Role of cyclin-dependent kinase (Cdk)6 in osteoblast, osteoclast, and chondrocyte differentiation and its potential as a target of bone regenerative medicine

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In the oral and maxillofacial region, conditions such as bone fracture, trauma-induced bone or cartilage defects, and tumor or birth defects such as cleft lip and palate (CLP) are common. To repair irreversible skeletal damage or defects, bone grafts are the current gold standard strategy. However, bone grafts present problems with respect to the availability of bone graft material, difficulties with the donor site etc. In order to establish new treatment strategies for such conditions, one of the most important goals is to clarify the molecular mechanisms that control the differentiation of cells. Accordingly, numerous studies have been conducted to investigate the role or involvement of factors which are thought to be crucial for differentiation of cells, including cell cycle factors, transcription factors, and growth factors. One of these factors is Cdk6, which has been reported to be involved in the commitment for osteoblast differentiation. Herein, we review the role of Cdk6 in differentiation of cells with a particular focus on osteoblast, osteoclast, and chondrocyte differentiation and discuss the potential of Cdk6 as a target of bone regenerative medicine.

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1. Introduction

In the oral and maxillofacial region, conditions such as bone fracture, trauma-induced bone or cartilage defects, and tumor or birth defects such as cleft lip and palate (CLP) are common. To repair irreversible skeletal damage or defects, bone grafts are the current gold standard strategy. However, bone grafts present problems with respect to the availability of bone graft material, difficulties with the donor site etc. In order to establish new treatment strategies for such conditions, one of the most important goals is to clarify the molecular mechanisms that control the differentiation of cells. Accordingly, numerous studies have been conducted to investigate the role or involvement of factors which are thought to be crucial for differentiation of cells, including cell cycle factors, transcription factors, and growth factors. Cell cycle factors in particular are considered to critically influence the differenti-
arrest complexes. By Fig. 1.

discovered p57KIP2 being (p16, p15, p18, p19 (INK4 family) p21, p27, p57 (Cip/Kip family) cycins, cyclin-dependent kinases (Cdk): Cdk4, 4, 6 Cdk inhibitor (Cki): p16, p15, p18, p19 (INK4 family) cyclins, cyclin-dependent kinases (Cdk): Cdk6, Ckis (p18, p21, p27, and p57) and Rb/phosphorylated-Rb proteins were analyzed by Western blotting. In this cell line, the amounts of Cdk6, Cdk2, and p18 increased and the amount of p21 decreased during serum stimulation, whereas the remaining factors were unchanged. When the cells were stimulated with BMP-2, induction of Cdk6 completely disappeared, while the levels of the remaining factors were indistinguishable from those obtained by serum stimulation. Since Cdk6 downregulation by BMP-2 was also seen in a culture of primary osteoblasts isolated from neonatal mouse calvariae, this phenomenon is not specific to a particular cell line, but is considered to be more general. In addition to downregulation of the protein, we found that cdk6 mRNA disappeared when MC3T3-E1 cells were stimulated with BMP-2. Because Cdk6 and Cdk4 are siblings and exert similar functions, and Cdk4 is known to be degraded by the ubiquitin–proteasome system [18], we studied the involvement of the same proteolytic system in the downregulation of Cdk6. MG132, a potent inhibitor of this proteolytic system, did not affect this downregulation of Cdk6, suggesting that the protein degradation of Cdk6 was not involved with the ubiquitin–proteasome system. These findings indicate that BMP-2 downregulated Cdk6 expression mainly by transcriptional repression. We investigated the signal pathway involved in Cdk6 downregulation by BMP-2. A differentiation signal transmitted by BMP-2 is known to be mediated by Smad proteins, particularly by R-Smads (Smad1, 5, 8) and Co-Smads (Smad4), while I-Smads, such as Smad6 and Smad7, inhibit this signaling [19]. When we overexpressed Smad6 in MC3T3-E1 cells by using an adenovirus vector, downregulation of Cdk6 by BMP-2 was completely cancelled. These data showed that Smads mediated downregulation of Cdk6 by BMP-2. Next, to determine whether downregulation of Cdk6 is essential for osteoblast differentiation, we constructed MC3T3-E1 cell clones stably expressing various levels of Cdk6 and compared differentiation markers among control cells and low (>–2-fold) and high (>10-fold) expressers. Induction of alkaline phosphatase (ALP) and production of osteocalcin mRNA by BMP-2 treatment was inhibited significantly in the low and completely in the high expressers, suggesting that downregulation of Cdk6 is essential for efficient osteoblast differentiation. Because Cdk6 promotes the G1–S transition, suppression of osteoblast differentiation by overexpressed Cdk6 could be caused by the promotion of cell proliferation. Therefore, we evaluated the cell cycle distribution or the proliferation rate of the cells determined by flow cytometric analysis or bromodeoxyuridine (BrdU) uptake, respectively. Overexpression of Cdk6 did not cause any significant changes in either the cell cycle distribution or the proliferation rate of the cells, implying that the inhibitory effect of Cdk6 on osteoblast differentiation was not

2. Cdk6 in osteoblast differentiation

Bone morphogenetic protein (BMP)-2 is a potent inducer of bone formation through its stimulation of osteoblast differentiation [17]. To identify cell cycle factors that critically regulate early differentiation, we analyzed the effect of BMP-2 on G1 cell cycle factors in the mouse osteoblastic cell line MC3T3-E1. The cells were arrested in quiescence by serum starvation, and stimulated with serum in the presence or absence of BMP-2. The amounts of cyclins (D1, D2, D3, and E), Cdk2, Cdk4, Cdk6, Ckis (p16, p21, p27, and p57) and Rb/phosphorylated-Rb proteins were analyzed by Western blotting. In this cell line, the amounts of Cdk6, Cdk2, and p18 increased and the amount of p21 decreased during serum stimulation, whereas the remaining factors were unchanged. When the cells were stimulated with BMP-2, induction of Cdk6 completely disappeared, while the levels of the remaining factors were indistinguishable from those obtained by serum stimulation. Since Cdk6 downregulation by BMP-2 was also seen in a culture of primary osteoblasts isolated from neonatal mouse calvariae, this phenomenon is not specific to a particular cell line, but is considered to be more general. In addition to downregulation of the protein, we found that cdk6 mRNA disappeared when MC3T3-E1 cells were stimulated with BMP-2. Because Cdk6 and Cdk4 are siblings and exert similar functions, and Cdk4 is known to be degraded by the ubiquitin–proteasome system [18], we studied the involvement of the same proteolytic system in the downregulation of Cdk6. MG132, a potent inhibitor of this proteolytic system, did not affect this downregulation of Cdk6, suggesting that the protein degradation of Cdk6 was not involved with the ubiquitin–proteasome system. These findings indicate that BMP-2 downregulated Cdk6 expression mainly by transcriptional repression. We investigated the signal pathway involved in Cdk6 downregulation by BMP-2. A differentiation signal transmitted by BMP-2 is known to be mediated by Smad proteins, particularly by R-Smads (Smad1, 5, 8) and Co-Smads (Smad4), while I-Smads, such as Smad6 and Smad7, inhibit this signaling [19]. When we overexpressed Smad6 in MC3T3-E1 cells by using an adenovirus vector, downregulation of Cdk6 by BMP-2 was completely cancelled. These data showed that Smads mediated downregulation of Cdk6 by BMP-2. Next, to determine whether downregulation of Cdk6 is essential for osteoblast differentiation, we constructed MC3T3-E1 cell clones stably expressing various levels of Cdk6 and compared differentiation markers among control cells and low (>–2-fold) and high (>10-fold) expressers. Induction of alkaline phosphatase (ALP) and production of osteocalcin mRNA by BMP-2 treatment was inhibited significantly in the low and completely in the high expressers, suggesting that downregulation of Cdk6 is essential for efficient osteoblast differentiation. Because Cdk6 promotes the G1–S transition, suppression of osteoblast differentiation by overexpressed Cdk6 could be caused by the promotion of cell proliferation. Therefore, we evaluated the cell cycle distribution or the proliferation rate of the cells determined by flow cytometric analysis or bromodeoxyuridine (BrdU) uptake, respectively. Overexpression of Cdk6 did not cause any significant changes in either the cell cycle distribution or the proliferation rate of the cells, implying that the inhibitory effect of Cdk6 on osteoblast differentiation was not...
exerted via cell cycle regulation. Finally, we investigated the association between transcription factors and the inhibitory effect of Cdk6, and the effects of this association on osteoblast differentiation. Rb, a repressor of the E2F-DP transcription factor, which is essential for the S phase onset, has been reported to act as a transcriptional co-activator for Runx2, a key transcription factor for osteoblast differentiation [20,21]. Because Rb is a direct target of Cdk6 in cell cycle control, we investigated the effects of overexpression of Cdk6 on the in vivo binding of Runx2 and Rb to the osteocalcin promoter during BMP-2 treatment. To this end, we performed a chromatin immunoprecipitation (ChIP) assay and compared the binding of Runx2 and Rb to the osteocalcin promoter among the empty vector–transfected MC3T3-E1 cells and the low and high Cdk6 expressers at day 1 and day 4 post-BMP-2 treatment. At day 1, Runx2 was slightly bound to the osteocalcin promoter only in the empty vector–transfected MC3T3-E1 cells, whereas no binding of Rb to this promoter was detected in any of these cells. At day 4, the binding of Runx2 to this promoter was clearly detected in the empty vector–transfected MC3T3-E1 cells and the low Cdk6 expresser, but not in the high Cdk6 expresser, in which osteoblast differentiation was completely blocked. Thus, there was a close correlation between osteoblast differentiation and the binding of Runx2 to the osteocalcin promoter in the Cdk6-induced inhibition of differentiation. In contrast, there was no apparent correlation between Runb binding and the Cdk6-induced inhibition of differentiation, though Rb is certainly required for efficient osteoblast differentiation of MC3T3-E1 cells. At day 4, the binding of Rb to the osteocalcin promoter was confirmed in all these cells, as reported previously [21]. However, Rb seemed to bind the promoter independently of Runx2, because in the high Cdk6 expresser, Rb bound the promoter without the binding of Runx2. The fact that the Runx2 binding to the osteocalcin promoter was lost during the Cdk6-induced differentiation inhibition raises the possibility that Cdk6 may inhibit osteoblast differentiation by interfering with the promoter-binding ability of the Runx2. These results indicate that Cdk6 is a critical regulator of BMP-2-induced osteoblast differentiation [22].

3. Cdk6 in osteoblast differentiation

The receptor activator of the nuclear factor-κB (NF-κB) ligand (RANKL) is a tumor necrosis factor (TNF)-related cytokine that stimulates osteoblast differentiation from hematopoietic precursor cells [23–25]. Cells of the mouse osteoblast precursor cell line RAW, differentiate to osteoblasts in response to stimulation with RANKL. By studying this in vitro model, we investigated the involvement of cell cycle factors that critically regulate osteoblast differentiation. We checked the protein expression levels of the G1 cell cycle factors, cyclins (D1, D2, D3, E), Cdk2, Cdk4, Cdk6, and Cdk inhibitors (p15 and p27) in RAW cells cultured in the presence/absence of soluble RANKL. The Cdk6 protein level was downregulated in RAW cells cultured in the presence of RANKL. Inhibition of the NF-κB pathway by the overexpression of dominant negative mutants of the IκB kinase 2 (IKK2DN) gene, using the adenovirus vectors [26], blocked Cdk6 downregulation by RANKL. RAW cells constitutively overexpressing Cdk6 resisted RANKL-induced osteoblast differentiation as determined by tartrate-resistant acid phosphatase (TRACP) staining. On the other hand, overexpression of Cdk6 did not alter the cell cycle regulation, as determined by BrdU uptake and flow cytometric analysis. The fact that the levels of Cdk6 overexpression did not change the cell cycle regulation suggested that the inhibitory effect of Cdk6 on osteoblast differentiation was not caused by promotion of proliferation. These results indicate that Cdk6 is a critical regulator of RANKL-induced osteoblast differentiation and that its NF-κB–mediated downregulation is important for efficient osteoblast differentiation [27]. However, there have been reports indicating that the role of Cdk6 in osteoblast differentiation is highly complex and diverse, and thus extensive studies will be needed to precisely clarify the role of Cdk6 in osteoblast differentiation [28,29].

4. Cdk6 in chondrocyte differentiation

Cells of the mouse prechondrocyte cell line ATDC5 differentiate to chondrocytes in response to stimulation signals evoked by insulin [30–32]. We investigated the involvement of cell cycle factors in the differentiation process of chondrocytes by using ATDC5 cells. We studied the amounts of cyclins (A, D1, D2, D3, and E), Cdk2, Cdk4, Cdk6, and Ckis (p15, p16, p18, p19, p21, p27, and p57) by Western blot analysis. Among these G1 cell cycle factors examined, both the protein and mRNA levels of Cdk6 were suppressed in ATDC5 cells cultured with insulin. Since proteasome inhibitors such as MG132 or lactacystin did not affect this downregulation of Cdk6, the protein degradation of Cdk6 was not involved in this phenomenon. Insulin is known to initiate cellular responses by binding to distinct cell-surface receptor tyrosine kinases which regulate diverse signaling pathways, such as those of MAPK and PI3K/Akt [33]. In addition, in chondrogenic differentiation, p38 MAPK, ERK-1/2, and PI3K/Akt [34–36], are reported to play an important role. We therefore investigated the involvement of these signal pathways in the downregulation of Cdk6. Among the inhibitors of p38 MAPK, ERK-1/2, and PI3K/Akt, only the p38 MAPK inhibitor SB203580 blocked the decrease in the Cdk6 protein level in cells cultured in the presence of insulin, indicating that the Cdk6 suppression was mediated by the p38 MAPK pathway. The expressions of the chondrocyte marker of type II collagen and type X collagen were markedly lower in ATDC5 cells constitutively expressing Cdk6. Concerning the key transcription factors in chondrogenic differentiation, expressions of Sox5 and Sox6, but not Sox9 were suppressed in ATDC5 cells constitutively expressing Cdk6.
Since Sox9 is essential for mesenchymal condensation that is prerequisite for the chondrogenic differentiation while Sox5 and Sox6 are needed thereafter as the co-activators, Cdk6 may contribute to the early stage of chondrogenic differentiation like commitment of precursors to the lineage. However, the Cdk6 overexpression did not alter the cell cycle regulation, as determined by BrdU uptake and flow cytometric analysis, suggesting that the inhibitory effect of Cdk6 on the differentiation was exerted by a mechanism largely independent of its cell cycle regulation. These results indicate that Cdk6 may regulate chondrocyte differentiation and that its p38-mediated downregulation may be involved in efficient chondrocyte differentiation [37].

5. Perspectives

5.1. The role of Cdk6 in skeletogenesis and the molecular mechanism of osteoblast differentiation controlled by Cdk6

We have found that Cdk6, a G1 cell cycle factor, plays a critical role in controlling osteoblast, osteoclast, and chondrocyte differentiation. A schematic presentation of the mechanism by which Cdk6 inhibits osteoblast, osteoclast and chondrocyte differentiation is shown in Fig. 2. It is worth noting that this finding is not specific to the differentiation of these three cell types. Matsushansky et al. reported a similar role of Cdk6 in erythroid differentiation of a murine leukemia cell line [38]. Fujimoto et al. reported that downregulation of Cdk6 is required for terminal granulocytic differentiation and that Cdk6 inhibits Runx1 activity by interfering with Runx1 DNA binding and Runx1-C/EBPalpha interaction [39]. Ericson et al. reported that expression of Cdk6, but not Cdk4 alters morphology of mouse astrocytes [40]. Slomiany et al. reported that forced Cdk6 expression in mouse astrocytes results in changes in the patterns of gene expression, changes in the actin cytoskeleton, and enhanced motility [41]. Kohrt et al. suggested that the subcellular distribution of Cdk6 is involved with this novel function of Cdk6 in differentiation [42]. Judging from these individual reports, it is certain that Cdk6 is one of the key regulators in the differentiation of multiple types of cells, at least in cell-based studies. However, as in the cases of p21CIP1-knockout mice [11] or P27KIP1-knockout mice [12], Cdk6-knockout mice did not display gross anatomical abnormalities [43], suggesting that the functions of Cdk6 can be compensated for in vivo by other cell cycle factors, possibly Cdk4 or Cdk2. With the purpose of uncovering the role of Cdk6 in skeletogenesis, we are now conducting a thorough investigation of the skeletal phenotype of Cdk6-knockout mice. In addition, we are trying to address the question of how Cdk6 controls osteoblast differentiation without influencing the cell cycle. One possibility is that Cdk6 directly controls a factor(s) critically involved in differentiation as demonstrated in the previous works [39,44,45]. Because the involvement of several transcription factors, such as Runx2, osterix, and LDL receptor protein 5, have been reported in bone formation [20,46,47], we assume these factors might be responsible for the BMP-2-invoked repression of Cdk6 transcription. In consideration of all these previous results [39,44,45], including our own, we propose a working hypothesis in regard to the regulatory mechanism of transcription factors controlled by Cdk6 (Fig. 3). However, identification of the transcriptional repressor as well as key targets for Cdk6 will absolutely be required for a deeper understanding of the molecular basis of cell differentiation.

5.2. Possibility of Cdk6 as a target of bone regenerative medicine

Finally, we would like to discuss the possibility of Cdk6 as a target in bone regenerative medicine. In bone regenerative medicine, BMPs have been clinically used for the treatment of long bone non-unions and for spinal fusion [48–51]. However, as the effective doses of BMPs required in humans are much higher than those used in smaller animals, the treatment requires a large amount of expensive recombinant BMPs [51]. The problem of treatment cost is one of the reasons why this method has not been widely adopted. Because downregulation of Cdk6 occurs downstream of BMP signaling and is important for efficient osteoblast differentiation, it is natural to wonder whether Cdk6 might have potential as a target of bone regenerative medicine. In order to address this question, we are studying the effects of Cdk6 inhibition on osteoblasts by overexpression of dominant negative (dn)Cdk6, silencing of Cdk6 through RNA interference or supplementation of Cdk6 inhibitors, and we are preparing for in vivo study as well. For the time being, it seems that Cdk6 could indeed be a candidate target in bone regenerative medicine (Ogasawara et al. unpublished data). Before long, we will be able to report our final conclusions, which we hope will contribute to bone regenerative medicine in the future.

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