR Kit (QIAGEN KK) with 100 ng of total RNA and primer pairs specific for bFGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene (TAKARA BIO Inc., Shiga, Japan) (Table 1) (16,17). RT-PCR conditions were reverse transcription at 50°C for 30 min, initial PCR activation at 95°C for 15 min, denaturation at 94°C for 1 min, primer-annealing at 60°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Twenty-five amplification cycles were used for both bFGF and GAPDH. PCR reaction products were subjected to electrophoresis using E-Gel® 2% (Invitrogen Corp., Carlsbad, CA, USA). RNA was illuminated with UV light, and photographed using a digital camera.

Influence of IL-1α pretreatment on release of bFGF

After cells had been arrested by DMEM-1 in a 24-well multiwell plate, semi-confluent cells were pretreated with 100 pg/ml IL-1α in DMEM-1 for 48 h and then collected with DPBS containing 0.25% trypsin and 0.02% EDTA. At this point, the whole cell bFGF level was almost maximal (Fig. 1, IL-1α treated for 48 h, 4.43 ± 0.86 ng/10^5 cells). Cells were washed three times with DPBS in order to exclude the influence of DMEM-1 and trypsin. Since

the extracellular fluid bFGF level in DMEM-1 was almost lower than the detectable range, i.e. less than 5 pg/ml, we carried out preliminary tests to find that PSS was suitable for this experiment. PSS contained (in mM) NaCl, 136.9; KCl, 15.4; $CaCl_2$, 1.5; $MgCl_2$, 1.0; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20; ethylenediamine-N, N, N', N'-tetraacetic acid (EDTA), 0.01; TPEN, 0.01; and glucose 5.5 (18). Cells were then suspended in PSS (containing 1.5 mM Ca^{2+}) or in Ca^{2+}-free PSS (lacking $CaCl_2$) and incubated for 0, 1, 3, 5, 10, 20, 30, 60 min at 37°C without or with 5 nM bradykinin or 1 μM thapsigargin. After the treatment, the cell suspension was centrifuged at 500 x *g* for 5 min at 4°C. The supernatant was collected and served as a sample for determining the extracellular fluid bFGF level. The residual cells in PSS were lysed in the same manner as described above. The lysates were kept at 4°C for 1 h and served as a sample for determining the whole cell bFGF level.

Statistical analysis of data

All data were expressed as the mean ± S.D. Statistical significance was determined by Dunnett's t test for multiple comparisons. Differences at $P < 0.05$ were considered to be significant.

Results

Effect of stimulants on total bFGF level

We have previously reported that IL-1α accelerates NIFr cell proliferation and suggested that it might play an important role in gingival overgrowth (2). Therefore it was of interest to investigate how IL-1α affects cell growth, focusing specifically on bFGF and Ca^{2+} mobilization. We studied the effect of IL-1α, and the Ca^{2+} mobilizing agents bradykinin and thapsigargin, on the level of total bFGF and the results are summarized in Fig. 1. Cells were treated with 100 pg/ml IL-1α, 5 nM bradykinin (BK) or 1 μM thapsigargin (TG) for 0, 3, 6, 12, 24, 48, and 72 h. IL-1α and thapsigargin significantly increased the total bFGF level ($P < 0.01$, $P < 0.05$, respectively), whereas bradykinin had no such effect. Nifedipine and exogenous bFGF also had no effect on the total bFGF level (data not shown). Since the levels of bFGF in extracellular fluid were below the limits of detection (<5 pg/10^5 cells), the values in Fig. 1 appear almost the same as the whole cell bFGF level.

Effect of IL-1α on expression of bFGF mRNA

Substances that play an active role in bFGF production (Fig. 1), IL-1α and thapsigargin, were investigated for their enhancement of mRNA expression in NIFr cells. Nifedipine was also studied because it is known to affect the growth of NIFr cells. After growth arrest, cells were treated for 12 h with 1 μM nifedipine, 100 pg/ml IL-1α, or 1 μM thapsigargin. As summarized in Fig. 2, IL-1α and thapsigargin enhanced the expression of bFGF mRNA, whereas nifedipine had no such effect.

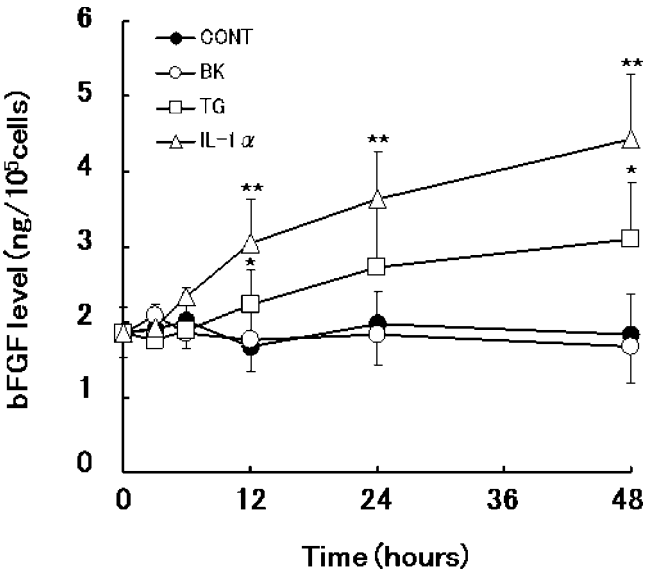

Fig. 1 Effect of stimulants on total bFGF (whole cell and extracellular fluid bFGF) production in NIFr cells. Cells arrested in DMEM-1 in 24-well culture plates were treated with 100 pg/ml IL-1α (IL-1α), 5 nM bradykinin (BK) or 1 μM thapsigargin (TG) for 0, 3, 6, 12, 24, and 48 h. Total bFGF in the absence of drugs served as a control. The bFGF level in the extracellular solution served as the extracellular fluid bFGF level and that of the whole cells served as the whole cell bFGF level. Cells cultured in the absence of drugs served as the control (CONT). The sum of whole cell and extracellular fluid bFGF levels was regarded as the total bFGF level. Statistical analysis: * $P < 0.05$; ** $P < 0.01$.

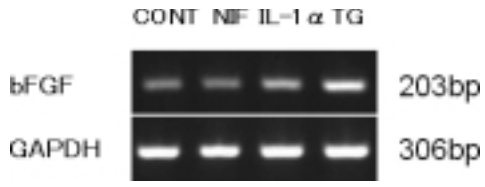

Fig. 2 Effect of stimulants on bFGF mRNA expression in NIFr cells. Cells were stimulated for 12 h with 1 μM nifedipine (NIF), 100 pg/ml IL-1α (IL-1α) and 1 μM thapsigargin (TG). RT-PCR reaction products for bFGF were subjected to electrophoresis using E-Gel® 2%, and then illuminated with UV light and photographed using a digital camera. GAPDH was used as an internal control.

Influence of IL-1α pretreatment on release of bFGF

As described above, the level of bFGF in extracellular fluid was less than 5 pg/10^5 cells. Since it is thought that released bFGF might be degraded or bound to extracellular matrix components such as heparan sulfate glycosaminoglycan on the outer cell membrane in DMEM-1, a different extracellular solution that does not affect the amount of bFGF in extracellular fluid was necessary in order to study the release of bFGF from the cells. For this purpose we used PSS as the extracellular solution and examined the release of bFGF from IL-1α-treated cells. As shown in Fig. 1, bFGF formation was almost maximal in cells treated with 100 pg/ml IL-1α for 48 h, and the cells

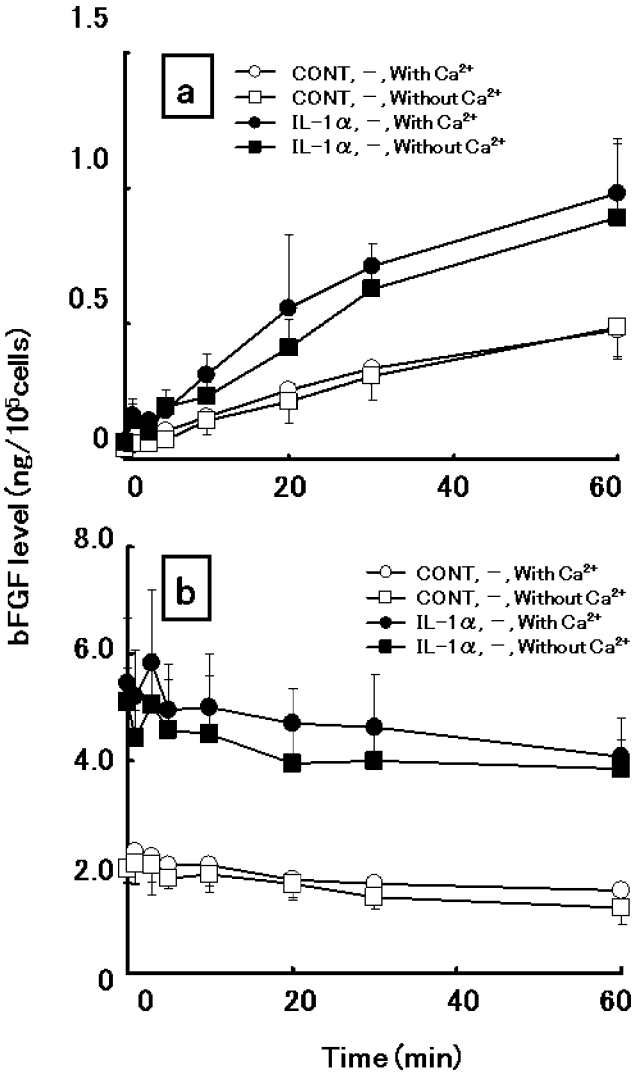

Fig. 3 bFGF release from NIFr cells in the presence or absence of Ca^{2+} after pretreatment with IL-1α. The bFGF level in untreated cells (CONT) served as a control. Semi-confluent cells were pretreated with 100 pg/ml IL-1α for 48 h and collected with DPBS containing 0.25% trypsin and 0.02% EDTA. A cell suspension in PSS (containing 1.5 mM Ca^{2+}) or Ca^{2+}-free PSS (omission of $CaCl_2$) was incubated without addition of stimulant (-) for 0, 1, 3, 5, 10, 20, 30, 60 min at 37°C. After the treatment, the cell suspension was centrifuged at 4°C and the supernatant (extracellular fluid) was collected. a, Extracellular fluid bFGF level; b, whole cell bFGF level.

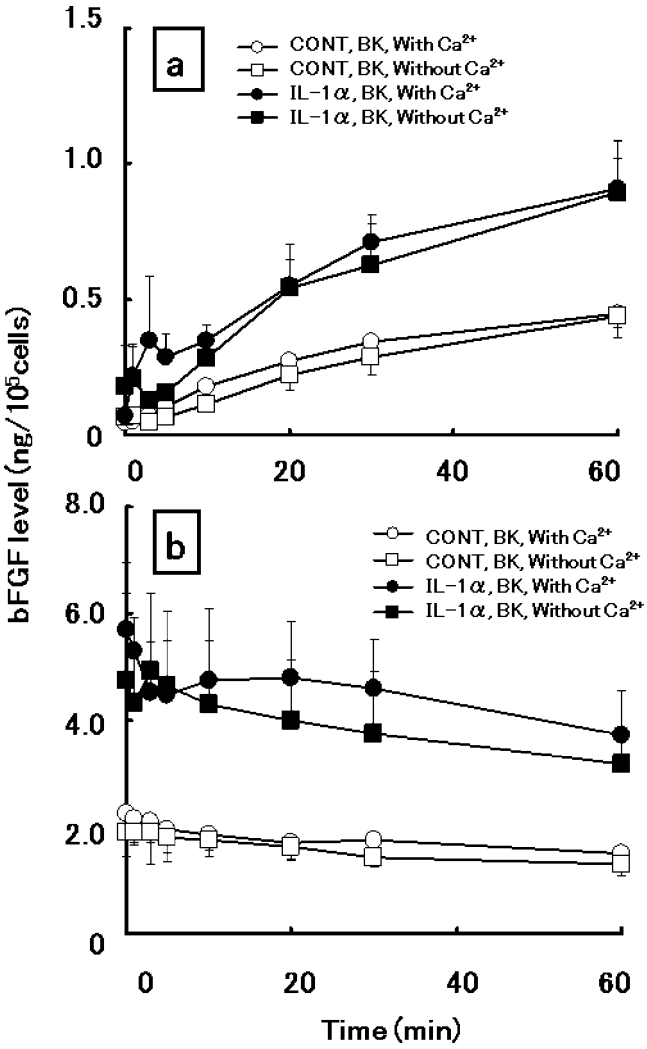

Fig. 4 bFGF release from NIFr cells treated with 5 nM bradykinin (BK) in the presence or absence of Ca^{2+} after pretreatment with IL-1α. Semi-confluent cells were pretreated with 100 pg/ml IL-1α for 48 h and collected with DPBS containing 0.25% trypsin and 0.02% EDTA. The bFGF level in the cells in the absence of IL-1α served as a control. A cell suspension in PSS (1.5 mM Ca^{2+}) or Ca^{2+}-free PSS was treated with 5 nM bradykinin (BK) for 0, 1, 3, 5, 10, 20, 30, 60 min at 37°C. After the treatment, the cell suspension was centrifuged at 4°C and the supernatant was collected. a, Extracellular fluid bFGF level; b, whole cell bFGF level.

had twice the amount of bFGF compared with non-treated cells. The bFGF level in extracellular fluid increased in a time-dependent manner (Figs. 3a, 4a, and 5a). IL-1α-pretreated cells released a significantly higher amount of bFGF into the extracellular fluid than non-pretreated cells. A small peak of bFGF release was found in bradykinin- and thapsigargin-treated cells within the first 5 min, perhaps indicating transient Ca^{2+} mobilization. In order to clarify the effect of the transient Ca^{2+} mobilization, differences in extracellular bFGF levels between control and bradykinin- or thapsigargin-treated cells in the presence of extracellular Ca^{2+} were calculated (Fig. 6). In bradykinin-treated cells, a transient peak was noted within 10 min. In thapsigargin-treated cells, a transient peak of Ca^{2+} was also obtained within 5 min, followed by a sustained increase. Furthermore, IL-1α-non-treated cells in the presence of extracellular Ca^{2+} and bradykinin- or thapsigargin-treated cells in the absence of extracellular Ca^{2+} showed only negligible changes (data not shown). The effect of transient and sustained Ca^{2+} mobilization was further studied by examining the effects of bradykinin and thapsigargin on the total amount of bFGF released in NIFr cells pretreated with 100 pg/ml IL-1α. The total amount of bFGF released during 30 min is shown in Fig. 7. The amount of bFGF released in both bradykinin- and thapsigargin-treated NIFr cells was higher in the presence, than in the absence, of extracellular Ca^{2+}, indicating that the influx of Ca^{2+} after transient Ca^{2+} mobilization might play an important role in these events. On the other hand, the total bFGF level was slightly decreased in the whole cells (Figs. 3b, 4b, and 5b, respectively), and the whole cell bFGF level was slightly decreased after pretreatment with IL-1α followed by treatment with or without bradykinin or thapsigargin (Figs. 3b, 4b, and 5b, respectively). Since the amount of bFGF released from whole cells was nearly equal to the

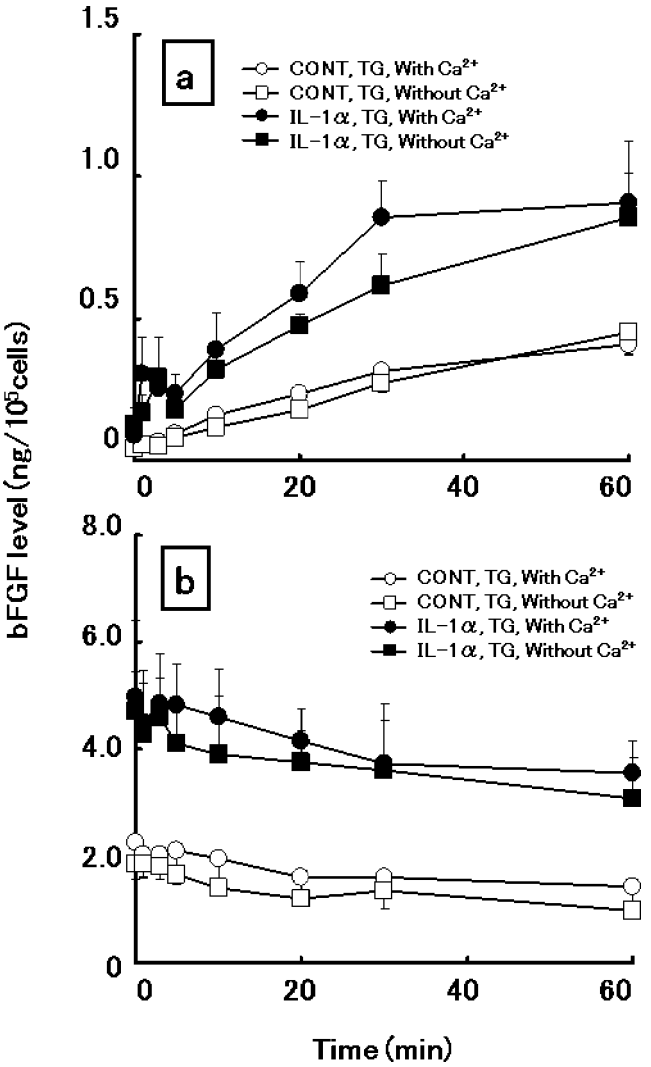

Fig. 5 bFGF release from NIFr cells treated with 1 μM thapsigargin (TG) in the presence or absence of Ca^{2+} after pretreatment with IL-1α. Semi-confluent cells were pretreated with 100 pg/ml IL-1α for 48 h and collected with DPBS containing 0.25% trypsin and 0.02% EDTA. The bFGF level in the cells in the absence of IL-1α served as a control. The cell suspension in PSS (1.5 mM Ca^{2+}) or Ca^{2+}-free PSS was treated with 1 μM thapsigargin (TG) for 0, 1, 3, 5, 10, 20, 30, 60 min at 37°C. After the treatment, the cell suspension was centrifuged at 4°C and the supernatant was collected. a, Extracellular fluid bFGF level; b, whole cell bFGF level.

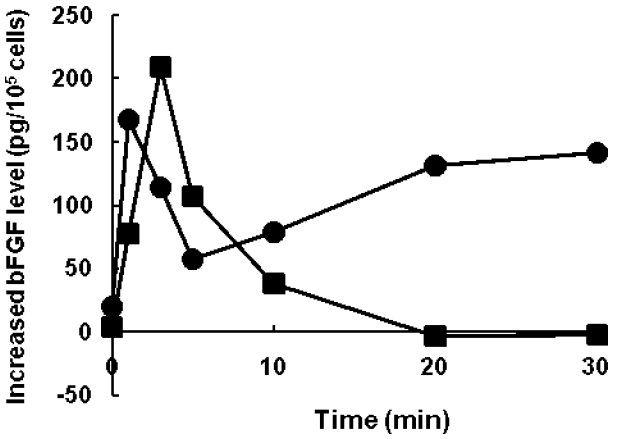

Fig. 6 Increased extracellular bFGF levels in bradykinin- and thapsigargin-treated NIFr cells pretreated with IL-1α. As shown above, transient bFGF release was noted in IL-1α-pretreated NIFr cells subsequently treated with bradykinin or thapsigargin in the presence of extracellular Ca^{2+}. These increased releases were calculated to obtain the differences in bFGF release between bradykinin- or thapsigargin-treated cells and the control. ■, bradykinin-treated cells. ●, thapsigargin-treated cells.

amount lost from the cells, formation of bFGF was considered not to occur in this system.

Discussion

IL-1α and thapsigargin, but not bradykinin, induced an accumulation of total bFGF in NIFr cells (Fig. 1). Nifedipine and exogenous bFGF failed to enhance the total bFGF level (data not shown). It has been reported that IL-1 induces synthesis of bFGF in human peritoneal mesothelial cells (8), rabbit corneal endothelial cells (9), and human osteoblasts (10). NIFr cells investigated in the present study were also found to produce bFGF when treated with IL-1α. FGF was originally identified by its ability to stimulate the growth of 3T3 fibroblasts (11). We have also reported that bFGF enhances the growth of NIFr cells (14). Therefore, it was thought that enhanced production of bFGF might play a role in the stimulation of NIFr cell growth by IL-1α. Thapsigargin releases intracellularly stored Ca^{2+} by inhibition of Ca^{2+}- ATPase in the sarcoplasmic reticulum and also stimulates Ca^{2+} influx through the plasma membrane (19). The increase in the intracellular level of Ca^{2+} in the presence of a sufficient extracellular Ca^{2+} concentration (1.0-2.0 mM) plays an important role in cell proliferation (20). It might also be speculated that the increase of $[Ca^{2+}]i$ induced by thapsigargin plays an important rols in acceleration of cell growth, although we believe that a downstream signal transduction pathway may be involved. Yokota et al. (21) have reported that bradykinin stimulates Ca^{2+} release from the inositol 1,4,5-trisphosphate (IP_3)-sensitive pool, although it is insufficient for prostaglandin E_2 release in human gingival fibroblasts. We have previously observed that sustained Ca^{2+} influx is greater when NIFr cells are treated with thapsigargin in comparison to bradykinin (15,18). Although the mechanism responsible for intracellular Ca^{2+} mobilization differs between bradykinin (Ca^{2+} release from the IP_3-sensitive pool) and thapsigargin (discharge of Ca^{2+} from the pool by inhibition of Ca^{2+}-ATPase), it seems reasonable to conclude that a sufficient amount of sustained Ca^{2+} influx might be important for acceleration of bFGF production. IL-1α and thapsigargin, but not nifedipine, enhanced the expression of bFGF mRNA (Fig. 2). Therefore, nifedipine does not directly affect bFGF production.

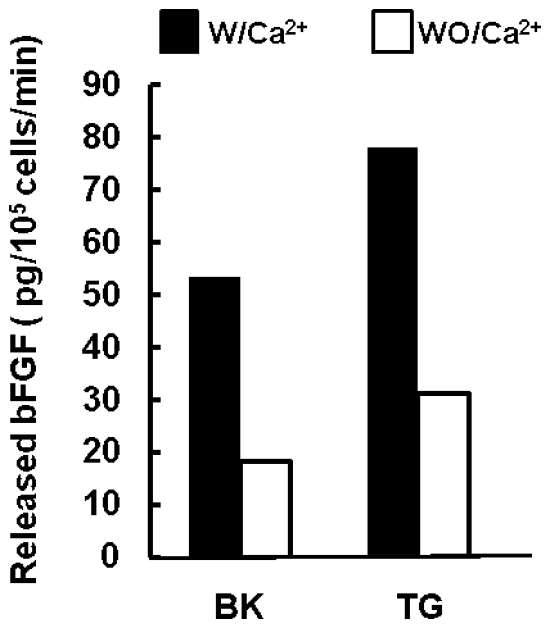

Fig. 7 Released bFGF in bradykinin- and thapsigargin-treated NIFr cells pretreated with IL-1α, with or without extracellular Ca^{2+}. The total bFGF released during 30 min was obtained in bradykinin (BK)- and thapsigargin (TG)-treated NIFr cells pretreated with 100 pg/ml IL-1α with (W) or without (WO) extracellular Ca^{2+}. ■, with extracellular Ca^{2+}. □, without extracellular Ca^{2+}.

Our study of bFGF release from NIFr cells treated with 100 pg/ml IL-1α for 48 h showed that they possessed more than twice the amount of bFGF compared with non-treated cells, and that the level of bFGF formation became almost maximal (Fig. 1). The release of bFGF increased in a time-dependent manner in control, bradykinin-treated and thapsigargin-treated cells. However, the release of bFGF from IL-1α-non-treated cells (CONT) was significantly lower. Thus, IL-1α might enhance not only the production, but also the release of bFGF. The release of bFGF in the presence of extracellular Ca^{2+} was slightly greater than that in its absence (Figs. 3-5). The extracellular concentration of Ca^{2+} is known to regulate hormonal secretion and cell differentiation in some types of cell (20,22). However, NIFr cells did not require extracellular Ca^{2+} for the release of bFGF. Bradykinin and thapsigargin caused a transient increase of $[Ca^{2+}]i$ and we noticed a small transient activation of bFGF release within less than 10 min (Fig. 6). Thapsigargin-treated cells also showed a sustained release of Ca^{2+}, suggesting that the sustained Ca^{2+} mobilization affected the release of bFGF. The influx of Ca^{2+} might be important for continuous bFGF release (Figs. 6 and 7). However, in the absence of extracellular Ca^{2+}, the transient increase of $[Ca^{2+}]i$ induced by thapsigargin or bradykinin had no influence on bFGF release (Fig. 4b. and 5b). The data for bFGF release induced by treatment with IL-1α alone (Fig. 3b), which does not affect the transient increase of $[Ca^{2+}]i$, agree with these findings.

In a previous study (2), we found that IL-1α enhanced cell growth and that the effect was further enhanced in the

presence of nifedipine. The effect of IL-1α, either alone or with nifedipine, in NIFr cells was greater than that in NIFn cells. In the present study, we found that NIFr cells produced a higher amount of bFGF after IL-1α treatment than non-treated control cells. Takeuchi et al. (14) demonstrated that cell growth and cell cycle progression in NIFr cells were more sensitive to bFGF in than in NIFn cells. Therefore, the enhancement of cell growth by IL-1α is related to bFGF production and release. Since nifedipine does not affect bFGF production, but does accelerate cell growth in the presence of IL-1α (2), nifedipine does not act via bFGF formation. Thus, the acceleration of cell growth by IL-1α with nifedipine might be an additive effect involving stimulation of bFGF production by IL-1α and another mechanism involving nifedipine.

IL-1α and thapsigargin induced bFGF production in NIFr cells, whereas neither nifedipine nor bradykinin had any such effect. IL-1α and thapsigargin, but not nifedipine, enhanced the expression of bFGF mRNA in comparison with the non-treated control. NIFr cells loaded with bFGF as a result of IL-1α pretreatment showed significantly greater bFGF release than untreated cells. Transient stimulation of bFGF release within less than 5 min was induced by the intracellular Ca^{2+}-mobilizing agents, bradykinin and thapsigargin. Since nifedipine did not enhance bFGF production, the previously reported acceleration of cell growth by IL-1α and nifedipine might be induced in a different manner.

Acknowledgments

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