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Original

The effect of basic fibroblast growth factor on cell cycle in human gingival fibroblasts from nifedipine responder and non-responder

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Abstract: It has previously been demonstrated that gingival fibroblasts derived from nifedipine-reactive patients (nifedipine responders) show a greater cell proliferation rate than those from nifedipine non-reactive patients (nifedipine non-responders) in the presence of 1 μM nifedipine. The aim of the present study was to characterize cell cycle differences between nifedipine responder and non-responder fibroblast cells and determine the effect of basic fibroblast growth factor (bFGF) on cell cycle progression. Further, the effect of bFGF on cyclins A, B1, D1, E, and CDKs 1, 2, 4, 6 mRNA expression in responder and non-responder cells was investigated. A population of nifedipine responder cells underwent progression to S and G2/M phases from G0/G1 phase in the presence of 10% fetal calf serum or 10 ng/ml bFGF was greater than nifedipine non-responder cells. mRNA expression of cyclins A, B1, D1, E and CDKs 1, 2, 4, 6 in the presence of 10 ng/ml bFGF was generally greater in nifedipine responder cells than non-responder cells. These results indicate that nifedipine responder cells may be more susceptible to growth factors such as bFGF with a resultant increase in expression of cyclins and CDKs in responder compared with non-responder cells. (J. Oral Sci. 46, 37-44, 2004)

Key words: nifedipine; cyclin; cyclin-dependent kinase (CDK); gingival fibroblast.

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Introduction

Gingival overgrowth in response to anti-epileptics (phenytoin), immunosuppressants (cyclosporin A), and calcium channel blockers (nifedipine, diltiazem, verapamil, nicardipine) is well-recognized (1). In the case of calcium channel blockers, gingival overgrowth is particularly marked with nifedipine (NIF). However, there is little information on the mechanism(s) by which NIF induces gingival overgrowth.

We have previously demonstrated that gingival fibroblasts derived from NIF responder (reactive patient, NIFr) exhibit greater proliferation rates, DNA synthesis and collagen synthesis than those from NIF non-responder (non-reactive patient, NIFn) in the presence of 1 μM of NIF (1). We have also shown that a mixture of NIF and interleukin-1 alpha (IL-1α) increases cell proliferation, suggesting that IL-1α may be a co-factor for NIF-induced gingival overgrowth (2). We have also demonstrated that IL-1α, but not NIF, accelerates the formation of intracellular basic fibroblast growth factor (bFGF) in cultured human gingival fibroblasts (3).

bFGF is a recognized mitogen for fibroblasts (4), keratinocytes (5), and epithelial cells (6), and also induces the progression of the cell cycle from G0/G1 to S phase in Schwann cells (7) and endothelial cells (8). Cell proliferation is dependent upon cell cycle progression, which is controlled by cyclins that activate cyclin-dependent kinases (CDKs). Therefore, it was proposed to determine whether bFGF plays a role in the growth of NIFr and NIFn cells. In the present study, the author compared the cell cycle progression and mRNA expression of cyclins A, B1, D1, E, and CDKs 1, 2, 4, 6 in the presence of bFGF in NIFr and NIFn cells.

Materials and Methods

Cell culture

Cultures of fibroblast-like cells were established from gingival specimens using methods described previously (9). The specimens were obtained during extraction of the remaining teeth from both a patient who had developed gingival overgrowth as a result of nifedipine medication (NIFr) and a patient who did not develop gingival overgrowth following nifedipine medication (NIFn). The protocol was approved by the Committee on Studies Involving Human Being of the Nihon University School of Dentistry at Matsudo (EC 099-001), and each patient gave informed consent for study participation. Cells were incubated in an atmosphere of 5% CO_2 - 95% air at 37°C in culture medium, DMEM-10 (Dulbecco's modified Eagle medium, DMEM, supplemented with 10% fetal calf serum (FCS), streptomycin 100 µg/ml, penicillin G 100 U/ml, amphotericin B 0.2 µg/ml), and routinely passaged with 0.25% trypsin and 1mM EDTA·4Na in Hanks solution (Invitrogen Corp., USA).

In order to synchronize the fibroblast cell cycle to G0/G1 phase, preliminary studies were performed to determine the optimal concentration of FCS and duration of incubation. These parameters were determined to be 0.5% FCS and 24 h pretreatment, respectively. Thus, cells were arrested by incubating in DMEM-0.5 (DMEM, supplemented with 0.5% FCS and antibiotics described as above) for 24 h.

Cell cycle analysis

Quiescent cells were stimulated to reenter the cell cycle by changing the culture medium to either DMEM-10 (FCS 10%) or 10 ng/ml bFGF in DMEM-0.5. More specifically, after cells had been arrested in G0/G1 phase by incubation in DMEM-0.5 for 24 h, cells were stimulated by incubation in DMEM-10 for 12, 15, 18, 21, and 24 h or 10 ng/ml bFGF in DMEM-0.5 for 18, 24, 30, 36, 42, and 48 h. Cells were then collected by trypsinization (0.25% trypsin and 1 mM EDTA·4Na in Hanks solution for 2 min at 37°C), pelleted (100 × g), washed twice in Dulbecco's phosphate-buffered saline (DPBS, Invitrogen Corp., USA) and resuspended in DPBS to a final concentration of approximately 1×10^6 cells/ml. Cell cycle analysis was performed using a CycleTEST PLUS DNA Reagent Kit (Becton Dickinson and Company, USA) and FACS Calibur (Becton Dickinson Immunocytometry Systems, USA). Data were analyzed using CellQuest software and ModFit LT (Verity Software House, USA), and the percentage of cells in G0/G1, S, and G2/M phases were determined.

Table 1 DNA primers applied in PCR analysis

Name	Primer sequences (5'→3')	Size (bp)
Cyclin A	TCCAAGAGGACCAGGAGAATATCA TCCTCATGGTAGTCTGGTACTTCA	466
Cyclin B1	GGACTGAGGCCAAGAACAGCTCTT CAGCTCCATCTTCTGCATCCACAT	365
Cyclin D1	AGCCATGGAACACCAGCTCCTGTG GATGGAGCCGTCGGTGTAGATGCA	399
Cyclin E	AGTTCTCGGCTCGCTCCAGGAAGA TCTTGTGTCGCCATATACCGGTCA	474
CDK1	CCGGGATCTACCATACCATTGACT GGAATCCTGCATAAGCACATGCTG	249
CDK2	ACGTACGGAGTTGTGTACAAAGCC GCTAGTCCAAAGTCTGCTAGCTTG	405
CDK4	CCAAAGTCAGCCAGCTTGACTGTT CATGTAGACCAGGACCTAAGGACA	193
CDK6	TGATGTGTGCACAGTGTCACGAAC CTGTATTCAGCTCCGAGGTGTTCT	737
GAPDH	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTGGTGAAGAC	306

RNA preparation and RT-PCR analysis

After arrest in the G0/G1 phase using DMEM-0.5 for 24 h, cells were stimulated for 3, 6, 12, 18, 24, 30, 36, 42, and 48 h with 10 ng/ml bFGF in DMEM-0.5. The total RNA was extracted from cells at each time point using the RNeasy Mini Kit (QIAGEN, Japan). RT-PCR was carried out using the OneStep RT-PCR Kit (QIAGEN, Japan) with 100 ng of total RNA, and primer pairs specific for cyclins A, B1, D1, E, CDKs 1, 2, 4, 6, and for the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (TAKARA BIO, Japan) (Table 1) (10,11). RT-PCR conditions were reverse transcription at 50°C for 30 min, initial PCR activation step at 95°C for 15 min, denaturation step at 94°C for 1 min, primer-annealing step at 64°C for 1 min, extension step at 72°C for 1 min, and final extension step at 72°C for 10 min. PCR reaction products were subjected to electrophoresis using 2% agarose gel. Gels were stained using 0.5 µg/ml of ethidium bromide, illuminated with UV light, photographed using a digital camera, and analyzed by computerized densitometric scanning of the images using Lane Analyzer (Rise & ATTO, Japan). Values were adjusted for assay variation by dividing the integrated optical density of cyclins A, B1, D1, E, and CDKs 1, 2, 4, 6 mRNA by the integrated optical density of GAPDH mRNA.

Statistical analysis

Differences between NIFr and NIFn groups were determined using the Student t test with the significant level at $P < 0.05$.

Results

Difference in cell growth between NIFr and NIFn cells in DMEM-10

To investigate the cell cycle-specific effect of FCS,

quiescent cells were stimulated to synchronously reenter the cell cycle in DMEM-10. Thus, cells were arrested by DMEM-0.5 for 24 h and then stimulated by changing FCS concentration to 10% (DMEM-10) for 12, 15, 18, 21, and 24 h. The percentages of cells in G0/G1, S, and G2/M phases were determined using flow cytometry. Fig. 1A shows a representative flow cytometric analysis of the cellular DNA content of NIFr and NIFn cells. Fig. 1B shows a summary of the difference from 0 h for G0/G1, S and G2/M phases at the indicated time point in proliferating NIFr and NIFn cells in DMEM-10. The percentage of NIFr cells in the G0/G1 phase slightly decreased more than in the case of the NIFn cells at 18, 21, and 24 h. The percentage of NIFr cells in the S phase slightly increased more than in the case of the NIFn cells at 18, 21, and 24 h. In the G2/M phase, the percentage of NIFr cells slightly increased more than in the case of the NIFn cells at 21 and 24 h. These results indicated that a population of NIFr cells

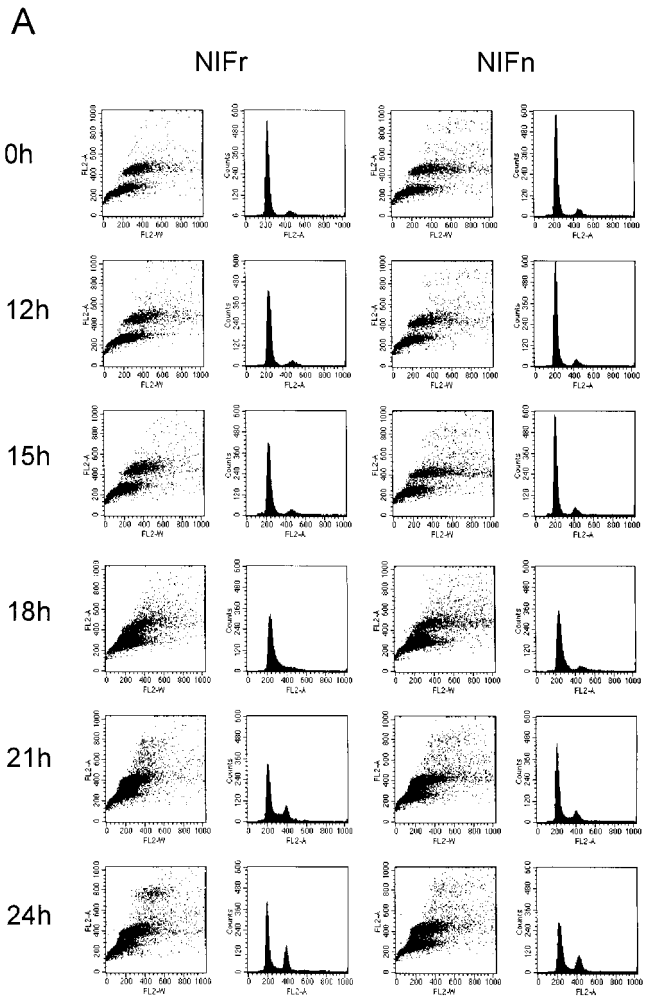

Fig. 1A DNA histogram: NIFr and NIFn cells were arrested by incubating in DMEM-0.5 for 24 h. Cells were then stimulated for 12, 15, 18, 21, and 24 h after changing the medium to DMEM-10. Propidium iodide fluorescence intensity corresponds to the DNA contents. The left column of each NIFr and NIFn cells shows the raw data while the right column of each NIFr and NIFn cells shows the relationship between the FL2-A channel and the number of cells. G0/G1 and G2/M cells were found at peaks of approximately 200 and 400 FL2-A channels, respectively, and the S cells were found between them. This figure is a representative data out of three trials.

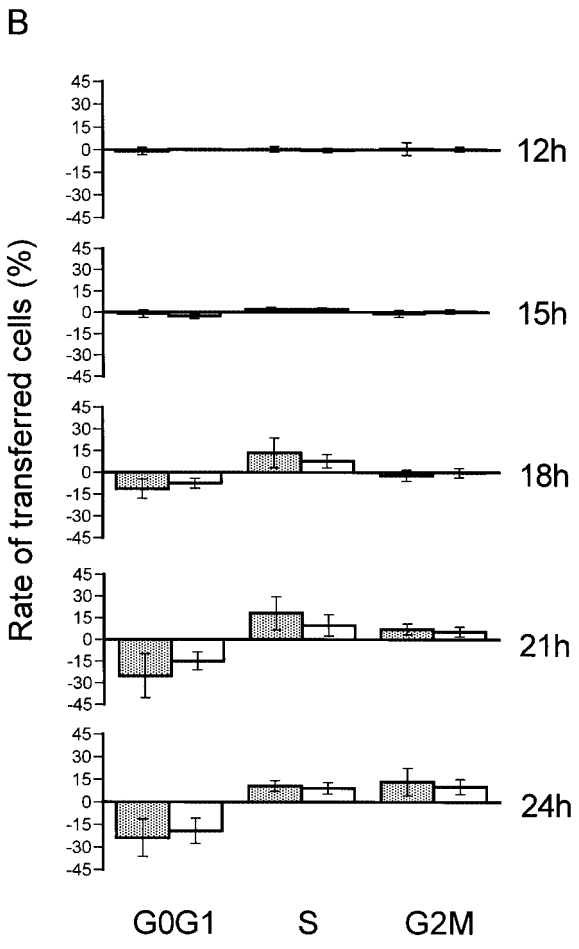

Fig. 1B Mean rates of cell transition for NIFr and NIFn cells in DMEM-10. Proportions of G0/G1, S and G2/M cells at 12, 15, 18, 21, and 24 h compared to 0 h were calculated from the data shown in Fig. 1A. Stippled bars, NIFr cells; open bars, NIFn cells. Vertical bar indicates SD.

underwent progression to S and G2/M phases in the presence of 10% FCS was larger than that of NIFn cells.

Difference in cell growth between NIFr and NIFn cells in DMEM-0.5 supplemented with bFGF

To investigate the cell cycle-specific effects of bFGF, quiescent cells were stimulated to synchronously reenter the cell cycle in DMEM-0.5 containing 10 ng/ml bFGF. Thus, cells were arrested by DMEM-0.5 for 24 h and then stimulated by DMEM-0.5 supplemented with bFGF (10 ng/ml) for 18, 24, 30, 36, 42, and 48 h. The percentage of cells in G0/G1, S, and G2/M phases were determined using flow cytometry. Fig. 2A shows a representative flow cytometric analysis of the cellular DNA content of NIFr and NIFn cells. Fig. 2B shows a summary of the difference from 0 h for G0/G1, S and G2/M phases at the indicated time point in proliferating NIFr and NIFn cells in DMEM-0.5 supplemented with bFGF (10 ng/ml). At 18 h, there was little change in the percentage of both NIFr and NIFn cells in the G0/G1 phase. At 24 h, 18.0% of NIFr cells and 10.4% of NIFn cells had moved from the G0/G1 to S

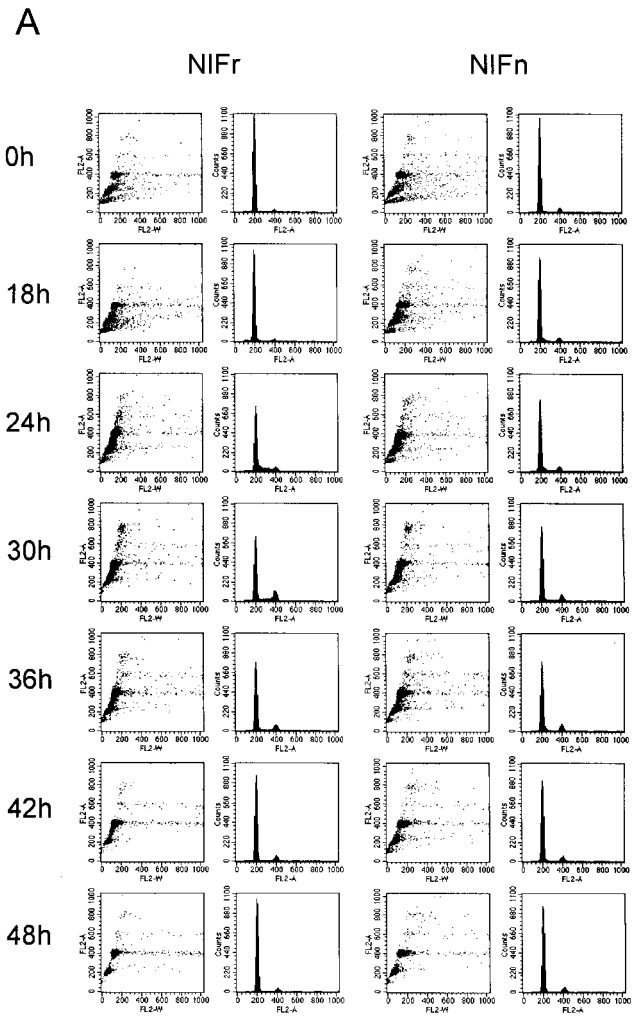

Fig. 2A DNA histogram: NIFr and NIFn cells were arrested by incubating in DMEM-0.5 for 24 h. Cells were then stimulated for 18, 24, 30, 36, 42, and 48 h after changing the medium to 10 ng/ml bFGF in DMEM-0.5. Propidium iodide fluorescence intensity corresponds to the DNA contents. The left column of each NIFr and NIFn cells shows the raw data while the right column of each NIFr and NIFn cells shows the relationship between the FL2-A channel and the number of cells. G0/G1 and G2/M cells were found at peaks of approximately 200 and 400 FL2-A channels, respectively, and the S cells were found between them. This figure is a representative data out of three trials.

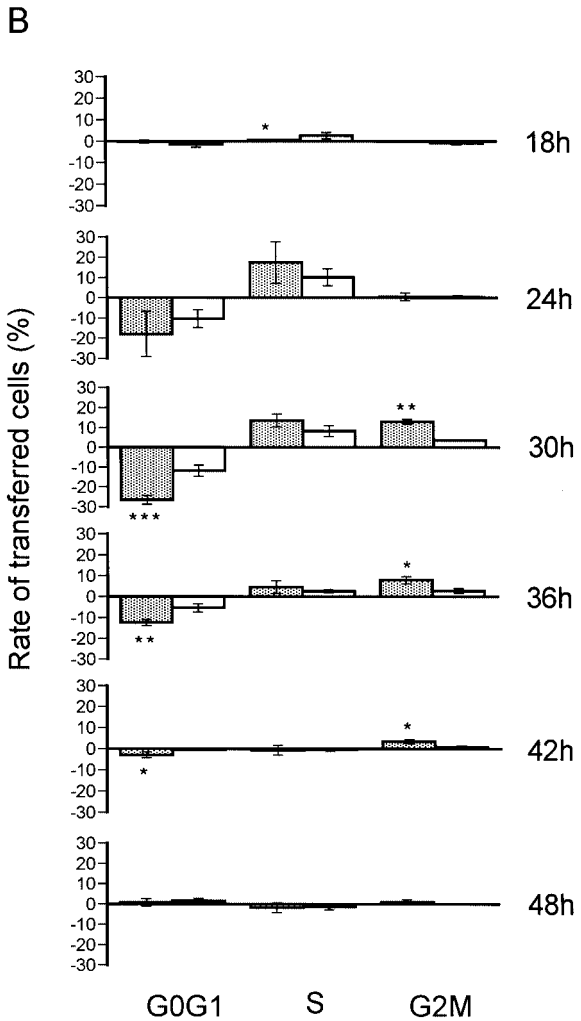

Fig. 2B Mean rates of cell transition for NIFr and NIFn cells in 10 ng/ml bFGF in DMEM-0.5. Proportions of G0/G1, S and G2/M cells at 18, 24, 30, 36, 42, and 48 h compared to 0 h were calculated from the data shown in Fig. 2A. Stippled bars, NIFr cells; open bars, NIFn cells. Vertical bar indicates SD. Statistical analysis; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

phase. At 30 h, 26.5% of NIFr cells in the G0/G1 phase and 11.2% of NIFn cells in the G0/G1 phase moved into the S and G2/M phase ($P < 0.001$), and a significantly higher portion of G2/M phase NIFr cells (12.9%) compared with NIFn cells (3.6%) were also seen ($P < 0.01$). Similar results were noted for 36 and 42 h. G0/G1 cells at 36 to 48 h may contain mother and daughter cells in the G0/G1 phase. These results indicated that a population of NIFr cells underwent to cell cycle was larger than that of NIFn cells in the presence of bFGF.

mRNA expression of cyclins D1, E, A, B1, and CDKs 4, 6, 2, 1 in NIFr and NIFn cells

Cells were arrested in DMEM-0.5 for 24 h and then stimulated with DMEM-0.5 supplemented with bFGF (10 ng/ml) for 3, 6, 12, 18, 24, 30, 36, 42, and 48 h. mRNA expression of cyclins D1, E, A, B1, and CDKs 4, 6, 2, 1 in NIFr and NIFn cells was determined by RT-PCR, analyzed using a Lane analyzer, and compared with GAPDH mRNA expression (Figs. 3A-3C). NIFr cells showed greater mRNA expression tested than NIFn cells. Cyclin D1 mRNA expression in both NIFr and NIFn cells was found at 3 h and reached a peak at 12-18 h, with a peak adjusted expression rate (relative to GAPDH mRNA expression) of 1.25 and 0.56, respectively (Fig. 3B). Cyclin E mRNA expression in NIFr cells was found from 18 h to 24 h with a peak adjusted expression rate of 1.02. There was no apparent increase in cyclin E mRNA expression in NIFn cells, with a peak adjusted expression rate of 0.32 (Fig. 3B). Cyclin A mRNA expression in both NIFr and NIFn cells was found at 18 h and peaked at 24 h with peak adjusted expression rates of 1.18 and 0.26, respectively (Fig. 3B). Cyclin B1 mRNA expression in both NIFr and NIFn cells was noted at 24 h and peaked at 36 h with peak adjusted expression rates of 1.64 and 0.95, respectively (Fig. 3B). CDK4 and CDK6 mRNA expression did not change much during the experimental period, 0 to 48 h (Fig. 3C). CDK2 mRNA expression in NIFr cells was noted at 18 h and peaked at 24 h with a peak adjusted expression rate of 1.48. There was no apparent increase in CDK2 expression in NIFn cells (Fig. 3C). CDK1 mRNA expression in NIFr and NIFn cells was found at 18 h and peaked at 24 h with peak adjusted expression rates of 1.70 and 0.48, respectively (Fig. 3C).

Discussion

In a preliminary study, it was demonstrated that the mean doubling time of NIFr cells was less than that of NIFn cells (12). The results of the present study confirmed that NIFr cells showed greater proliferation than NIFn cells and the doubling time of NIFr cells was shorter than that of NIFn cells (Figs. 1A and 1B). The proportion of NIFr cells in the G0/G1 phase decreased more than in the case of the NIFn cells, indicating that a proportion of NIFr cells progressed to the S and G2/M phases was greater in the presence of 10% FCS. This manner was indicated to be applicable to find that the same trend was demonstrated when cells were stimulated with 10 ng/ml bFGF in DMEM-

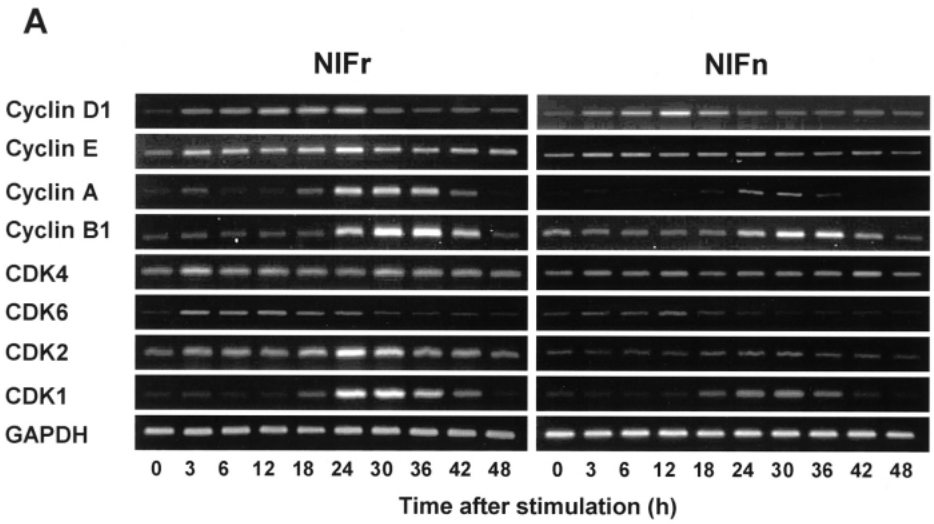

Fig. 3A mRNA expression of cyclins D1, E, A, B1, and CDKs 4, 6, 2, 1, and GAPDH in NIFr and NIFn cells. Specific primers (Table 1) were used in RT-PCR to detect each mRNA. RT-PCR for GAPDH (bottom lane) was performed as an internal control. Cells were stimulated for 3, 6, 12, 18, 24, 30, 36, 42, and 48 h with 10 ng/ml bFGF in DMEM-0.5. PCR reaction products were subjected to electrophoresis using 2% agarose gels, which were then dyed using 0.5 μg/ml of ethidium bromide, illuminated with UV light, and photographed using a digital camera. This figure is a representative data out of three trials.

0.5 (Figs. 2A and 2B).

When cells in culture are exposed to environmental conditions that are suboptimal for proliferation, the cellular response varies depending on cell type. Nontransformed cells usually cease to proliferate and are arrested in the G1 phase or enter a state of quiescence (G0) from G1 after deprivation of serum growth factors or nutrients (13). As shown in Figures 1A and 2A, more than 95% of cells were in G0/G1 phase after deprivation of serum (0.5%, for 24 h).

In general, the presence of teeth as well as gingival inflammation is essential to induce gingival overgrowth. Therefore, inflammatory mediators, such as IL-1, present in gingival crevicular fluid play an important role in the development of gingival overgrowth. It has been reported that IL-1α may be important for NIF-induced gingival overgrowth and that IL-1α induces intracellular bFGF in cultured human gingival fibroblasts (2,3). In the present study, the cell cycle of NIFr and NIFn cells was not changed by stimulation with bFGF until after 18 h (Fig. 2A). At 24 h after stimulation with bFGF, however, cells moved from the G0/G1 to S phase, and the rate of transition was higher in NIFr cells than in NIFn cells (Fig. 2B). At 30 h after stimulation with bFGF, a greater number of NIFr than NIFn cells had entered the G2/M phase (Fig. 2B), suggesting that NIFr cells might be more sensitive than NIFn cells to bFGF.

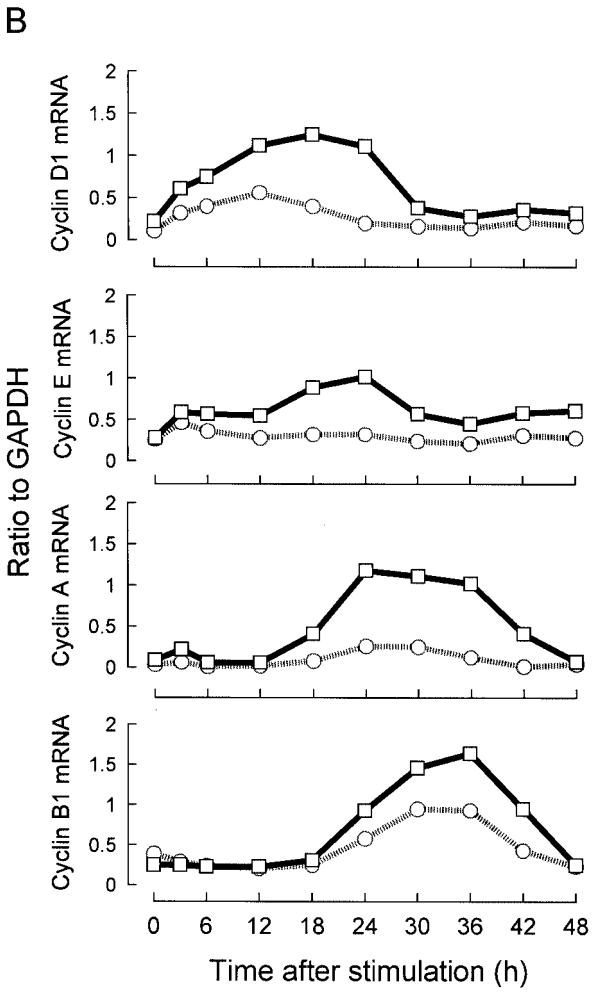

Fig. 3B Relative mRNA expression of cyclins D1, E, A, and B1 in NIFr and NIFn cells. The data shown in Fig. 3A for cyclin mRNAs were analyzed by computerized densitometric scanning of the images using Lane Analyzer. Values were adjusted for assay variation by dividing the integrated optical density of cyclins D1, E, A, and B1 mRNA by the integrated optical density of GAPDH mRNA. □, NIFr cells; ○, NIFn cells.

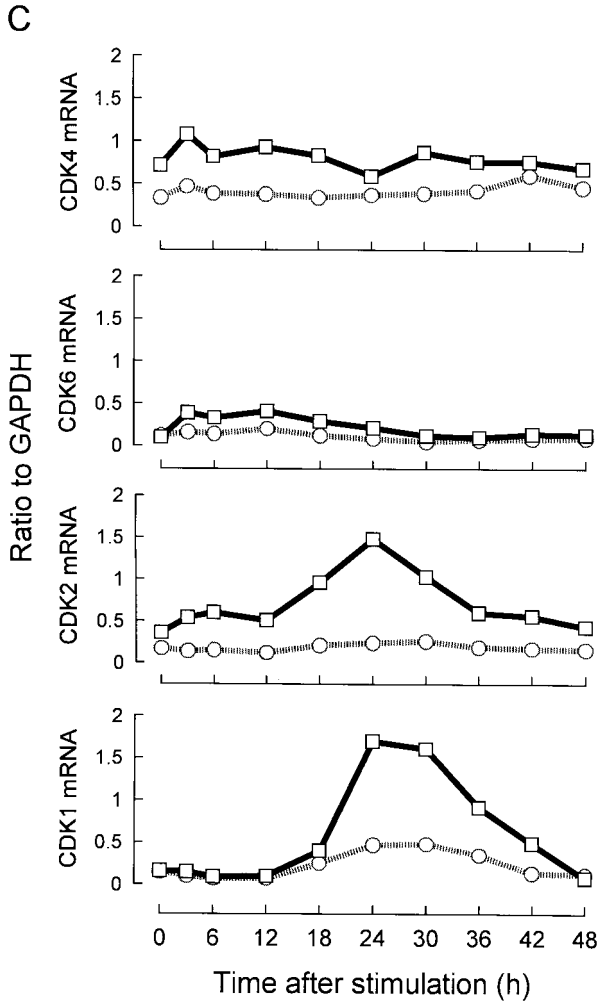

Fig. 3C Relative mRNA expression of CDKs 4, 6, 2, and 1 in NIFr and NIFn cells. The data of Fig. 3A for CDK mRNAs were analyzed by computerized densitometric scanning of the images using Lane Analyzer. Values were adjusted for assay variation by dividing the integrated optical density for cyclins D1, E, A, and B1 mRNA by the integrated optical density of GAPDH mRNA. □, NIFr cells; ○, NIFn cells.

Cyclins play a fundamental role in regulating cell cycle events in all eukaryotic cells. Cyclins D1 and E play a role in G1/S transition (14,15), while cyclin A is important in progression to the S phase (16). Cyclin B plays a role in initiation and progression of M phase (17-19). In order to clarify the effect of bFGF on the cell cycle in NIFr and NIFn cells, mRNA expression of cyclins D1, E, A and B1 was determined over time to find that NIFr cells expressed greater amount of cyclin mRNAs than NIFn cells at each identical peak time of cyclins D1, E, A and B1 mRNAs (Figs. 3A and 3B).

The vast majority of growth factors including bFGF act during the G1 phase and eventually promote cell cycle progression through the G1/S phase transition. CDK4 and CDK6, in association with cyclin D, and CDK2 in association with cyclin E, possibly regulate this transition by phosphorylating the retinoblastoma protein, which in turn results in the release of E2-promotor-binding factor-related proteins and enhanced transcription of genes participating in cell cycle progression (20). S phase transition is induced by CDK2 and CDK1 in association with cyclin A while M phase transition is induced by CDK1 in association with cyclin B. In the present study, CDK mRNA expression was greater in NIFr cells than in NIFn cells in the presence of 10 ng/ml bFGF in DMEM-0.5. Thus, in addition to cyclin mRNA expression, the corresponding CDK mRNA expression was greater in NIFr cells than in NIFn cells. Jones et al. (21) reported that 50 ng/ml bFGF stimulated cyclin D1 mRNA expression and increased CDK6 protein levels in uterine stromal cells. The present results, demonstrating that CDK2 and CDK1 mRNA expression was greater in NIFr cells than in NIFn cells, is in accord with the results of Jones et al. (21).

In conclusion, a proportion of NIFr cells underwent progression to S and G2/M phases from the G0/G1 phase in the presence of 10% FCS and 10 ng/ml bFGF was larger than NIFn cells. In most cases, mRNA expression of cyclins A, B1, D1, E and CDKs 1, 2, 4, 6 was greater in NIFr cells than NIFn cells in the presence of bFGF. These results indicate that NIFr cells may be more susceptible to the growth factors in FCS as well as bFGF than NIFn cells.

Acknowledgments

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