Role of c-jun N-terminal kinase (JNK) Signaling in Hypertrophic Chondrocyte Differentiation

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Introduction

The majority of the vertebrate skeleton develops through the process of endochondral ossification and involves chondrogenesis, chondrocyte proliferation, and hypertrophic chondrocyte differentiation. Hypertrophic differentiation is marked by expression of the type X collagen (COL10), as well as induction of MMP-13, alkaline phoshatase, and osteocalcin. Although multiple extracellular signaling molecules and nuclear transcription factors are implicated in hypertrophic chondrocyte differentiation, the precise intracellular signaling pathways connecting extracellular signaling molecules to transcriptional activation are poorly understood.

The c-jun N-terminal kinase (JNK) pathway is one of three major mitogen-activated protein kinase (MAPK) signaling pathways influencing cell proliferation and differentiation (1). Nakajima et al. have shown that JNK functions as a negative regulator of early-stage chondrogenesis, and as a positive regulator that modulates hypertrophic chondrocyte differentiation through effects on COL10 and MMP-13. The experimental plan compared effects of JNK inhibition on induction of hypertrophic chondrocyte differentiation to effects of inhibiting extracellular signal-regulated kinase ½ (ERK1/2), and p38, the two additional members of the MAPK pathways.

Materials and methods

A JNK inhibitor (SP600125), an ERK inhibitor (PD98059), and a p38 inhibitor (SB202190) were purchased from Calbiochem (San Diego, CA, U.S.A.). ATDC5 cells were plated on day 0. On day 3, the culture medium was changed to DMEM/F12 containing 5% FBS, ITS (10 µg/ml insulin, 5.5 µg/ml transferrin and 5.0 ng/ml sodium selenite) (Sigma-Aldrich, St. Louis, MO), and ascorbic acid 2-phosphate (50 µg/ml) to induce chondrogenesis. On day 14, cells were treated with vehicle, SP600125 (5, 10, and 20 µM), PD98059 (5, 10, and 40 µM), or SB202190 (5, 10, and 20 µM). Total cellular RNA was extracted using guanidinium isothiocynate (Tri-Reagent, Sigma Chemical) and cDNA was prepared using TaqMan reverse transcription (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR (TaqMan PCR) was carried out using a PE Applied Biosystems 7900 Sequence Detector with specific probes for the α1 chain of type X collagen (col10a1), mmp-13, the α1 chain of type II collagen (col2a1), alkaline phosphatase-1 (akp1), and osteocalcin. Cell lysates from untreated and treated cells were prepared and subjected to Western blot analysis of col10 expression. ALP enzymatic activity was measured by means of p-nitrophenyl phosphate (PNPP) method and normalized to total protein determined using the Bio-Rad protein assay solution (Bio-Rad Laboratories, Hercules, CA). The formation of mineralized matrix nodules was assessed by Alizarin Red-S staining.

Results

ATDC5 cells were treated with inhibitors for JNK, ERK, and p38 from day 14, and harvested on day 18. As shown in Figure 1, SP600125, a JNK inhibitor, dose-dependently increased both col10a1 and mmp-13 mRNA expression but not col2a1. On the other hand, PD98059, an ERK inhibitor, did not statistically influence any of the genes, whereas SB202190, a p38 inhibitor, strongly inhibited col10 and MMP-13 gene expression at all concentrations and type II collagen at the highest concentration (Figure 1). Western blot analysis showed that col10 was up-regulated at the protein level by the treatment with the JNK inhibitor, SP600125 (Figure 2). To test whether or not inhibition of JNK signaling involved other phenotypic markers of hypertrophic chondrocytes, ALP enzymatic activity and Alizarin red-S staining were quantified. The results showed that SP600125 at all concentrations tested did not influence ALP activity or the formation of mineralized matrix nodules. ERK inhibition also failed to influence either ALP activity or Alizarin red-S staining. In contrast, the p38 inhibitor strongly inhibited ALP activity and mineralization in a dose-dependent manner. In order to rule out the possibility that 96 hr was an insufficient time period to induce these phenotypic markers of hypertrophic chondrocyte differentiation, ATDC5 cells were treated with each of the MAPK inhibitors from day 14 to day 24 prior to analysis. TaqMan PCR revealed that the long-term (ten days) treatment with SP600125 did not enhance mRNA expression of akp1 and osteocalcin compared to the control cultures whereas col10a1 and mmp-13 mRNA signal levels remained elevated in the presence of the inhibitor.

Discussion

Our results showed for the first time that JNK signaling pathway acts as a negative regulator in terms of col10a1 and mmp13 expression during hypertrophic chondrocyte differentiation. The up-regulation of col10a1 and mmp13 mRNA was observed at 96 hr after the treatment with SP600125. The effect of JNK inhibition was also evident for these genes following a longer period of exposure (ten days). The role of JNK as a negative regulator of signaling pathways has precedence based on effects documented for early stages of chondrogenesis (2) and recent genetic studies where loss of JNK increased hormonal sensitivity. In our study, expression of akp1 and osteocalcin mRNA, ALP activity and the formation of mineralized matrix nodules were not significantly influenced by treatment with SP600125. Further studies will be needed to elucidate whether JNK regulation might be limited to col10a1 and mmp13 expression during the initiation steps leading to hypertrophic chondrocyte differentiation.

References

(1) Raman M, et al., Oncogene. 2007, 26(22):3100-12
(2) Nakajima M, et al., Biochem Biophys Res Commun. 2004; 320: 1069-1075

Fig.1 Effects of MAPK inhibitors, SP600125 for JNK, PD98059 for ERK, and SB202190 for p38, on expression of col10a1, mmp13, and col2a1 in ATDC5 cells induced hypertrophic differentiation.

Fig.2 Western blot: Type X collagen dose-dependently induced by the treatment with SP600125.