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Expression Patterns of Bone-Related Proteins During Osteoblastic Differentiation in MC3T3-E1 Cells

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Abstract Bone formation involves several tightly regulated gene expression patterns of bone-related proteins. To determine the expression patterns of bone-related proteins during the MC3T3-E1 osteoblast-like cell differentiation, we used Northern blotting, enzymatic assay, and histochemistry. We found that the expression patterns of bone-related proteins were regulated in a temporal manner during the successive developmental stages including proliferation (days 4–10), bone matrix formation/maturation (days 10–16), and mineralization stages (days 16–30). During the proliferation period (days 4–10), the expression of cell-cycle related genes such as histone H3 and H4, and ribosomal protein S6 was high. During the bone matrix formation/maturation period (days 10–16), type I collagen expression and biosynthesis, fibronectin, TGF-β1 and osteonectin expressions were high and maximal around day 16. During this maturation period, we found that the expression patterns of bone matrix proteins were two types: one is the expression pattern of type I collagen and TGF-β1, which was higher in the maturation period than that in both the proliferation and mineralization periods. The other is the expression pattern of fibronectin and osteonectin, which was higher in the maturation and mineralization periods than in the proliferation period. Alkaline phosphatase activity was high during the early matrix formation/maturation period (day 10) and was followed by a decrease to a level still significantly above the baseline level seen at day 4. During the mineralization period (days 16–30), the number of nodules and the expression of osteocalcin were high. Osteocalcin gene expression was increased up to 28 days. Our results show that the expression patterns of bone-related proteins are temporally regulated during the MC3T3-E1 cell differentiation and their regulations are unique compared with other systems. Thus, this cell line provides a useful in vitro system to study the developmental regulation of bone-related proteins in relation to the different stages during the osteoblast differentiation.

Key words: osteocalcin, osteonectin, collagen, TGF-β1, histone, fibronectin, alkaline phosphatase, ribosomal protein S6, differentiation, MC3T3-E1 cells

Bone formation is a complex biological process and involves several tightly regulated gene expression patterns of bone-related proteins [Stein and Lian, 1993]. The expression of bone-related proteins during the bone formation has been studied both in vivo and in vitro systems. As in vivo models, developing rat bone [Yoon et al., 1987; Weinreb et al., 1990; Chen et al., 1992], mandibular condyle [Strauss et al., 1990], developing human calvarial bone [Sandberg et al., 1988], and mouse embryo [Nomura et al., 1988] have been studied for the expression of bone-related proteins. The expression patterns of bone-related proteins in in vivo systems are a temporal manner. As in vitro models, the primary explant cultures of the fetal rat calvaria or the bone marrow cells in the presence of the organic phosphate and ascorbic acid have been extensively studied for the expression of bone-related proteins during the bone formation. The expression patterns of bone-related proteins in in vitro systems are also a temporal manner. For example, the expressions of cell cycle or growth-related genes (histone, *c-fos*, and *c-myc*) and extracellular matrix genes (type I collagen, fibronectin, and transforming growth factor-β1 [TGF-β1]) were increased and followed by the bone mineralization genes such as osteocalcin, osteopontin, and bone sialoprotein during the bone

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formation [Stein and Lian, 1993]. Therefore, it is reasonable to speculate that there are common expression patterns of bone-related proteins for the osteoblast differentiation. However, the expression patterns of some bone-related proteins are different among the culture systems. For example, the expression of osteocalcin gene, which is expressed at a later stage of the bone cell differentiation than alkaline phosphatase in the rat calvarial osteoblasts [Stein and Lian, 1993], parallels the expression of alkaline phosphatase in the chicken osteoblasts [Gerstenfeld et al., 1987]. On the basis of these expression patterns, we hypothesize that each osteoblast culture system has its own specific expression patterns of bone-related proteins during the osteoblast differentiation.

Mouse calvaria-derived MC3T3-E1 osteoblast-like cells have been extensively studied with respect to the bone matrix accumulation, mineralization, effects of growth factors, and changes in morphology and metabolism [Kumegawa et al., 1983; Sudo et al., 1983; Kurihara et al., 1984; Hurley et al., 1993]. Although there have been several reports about the expressions of collagen, osteocalcin, and other osteoblast markers in this cell line [Franceschi and Iyer, 1992; Quarles et al., 1992; Sherbina and Bornstein, 1992; Dean et al., 1994; Majeska et al., 1994], a more comprehensive understanding of the expression patterns of bone-related proteins during the osteoblast differentiation in MC3T3-E1 cells remains to be established.

The purpose of this study was to determine the expression patterns of bone-related proteins during the osteoblast differentiation in MC3T3-E1 cells. We studied the cell cycle and differentiation associated genes such as histone H3 and H4, ribosomal protein S6, type I collagen, TGF-β1, fibronectin, osteonectin, and osteocalcin during the osteoblast differentiation in MC3T3-E1 cells. We also determined the activity of alkaline phosphatase (ALP) and the collagen synthesis rate. We used Northern blotting, enzymatic assay, and histochemical method to analyze the expression of bone-related proteins and nodule formation. Here we report that MC3T3-E1 cells undergo a unique temporal expression of bone-related proteins during the osteoblast differentiation.

MATERIALS AND METHODS

Cell Culture

MC3T3-E1 cells, a generous gift from Dr. Hee-Moon Kyung (Kyungpook University, Taegu, Korea), were cultured in alpha-minimum essential medium (α-MEM, Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) 100 U/ml of penicillin and 100 μg/ml of streptomycin [Kodama et al., 1981; Sudo et al., 1983]. Cells were seeded at an initial density of 2×10^4 cells/cm^2 and were grown in 10% FBS containing 10 mM β-glycero-phosphate (β-GP, Sigma, St. Louis, MO) and 50 μg/ml of ascorbic acid in 100 mm dishes or 24-well plates (Corning, Corning, NY). The medium was changed every 3 days.

Histochemical Analysis

To assess the time of nodule formation, cells were cultured in 100 mm dishes and maintained for 30 days. To detect calcium deposits, cells were rinsed twice with the ice-cold phosphate buffered saline (PBS) and incubated for 1 h in 0.1% Alizarin Red S solution at room temperature. Then, cells were stained with 0.1% light green SF solution for 30 min. Before light microscopy, cells were serially washed with 1% acetic acid and absolute ethanol [Dahl, 1952].

Northern Blot Hybridization

After 24 h of media changes (days 4, 7, 10, 16, 22, 28), cells were harvested with guanidinium thiocyanate solution and stored at −70°C. Total RNA was isolated by the method of Chomczynski and Sacchi [1987]. Total RNA (10 μg) was fractionated by electrophoresis on 0.8% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond N plus nylon (Amersham, Arlington Heights, IL) [Sambrook et al., 1989]. RNA was cross-linked to membrane by UV irradiation for 1 min. The DNA probes used for hybridizations were obtained from the following sources. Rat osteocalcin cDNA, pOC3.4 [Lian et al., 1989], and histone H3 and H4 [Grimes et al., 1987] were generously provided by Dr. G.S. Stein (Massachusetts Medical School, Worcester). Osteonectin [Mason et al., 1986], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Tso et al., 1985], and human fibronectin, pFH1 [Kornblihtt et al., 1983] were obtained from ATCC (Rockville, MD). Mouse ribosomal protein S6 cDNA [Lalanne et al., 1987] was a kind gift from Dr. Jung-Chul Kim (Kyungpook University, Taegu, Korea). Mouse TGF-β1 cDNA [Derynck et al., 1986] was generously provided by Dr. B. deCrombrugghe (M.D. Anderson Cancer Center, Houston, TX). Human α1(I) procollagen cDNA, Hf677 [Chu et al., 1982], was obtained

from Dr. Y. Nagai (Tokyo Medical and Dental University, Tokyo, Japan). Labeling of cDNAs with [α-^{32}P] dCTP was performed using the random primed DNA labeling kit based on the method by Feinberg and Vogelstein [1983] to a specific activity of 1–2 $\times$ 10^9 cpm/μg of DNA. The membrane was hybridized with cDNA probes [Sambrook et al., 1989]. The filters were then washed with 2 $\times$ SSC, 0.1% SDS at room temperature for 20 min, three times with 0.2 $\times$ SSC, 0.1% SDS at 68°C for 20 min, and exposed to film at −70°C. After autoradiography, the filter was boiled for 10 min in 0.5% SDS to remove bound radioactivity and reprobed with the GAPDH or other cDNAs. Autoradiograms were quantitated using the NIH image analysis program [Masters et al., 1992]. Results were normalized to GAPDH to account for variations in RNA quantitation.

Analysis of Collagen Production

Cells were cultured using the same condition as described above. To determine collagen synthesis, medium was replaced with 1 ml of conditioning medium containing 1% FBS and 50 μg/ml ascorbic acid at selected time points (5, 11, 17, 23, 29 days) throughout 30 days and incubated for 24 h with 2 μCi of [^{3}H] proline (NEN, Boston, MA). At the end of labelling, the media and cell layers were added to proteinase inhibitors to yield the following concentrations: phenylmethylsulfonyl fluoride (PMSF), 0.2 mM; N-ethylmaleimide 5 mM. The amounts of radioactive collagen were determined by the method of Peterkofsky et al. [1982], which was modified from the previous method [Choi et al., 1995]. Percentage of collagen synthesis (percent collagen) as a collagenase-digestible protein (CDP) synthesis against total protein production was calculated by the following formula [Peterkofsky et al., 1982]: percent collagen (%) = (d.p.m. in CDP $\times$ 100)/(d.p.m. in noncollagen protein $\times$ 5.4 + d.p.m. in CDP). The amounts of DNA in each well were determined by the method of Labarca and Paigen [1980]. Data were expressed as d.p.m./ng of DNA and summarized as mean $\pm$ SE. Statistical analysis (SPSS) was performed using analysis of variance for multiple comparison (Scheffe test).

Determination of Alkaline Phosphatase Activity

Cells were cultured in 24-well plates at a density of approximately 4 $\times$ 10^4 cells per well. The medium was changed every 3 days during the culturing period up to 28 days. Cells were washed with PBS and lysed in 1 ml of 0.02% Nonidet P-40 (Sigma). The lysates were sonicated for 15 s with an ultrasonicator (Fischer, Rockville, MD) adjusted for 30% output. The sonicated lysates were centrifuged for 15 min at 12,000*g*. The supernatant was kept at −20°C before the assay was performed. The activity of ALP in lysates was measured by using p-nitrophenyl phosphate as the substrate and the optical density at 410 nm was determined as previously described [Bessay et al., 1946]. Protein concentration was estimated by the method of Lowry et al. [1951] with bovine serum albumin as the standard. The activity of ALP was expressed as nmol/min/mg of protein.

RESULTS

Nodule Formation During the MC3T3-E1 Cell Differentiation

Bone nodule formation is one of the markers of the bone cell differentiation. To determine the time of nodule formation, cells were stained with Alizalin Red S at the indicated time (Fig. 1). As shown in Figure 1, the nodules were observed after 16 days in this culture system and the number of Alizalin Red S positive nodules increased during the MC3T3-E1 cell differentiation. To confirm that this culture condition is important for the formation of the bone nodules, MC3T3-E1 cells were cultured for 30 days in α-MEM containing 10% FBS without the ascorbic acid and β-GP. We found that no Alizarin Red S positive nodules were observed (data not shown). We also used the same serum lot of FBS to rule out the effects of the serum variation. The increase of the nodule formation according to the time course indicates that this culture condition is favorable to show the expression patterns of bone-related proteins.

Expression of mRNAs for Bone-Related Proteins During the MC3T3-E1 Cell Differentiation

To determine the expression of bone-related proteins during the bone cell differentiation, we determined the steady-state level of their mRNAs. Firstly, we determined the amounts of histone H4 and osteocalcin mRNAs because both were well known as showing the reciprocal expression and the markers for the proliferation and the mineralization stages in the primary cultured rat calvarial osteoblasts, respectively [Aronow et al., 1990; Shakoori et al., 1992] (Fig. 2A,B). The expression of histone H4 was highly active for the early period (days 4–10), then

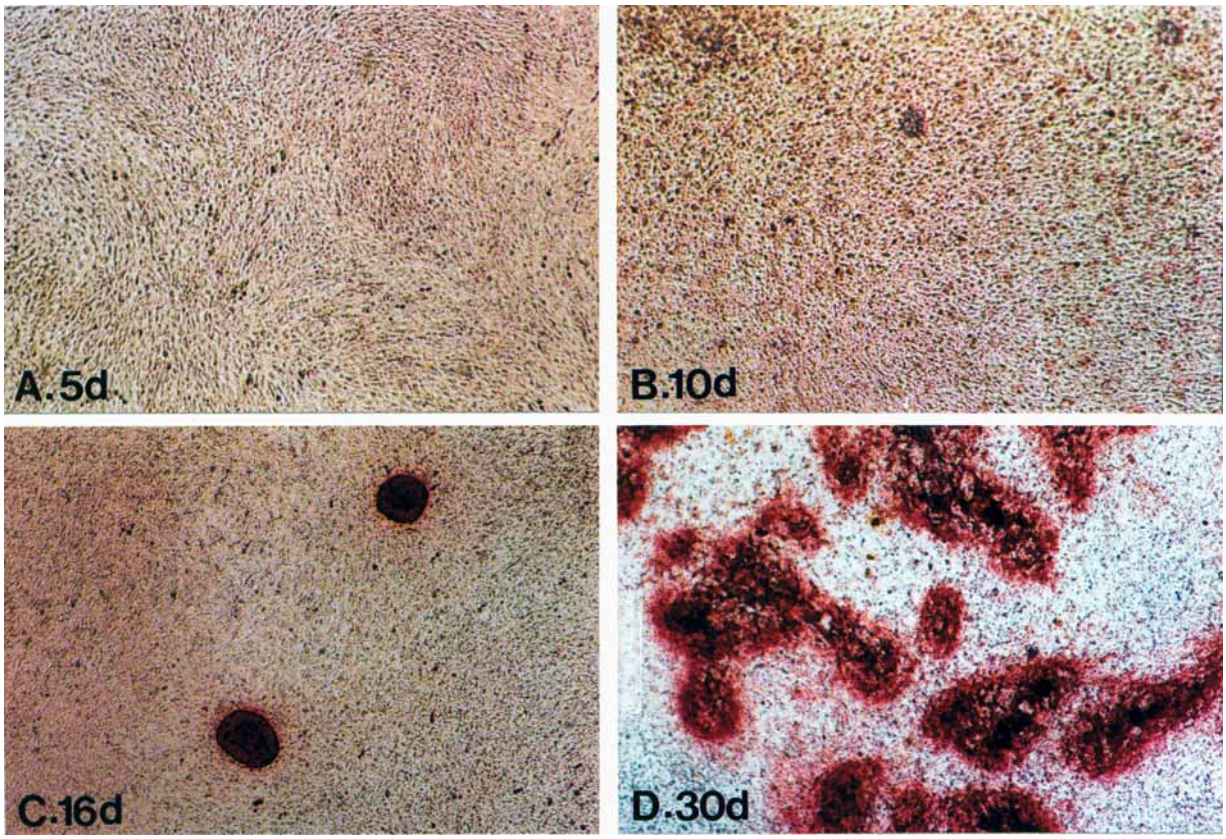

Fig. 1. Histochemical changes during differentiation of MC3T3-E1 osteoblastic cells. Phase contrast micrographs (×40) were taken by Alizarin Red S staining after 5 d (**A**), 10 d (**B**), 16 d (**C**) and 30 days (**D**), respectively. Cells were cultured with α-MEM containing 10% FBS, 50 μg/ml of ascorbic acid, and 10 mM β-glycerophosphate.

declined to 30% of the maximal level at day 28. As the proliferation was downregulated, osteocalcin mRNA was highly expressed. Osteocalcin mRNA was not detectable prior to day 7 and its expression initiated at day 10. Osteocalcin gene expression at day 28 was about 7-fold higher than day 10. The active expression of the osteocalcin gene for the late period (days 16–28) correlates with the formation of bone nodules (Fig. 1). These results indicate that the expression patterns of the markers for the proliferation (histone H4) and the mineralization (osteocalcin) of bone cell are mutually exclusive.

To confirm the presence of the proliferation period during the MC3T3-E1 cell differentiation, we determined the mRNA level for histone H3 that is known as the replication-dependent gene [Larson et al., 1989] (Figs. 3 and 4). Histone H3 mRNA was highly expressed during the early period of the differentiation and gradually declined to 60% of the maximal level by day 28. This result indicates that the decrease of histone H3 is less prominent than histone H4 in this culture system. The expression of the ribosomal protein S6 gene was also active for the early period (days 4–10) and throughout the subsequent differentiation the transcript level decreased to 20% of the maximal level seen in this culture system by day 22 (Figs. 3 and 4). The expression patterns for the histone H3 and ribosomal protein S6 indicate that these proteins are expressed in the proliferation period during the osteoblast differentiation.

In addition to the presence of the proliferation period, several reports indicate that the proper deposition of extracellular matrix is required for the bone cell differentiation. For example, if the proliferation is inhibited by TGF-β1, cells do not differentiate normally [Breen et al., 1994]. In addition, if collagen is not produced properly, mineralization is not observed [Owen et al., 1990; Tassinari et al., 1991]. In this context we determined the expressions of bone matrix proteins such as type I collagen, TGF-β1, fibronectin, and osteonectin. The expression of type I collagen was active for the first 16 days and was

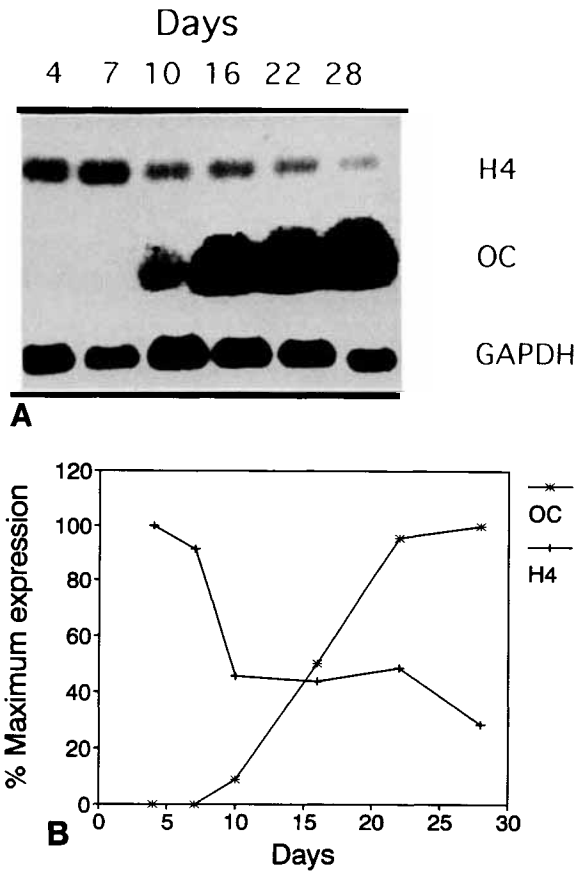

Fig. 2. Histone 4 and osteocalcin mRNA levels during differentiation of MC3T3-E1 osteoblastic cells. **A:** Northern blots of histone 4 (H4), osteocalcin (OC), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **B:** Expression of histone H4 (H4) and osteocalcin (OC) genes were analyzed by Northern blot on the indicated days from MC3T3-E1 cells. Values were presented as the percent of the maximal expression for each transcript. It was determined by the NIH image analysis program and was normalized to the levels of GAPDH mRNA.

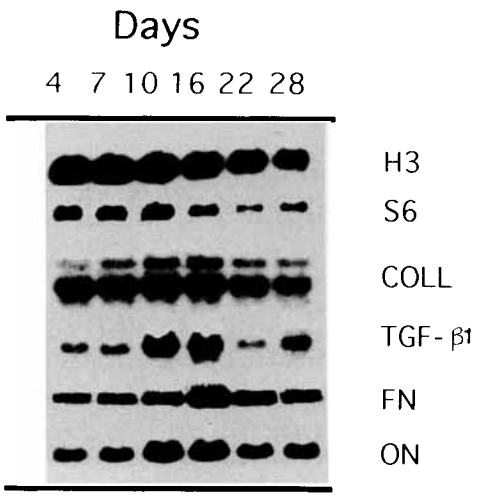

Fig. 3. Northern blots of histone H3 (H3), ribosomal protein S6 (S6), α1(I) procollagen (COLL), transforming growth factor-β1 (TGF-β1), fibronectin (FN), and osteonecton (ON) during the differentiation of MC3T3-E1 osteoblastic cells. Cells were cultured in α-MEM containing 10% FBS, 50 μg/ml of ascorbic acid, and 10 mM β-glycerophosphate. Culture media were changed every 3 days. Total RNA was extracted at the indicated time (4, 7, 16, 22, 28 days) and analyzed by Northern transfer as described in Materials and Methods.

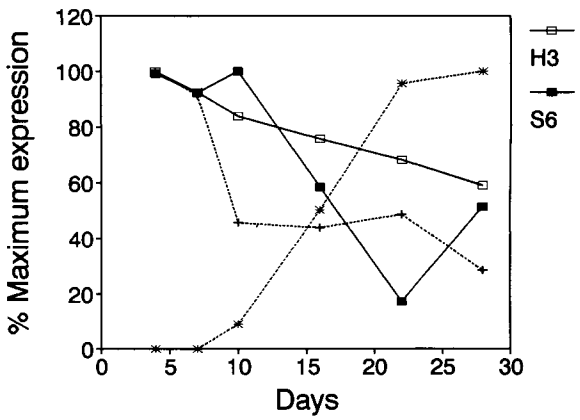

Fig. 4. Expression of histone H3 and ribosomal protein S6 during the differentiation of the MC3T3-E1 osteoblastic cells. Levels of expression of histone H3 (H3) and ribosomal protein S6 (S6) were analyzed by Northern blot and were presented as the percent of the maximal expression during the osteoblast differentiation. Value for the cell growth and the differentiation markers histone H4 (+) and osteocalcin (*) are identical to those presented in Figure 2B and serve as the references for the proliferation and mineralization stages, respectively.

followed by a decrease to 20% of the maximal level observed during the time course (Figs. 3 and 5). The expression pattern of TGF-β1 was similar to type I collagen by day 22 and both were highly active at day 10 and day 16 (Figs. 3 and 5). Next, the same blot was reprobed with fibronectin probe and fibronectin is an adhesion molecule present within the extracellular matrix (Figs. 3 and 6). Fibronectin mRNA level gradually increased until day 22 and then decreased to about 80% of the maximal level seen by day 28. The expression of osteonectin mRNA, another adhesion molecule in the bone tissue, was also active at day 10 and maintained its level of expression throughout the culturing period (up to day 28) (Figs. 3 and 6). The expression of these bone matrix proteins is very active in the intermediate period that is between the proliferation (histone H4) and the mineralization (osteocalcin) stages. However, the expression patterns of bone matrix proteins are two types: One is the expression of type I collagen and TGF-β1 and the other is the expression of fibronectin and osteonectin. The difference of

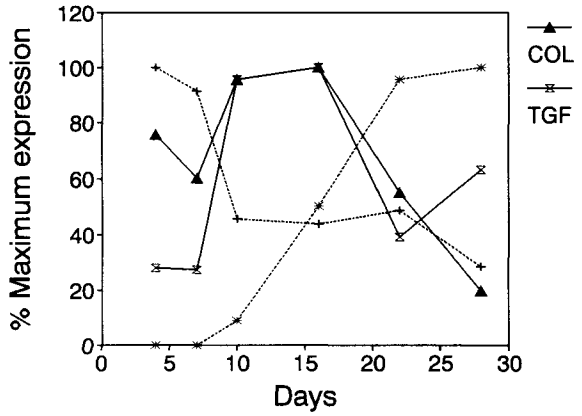

Fig. 5. Expression of type I collagen and TGF-β1 during the differentiation of the MC3T3-E1 osteoblastic cells. Levels of expression of type I collagen (COL) and TGF-β1 (TGF) were analyzed by Northern blot and were presented as the percent of the maximal expression observed during the osteoblast differentiation. Values for the cell growth and the differentiation markers histone H4 (+) and osteocalcin (*) are the same as those presented in Figure 2B.

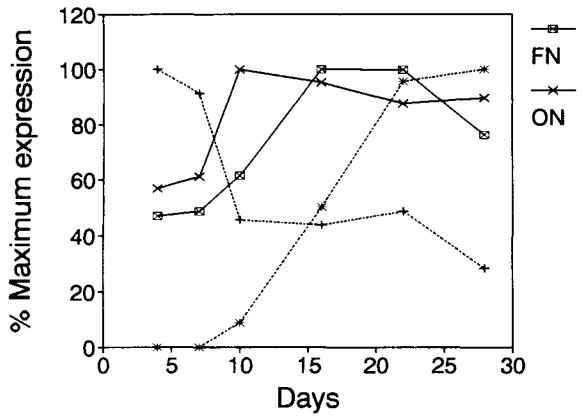

Fig. 6. Expression of fibronectin and osteonectin during the differentiation of the MC3T3-E1 osteoblastic cells. Levels of expression of fibronectin (FN) and osteonectin (OC) were analyzed by Northern blot and were presented as the percent of the maximal expression during the osteoblast differentiation. Values for the cell growth and the differentiation markers histone H4 (+) and osteocalcin (*) are the same as those presented in Figure 2B.

expression patterns is especially apparent in the late stage of differentiation (Figs. 3, 5, and 6).

Collagen Production and Alkaline Phosphatase Activity During the MC3T3-E1 Cell Differentiation

After we determined the expression of type I collagen mRNA, we tested whether the mRNA level of type I collagen is parallel to its protein level. As shown in Figure 7, the noncollagen protein production (NCP) significantly increased during the osteoblast differentiation. The collagenase-digestible protein (CDP) synthesis significantly increased for the first 11 days and was followed by a decrease to a level still above the baseline (day 5) by day 29. These results show that the collagen and noncollagen protein productions were active even during the late stages of the differentiation in this culture system. Thus, the percentage of collagen production (percent collagen), which was determined by the ratio of the collagen synthesis to the total protein synthesis, increased up till day 11 and then decreased to a level below at day 5 and this decreasing effect was significant at day 23 and day 29. When we compared the percent collagen with type I collagen RNA level (Figs. 3 and 5), there was a discrepancy for the late period (days 17–29) of the differentiation. The percent collagen was not significantly changed in this period (days 17–19), however, the RNA levels of type I collagen were gradually decreased. This is possibly due to the marked increase of type I collagen translation. However, Gerstenfeld et al. (1988) show that the collagen mRNA translation is decreased during the osteoblast differentiation. Although we do not know the exact mechanism of this discrepancy at present. It may result from the difference of the species and the culture conditions. In this study, DNA amounts were also determined together with the collagen synthesis. The DNA amounts were about 50% at day 5 compared with the maximal level (day 29) and were above 90% for days 10–22 (data not shown). As another marker of the osteoblast differentiation, we determined the activity of ALP during the MC3T3-E1 cell differentiation. The activity of ALP increased significantly and reached the maximal level around day 10 and subsequently its activity dropped to a level that was still higher than day 4 (Fig. 8). It shows that the maximal level of the activity of ALP is before the expression of osteocalcin and the formation of nodules. This pattern of ALP activity is similar to the rat calvarial osteoblasts [Bronkers et al., 1987; Aronow et al., 1990; Owen et al., 1990].

DISCUSSION

The biochemical and histochemical data obtained in this study indicate that the expression patterns of bone-related proteins, which are regulated in a temporal manner during successive developmental stages including proliferation, bone matrix formation/maturation, and

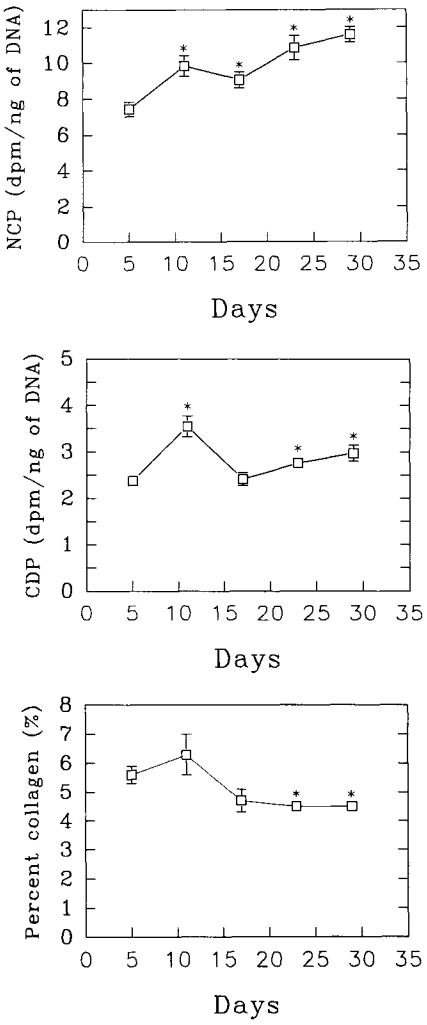

Fig. 7. Collagen and noncollagen protein synthesis during the differentiation of MC3T3-E1 osteoblastic cells. Cells were cultured in 24 well plates with 50 μg/ml of ascorbic acid and 10 mM of β-glycerophosphate. Confluent cultures of the cells were labeled with 2 μCi/ml of [^{3}H] proline for 24 h. Collagenase-digestible protein (CDP), noncollagen protein (NCP), and percentage of CDP against the total protein synthesis (percent collagen) were determined as described in Materials and Methods. Each value represents the mean ± SE of six determinations. *$P < 0.05$ vs. control (day 5).

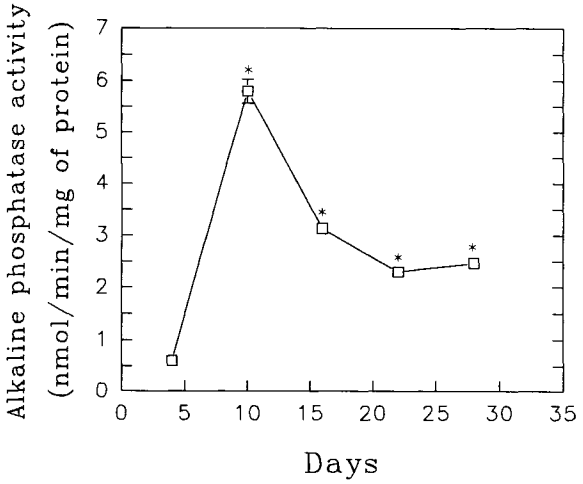

Fig. 8. Alkaline phosphatase activities during the differentiation of the MC3T3-E1 osteoblastic cells. Cells were cultured in 24 well plates with 50 μg/ml of ascorbic acid and 10 mM of β-glycerophosphate. ALP activity in cell layers was determined at the indicated times (4, 10, 16, 22, 24 days) described in Materials and Methods. Data were expressed as mean ± SE of six determinations. *$P < 0.05$ vs. control (day 5).

mineralization, are unique in the MC3T3-E1 cell culture system compared with other systems.

One evidence for the temporal expression of bone-related proteins during the MC3T3-E1 cell differentiation is the presence of an active proliferation stage. Its presence has been demonstrated in two ways. First, the lack of expression of osteocalcin and the formation of bone nodules is consistent with the concept of the presence of proliferation stage in other systems such as rat calvarial osteoblasts [Stein and Lian, 1993] and bone marrow derived osteoblasts [Aubin et al., 1993]. Second, we found that the expression of histones and ribosomal protein S6 gradually decreased during the MC3T3-E1 cell differentiation. The downregulation of histone genes during the cellular differentiation are similar to the other studies conducted in a variety of cell types, including HL-60 promyelocytic cells [Stein et al., 1989], rat [Owen et al., 1990, 1991] and chicken [Shalhoub et al., 1989] osteoblasts, rat myoblasts [Bird et al., 1985; Larson et al., 1989], and adipocytes [Ramsey-Ewing et al., 1995]. However, a surprising finding was that the expression of ribosomal S6 protein decreased during the osteoblast differentiation. Although ribosomal protein S6 is involved in protein translation [Jefferies et al., 1994], we do not know the meaning of its decrease during the MC3T3-E1 cell differentiation at this time. We believe that the proliferation stage is from day 4 to day 10 in this culture system because the nodule formation and the expression of osteocalcin are absent while the expression of proliferation-related histone genes is high during this period.

Further proof for the temporal expression of bone-related proteins during the MC3T3-E1 cell differentiation is the presence of a bone matrix formation/maturation period. The maximal levels of the bone matrix protein mRNAs such as

type I collagen, TGF-β1, fibronectin, and osteonectin are shown during the intermediate period (days 10–16). We call this intermediate period the bone matrix production/maturation stage. During this stage the expression patterns of several bone-related proteins show several differences compared with the other osteoblasts. First, the rat calvarial osteoblasts produce the bone matrix proteins maximally during the proliferation stage [Stein and Lian, 1993]. However, the expression of these proteins in MC3T3-E1 cells does not coincide with the proliferation stage and is also not clearly divided between the proliferation, the bone matrix production/maturation and mineralization stages like the rat calvarial osteoblasts. Second, the maximal expression of bone matrix proteins in MC3T3-E1 cells was not concomitant with that in the rat calvarial osteoblasts [Stein and Lian, 1993]. The expression patterns of the bone matrix genes may fall into the two categories: one is the expression pattern of type I collagen and TGF-β1 and the other is the expression pattern of fibronectin and osteonectin. It is likely that the expression of type I collagen and TGF-β1 in the bone formation may have an important role in the early and middle stages, whereas the expression of the fibronectin and osteonectin may have an important role in the late stage of the osteoblast differentiation. Third, the relative levels of the activity of ALP with respect to the synthesis of collagen in the MC3T3-E1 cells are different from that found in the rat calvarial osteoblasts. The pattern of the activity of ALP parallels the synthesis of collagen during the MC3T3-E1 cell differentiation. A high level of the expression of collagen during the proliferation period in the rat calvarial osteoblasts is followed by the increase of the ALP activity [Aronow et al., 1990; Owen et al., 1990]. Finally, our results also differ from those of the chicken osteoblast cultures in which the activity of ALP increased concurrently with the osteocalcin and the calcium deposition [Gerstenfeld et al., 1987].

The third piece of evidence to support the existence of these stages during the MC3T3-E1 cell differentiation is the formation of the nodules and the expression of osteocalcin gene. We call this period the mineralization stage. The nodule formation is observed only for the late mineralization stage (days 16–30) of this culture system. This result confirms the separate observations of the previous studies in MC3T3-E1 [Sudo et al., 1983; Franceschi and Iyer, 1992; Dean et al., 1994] and several other cells [Gerstenfeld et al., 1987; Bellows et al., 1991; Stein and Lian, 1993; Malaval et al., 1994]. In addition, we did not observe the nodule formation when cells were cultured without the ascorbic acid and β-GP. It is well correlated with other reports [Franceschi and Iyer, 1992; Dean et al., 1994]. We also observed that osteocalcin was expressed during the late stage of differentiation. The osteocalcin expression is well correlated with the time of the nodule formation and it supports the notion that osteocalcin may act as a facilitating factor in the mineralization of the bone matrix [Price et al., 1981; Collin et al., 1992]. This finding is in accordance with the results of studies in the rat calvarial osteoblasts [Aronow et al., 1990] and the bone marrow cells [Malaval et al., 1994]. At this time, we can not explain the differences we described above. These differences may partly result from the difference of the culture condition such as cell plating number and serum lot.

Taken together, our results demonstrate that the expression patterns of bone-related proteins are regulated in a temporal manner during the successive developmental stages including proliferation, bone matrix formation/maturation, and mineralization such as the rat calvarial osteoblast system [Stein and Lian, 1993]. However, the temporal expression patterns of some bone-related proteins are unique in this culture system compared with other systems. Thus, we suggest that this uniqueness is partly the result from the difference of the culture conditions. However, it needs further study to clarify whether the uniqueness of the expression patterns of the bone-related proteins is the result of the difference of the culture conditions or the difference of the differentiation pathway. Furthermore, the MC3T3-E1 cell culture system represents a very useful model for studying the developmental regulation of the bone-related proteins in relation to the different stages during the osteoblast differentiation and the molecular mechanisms associated with the bone formation.

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