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c-fos m-RNA expression after treatment with fetal bovine serum and epidermal growth factor for osteoblastic MC3T3-E1 cells cultured in a low calcium environment

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SUMMARY: This paper examines the existence of c-*fos* mRNA expression in osteoblastic MC3T3-E1 cells cultured in a low calcium environment. The MC3T3-E1 cells were placed in a serum free medium for 24 hours after subconfluence and then the cells were treated with fetal bovine serum and epidermal growth factor for 0, 5, 10, 15, 30, or 60 minutes. The *c-fos* m-RNA expression was found in the MC3T3-E1 cells cultured in both normal medium (control group) and low Ca medium (low Ca groups). The degree of expression was significantly higher in the low Ca group than in the control group in each treatment period ($p<0.01$) and in both groups, after the treatment with FBS or EGF, the expression levels increased with time at 5 and 10 minutes but decreased at 15 minutes. Thereafter, levels increased again to reach to a maximum at 30 minutes after which it decreased rapidly. On the other hand, in both groups, after the treatment with EGF, the expression increased at 30 minute. This suggests that the MC3T3-E1 cells placed in a low calcium environment react to restore cell functioning to normal at the gene level.

In vivo experiments with low Ca diets have shown osteoporosis and bone atrophy (1-3), with lower plasma calcium and higher plasma alkaline phosphatase levels

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(4,5). This is reminiscent of the phenomenon reported in hypocalcemic environments *in vitro* (6).

In these ten years, it has been reported that an intracellular signal transduction system is important to maintain a normal cell function (7-9). We investigated this system to clarify the relationship between osteoporotic diseases and low Ca environments, and reported that the intracellular free calcium ($[Ca^{2+}]i$) (10) and phosphatidylinositol-1,4,5-triphosphate (IP_3) contents(11) were lower in calvarial bone cells cultured in low Ca environments. In addition, protein kinase C activity was also lower in those cells, and the sensitivity to protein kinase C of the cell appeared to increase (12).

Recently, there are numerous reports at the gene level due to advances in biotechnology (13-16). In addition, it is also important to determine the relationship between the intracellular signal transduction system and intranuclear genes.

It is still unclear how different genes behave in osteoblasts when cultured in a low-calcium environment.

The present study examined the movement of c-*fos* m-RNA expression (17) for fetal bovine serum and epidermal growth factor which is one of cytokines in osteoblastic MC3T3-E1 cells cultured in a low calcium environment with the Northern blot analysis.

MATERIALS AND METHODS

1. Cell culture

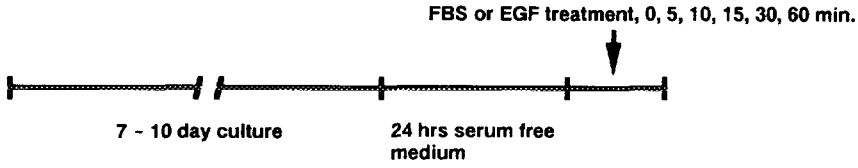
1) Cell

Osteoblastic cells, clone MC3T3-E1 (18,19) derived from C57B2/6 of newly born mouse calvaria, were donated by DR. H Kodama, Ohu University, Fukushima, Japan.

2) Culture method

The osteoblastic MC3T3-E1 cells were cultured in plastic petri dishes (90mmø) with a control medium (control group) and a low calcium medium (low Ca group) for 7～10 days. The medium was changed twice a week, and temperature was maintained at 37℃ under 5% CO_2-95% air in αMEM with 10% fetal bovine

serum (Gibco. Co.), Kanamycin (6mg/ml) (Sigma Co.). The cells were put in serum free culture media 24 hrs after the 7~10 day-culture period, and then, the cells were treated with fetal bovine(FBS) or epidermal growth factor(EGF) (Takara. Co.) for 0, 5, 10, 15, 30, 60 minutes. respectively. The concentration of calcium in the αMEM culture medium was 1.85 ± 0.34 mM(n=10), in the control medium, and 0.32 ± 0.02 mM(n=10) in the low Ca medium. The experimental protocol is as follows.



2. Northern blot analysis

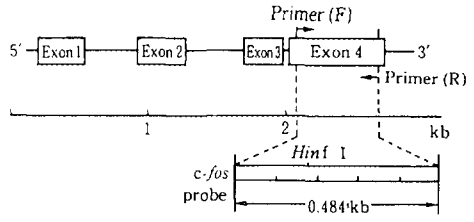
To extract total RNA, 10^8 MC3T3-E1 cells in the both groups were used to extract total RNA. Total RNA was extracted with a acid guanidium-phenol-chloroform method. The ratio of the 260 nm to 280 nm wave lengths were 1.80~2.00. The total RNA (20μg) per lane was denatured and separated in 1.1% agarose gel containing deionized formamide and formaldehyde. The RNA was transferred from the gel to a nylonmembrane (GeneScreen Plus membrane, DuPont Co.) overnight. The filters were baked for 2hrs at 80℃.

Prehybridization was carried out in 5×SSPE, 50% deionized formamide, 5× Denhardt's, 1% SDS, and 0.1 mg/ml denatured salmon sperm DNA for 18 hrs at 42 ℃.

Hybridization was carried out in a solution with labelled probe added to the prehybridization solution for 24 hrs at 42℃.

The hybridized filter was washed with 2× SSPE at room temperature for 15 min., two times; 0.1×SSPE at room temperature for 15 min., two times. The filter was autoradiographed with Fuji X ray films(Fuji Film Co.) for one day at -70℃ with intensifying screens.

The c-*fos* gene (0.484 kb) (20) of oligonucleotides was used as a probe(Fig. 1), and β actin(Oncogene Sci. Co.) as a control probe. Oligonucleotides were labeled with



Primer (F) 5' GAGATTGCCAACCTGCTGAA 3'
Primer (R) 5' AGACGAAGGAAGACGTGTAA 3'

Fig. 1. Restriction enzyme map of c-*fos* gene.

[α-^{32}P]dCTP (Bresatec Co.), using the Random Primer Labeling System(Takara Co.).

3. Analysis by densitometer

The degree of each spot density was examined, using the Densito-Pattern Analyzer (Model : EPA-3000, Maruzen Petrochemical Co.)

4. Measurement of medium calcium

The contents of medium calcium were measured with atomic absorbtion spectrophotometry(Model:AA-1475, Varian).

5. Statistical analysis

Student's *t* test was used for the analysis of the data.

RESULTS

We examined the effect of FBS and EGF on c-*fos* mRNA expression in osteoblastic MC3T3-E1 cells.

1. FBS treatment

As shown in Fig. 2, the c-*fos* mRNA expression and β actin expression for FBS was found in the MC3T3-E1 cells cultured in both normal and low Ca medium.

The degree of expression was higher in the low Ca group than in the control group at each treatment periods as FBS. After the treatment with FBS, the expression levels in both groups increased with time at 5 and 10 minutes, but decreased at 15 minutes. Thereafter, levels increased again to reach to a maximum at 30 minutes, after which they decreased rapidly. As shown in Fig. 3, the analysis with densitometer showed that the density of the spot was clearly higher in the low Ca

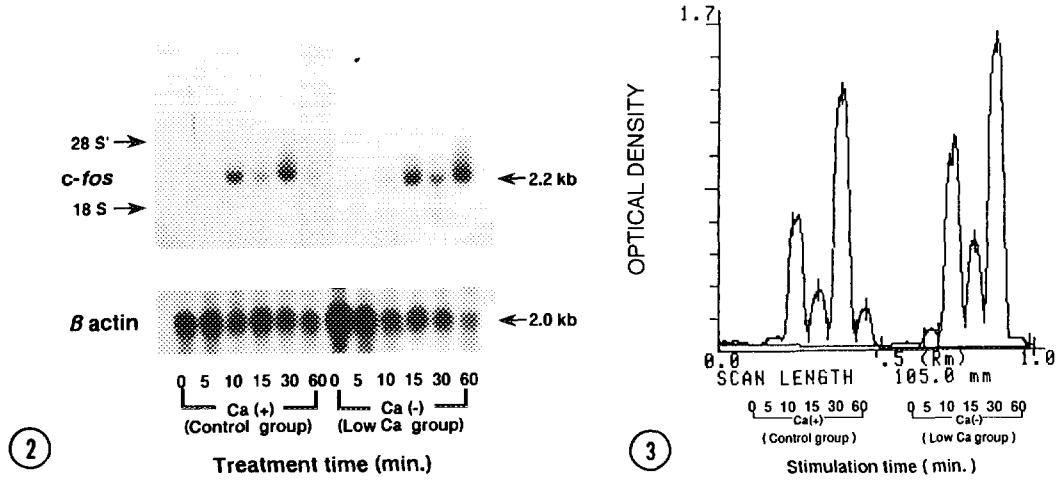


Fig. 2. Northen blot analysis of c-*fos* mRNA expression and β actin expression after the treatment with FBS in osteoblastic MC3T3-E1 cells cultured in a low calcium environment. The c-*fos* mRNA expression seems higher in the low Ca group than in the control group.

Fig. 3. Densito-scanning analysis of autoradiograms. There is an increase until 10 minutes after the treatment with FBS, a decrease at 15 minutes, and the maximum is reached at 30 minutes.

group than in the control group at all lengths of treatment and the density in 30 minute after the treatment with FBS was strongest as shown in Fig. 2.

It was confirmed that the results of the spot-size in the autoradiogram corresponded to those obtained by the densitometer analysis.

As shown in Fig. 4, the tendency of areas of densito-scanning patterns in the autoradiogram was similar to that in Fig. 3.

As shown in Table 1, the ratios of c-*fos* mRNA expression to the β actin expression (Fig. 2) was significantly higher in the low Ca group than in control group in all the treatment periods($p<0.01$).

2. EGF treatment

As shown in Fig. 5, the c-*fos* mRNA expression and β actin expression for EGF was found in the MC3T3-E1 cells cultured in both normal and low Ca medium.

The degree of expression was higher in the low Ca group than in the control group

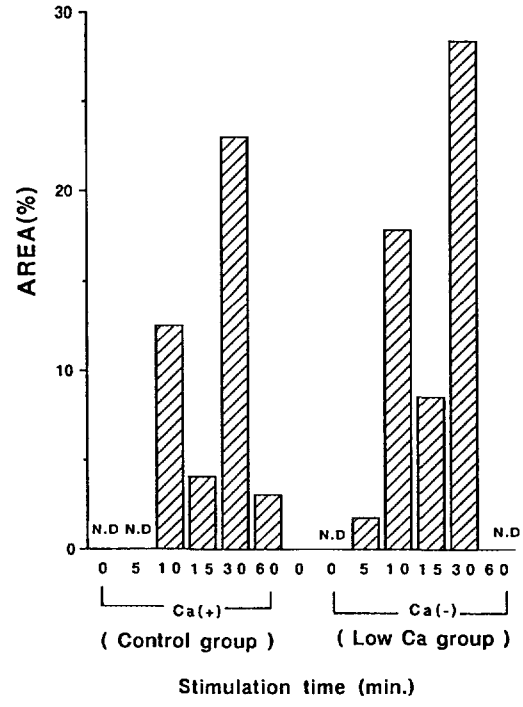


Fig. 4. Area of densito-patterns in Fig. 3. The changes corresponded to that in the densito-pattern analysis, and the areas in the relevant columns are higher in the low Ca group than in the control.

at each treatment periods. After the treatment with EGF, the expression levels in both groups increased at 30 and 60 minutes. The degree was higher at 30 minutes than at 60 minutes. As shown in Fig. 6, the analysis with densitometer showed that

Table 1. The ratios of c-*fos* mRNA expression to β-actin mRNA expression

Length of treatment (min)	Control group	Low Ca group
0	—	—
5	—	0.026 ± 0.002
10	0.32 ± 0.02	0.46 ± 0.05*
15	0.12 ± 0.02	0.24 ± 0.03*
30	0.54 ± 0.05	0.69 ± 0.06*
60	0.10 ± 0.01	—

Each value that was obtained from optical density in each group shows mean ± S.D (n=3).

* Significantly different from control value ($p<0.01$).

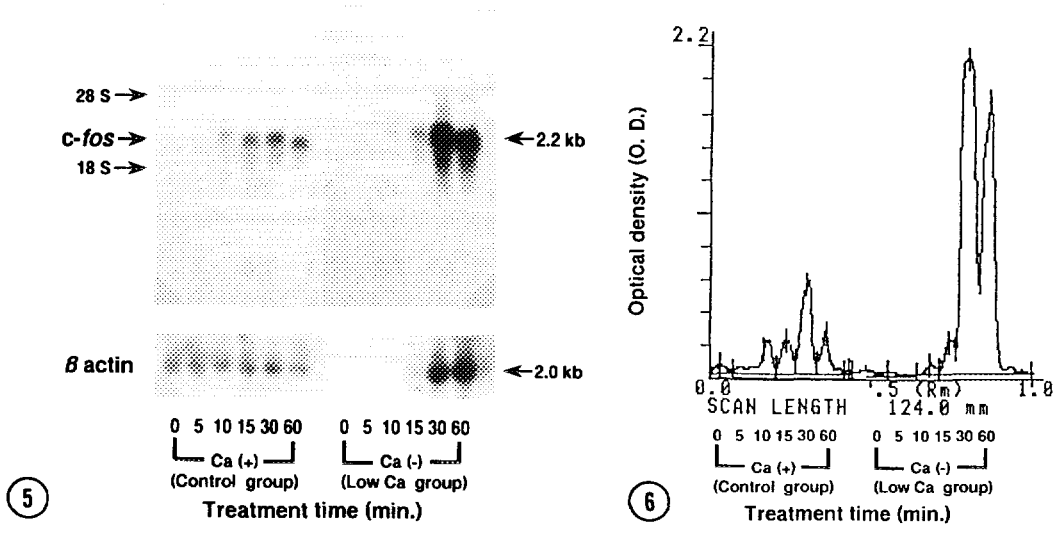


Fig. 5. Northern blot analysis of c-*fos* mRNA expression and β actin expression after the treatment with EGF in osteoblastic MC3T3-E1 cells cultured in a low calcium environment. The c-*fos* mRNA expression seems higher in the low Ca group than in the control group.

Fig. 6. Densito-scanning analysis of autoradiograms. There is an increase at 30 and 60 minutes after the treatment with EGF. The degree is higher in 30 minute treatment than in the 60 minute one.

the density of the spot was higher in the low Ca group than in the control group at 30 and 60 minutes treatment and the density in 30 minute after the treatment with EGF was higher as shown in Figs. 5 and 6. It was confirmed that the results of the spot-size in the autoradiogram corresponded to those obtained by the densitometer analysis.

As shown in Table 2, total RNA quantity applied to each lane corresponding to each treatment time was small one with the exception of 30 and 60 minute-trearment time in the low Ca group.

As shown in Table 3, the ratios of c-*fos* mRNA expression to the β actin expression (Fig. 5) was significantly higher in the low Ca group than in control group in 30 and 60 minutes treatment periods($p<0.01$).

Table 2. Total RNA quantity (μg) applied to each lane corresponding to each treatment time in agarose gel electrophoresis

	Treatment time (min.)					
	0	5	10	15	30	60
Control group	7.1	7.1	7.1	7.2	7.3	7.0
Low Ca group	2.9	2.1	3.4	4.6	20.0	20.2

DISCUSSION

The changes of c-*fos* mRNA expression in the osteoblastic MC3T3-E1 cells cultured in a low calcium environment were examined in the present study. Recently, the interrelationship between intracellular constituents related to cell function and various genes or their mRNA expressions have been investigated (13,21,22) . In the study here, the mRNA expressions at all the treatment periods with FBS and EGF was clearly higher in the low Ca group than in the control group (Figs. 2～5, Tables 1～2).

This phenomenon may show that the MC3T3-E1 cells cultured in a low-calcium environment cope with the environmental calcium deficiency around cells.

The present study corroborated the finding that a compensation mechanism seemed

Table 3. The ratio of c-*fos* mRNA expression to *β* actin expression after treatment with EGF(50ng/ml) in MC3T3-E1 cells cultured in a low calcium environment

	n	Treatment time (min.)	Ratio
Control group	3	30	1.09 ± 0.12
		60	1.06 ± 0.09
Low Ca group	3	30	1.37 ± 0.14*
		60	1.28 ± 0.11*

Mean ± S. D. is shown.
*Significantly different from the relevant control value ($p<0.01$).

to operate more strongly in the low Ca group than in control group (6,11,12) .

At all treatment times, the evidence that the degree of c-*fos* mRNA expression was higher in the low Ca group than in the control group may be dependent on the operation of the physiological compensation mechanism. It may be sufficient that the function of the osteoblastic MC3T3-E1 cells placed in a low-calcium environment make a greater contribution to a restoration of normal functioning or status.

The c-*fos* gene is a growth-regulating one and plays an important role in the cell differentiation and proliferation and in the process from G_0 to G_1 of the cell cycle (23-27).

We found that the degree of c-*fos* mRNA expression. This is an interesting development. At the beginning of the G_0 stage, there may be a rhythm in the operation of the cell function, and the phenomenon observed here may be intimately related to the cell cycle process. At 15 minute of the treatment with FBS, the cell cycle process may be in the S, G_2, or M stage. However, as we did not treat a synchronous culture, further experiments will be required to clarify the many problems associated with cell rhythm and cell cycles.

At the present experiment, the FBS was used to treat the cells, and FBS includes various factors including numerous cytokines (TGFβ, TGFα, EGF, platelet derived growth factor), PGs, LTs, PTH, calcitonin, hormones, vitamins and others. Since EGF is closely involved in the growth and development(28~31) and discussed about the relation to the tyrosine kinase in the mediation of EGF receptor(32), we planned to use EGF as a candidate of the stimulator of the cells and tried whether this EGF shows the same behavior as that of FBS or not. As the results of this experiment we could know that EGF treatment also enhances c-*fos* mRNA expression in the cells cultured in a low-calcium environment(Table 2, 3).

This evidence may show that gene expression in the cells treated with other cytokines under the low-calcium environment is enhanced. From this view-point, it will be subsequently, necessary to administer many kinds of agents to cells to establish which factor is most important in maintaining normal cell function.

Clinically, patients with osteoporosis and periodontal ailments are recently increasing and the establishment of therapeutic methods are urgently necessary.

The present experiments may show that the function of the c-*fos* gene in the osteoblast temporarily accelerates in osteoporotic ailments. This response may depend on a compensation mechanism to restore abnormali cell-functions to normality. However, we need much knowledge to be able to apply experimental results to the patients.

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