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EXPRESSION OF BONE MORPHOGENIC PROTEIN 7 mRNA IN MDCK CELLS

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SUMMARY Recently, a family of proteins, bone morphogenic proteins (BMPs), have been identified, which promote osteoblast differentiation and bone mineralization. One of them, BMP7, has been shown to be expressed at high levels in the kidney. We detected the message in a kidney tubular cell line, Madin-Darby canine kidney (MDCK) cells. After serum starved for 48h, 8-bromo-cAMP enhanced BMP7 mRNA at 3h and forskolin enhanced it at 6h to 24h in MDCK cells. PMA increased BMP7 mRNA at 6h but down-regulated it at 12h and 20h. The result suggests that BMP7 gene expression may be regulated in MDCK cells by PKA and PKC. MDCK cells can be used in further studies of BMP7 regulation.

Recently, a family of proteins, bone morphogenic proteins (BMPs), have been identified, which promote osteoblast differentiation and bone mineralization (1). BMPs are superfamily of TGFß except BMP1. BMPs also work on embryogenesis and developmental pattern formation, suggesting much wider function beyond osteogenesis (2). One of them, BMP7, has been shown to be expressed at highest level in the kidney (3, 4). As the kidney plays an important role in bone mineralization through regulation of calcium and phosphate metabolism as well as activating vitamin D_3, BMP7 may work as a mediator of renal effect on bone modulation in an endocrine manner. The cause of renal osteodystrophy in chronic renal failure is attributed to the failure to activate vitamin D_3 by the kidney. However, the supplement with active form of vitamin D_3 does not fully correct renal osteodystrophy. And recently a low turnover bone disease has been observed in patients without aluminium accumulation and this incidence seems to be increasing (5). Other factors which are missed in renal failure may be necessary to normalize bone formation. BMPs will be a good candidate for one of these missed factors. We found BMP7 mRNA in a renal epithelial cell line, MDCK cells, which was upregulated by PKA and PKC.

METHODS

Materials: A multiprime DNA labeling kit was obtained from Takara Shuzou. Nylon membrane (Magna Nylon) was from Micron Separation Inc. [α-^{32}P]dCTP(3000 Ci/mmol) was from New England Nuclear, X ray film (RX) was from Fuji Photo Film Co. cDNA

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synthesis kit was from Boehringer Mannheim, Gene Amp DNA amplification kit from Perkin Elmer Cetus, and DNA sequencing kit from United States Biochemical Corporation.

Cell culture: OK cells, LLCPK cells and MDCK cells were maintained in DMEM (Gibco Laboratory, Grand Island, New York), 10% fetal calf serum; $NaHCO_3$ 3.7 g/l; Hepes 20 mM; penicillin 100U/ml; streptomycin 100 μg/l; in 5%CO_2/95%air. After reaching subconfluent, cells were cultured in FCS free medium for 24-48 h. Then, the medium was changed to FCS free medium with PMA, forskolin and 8-bromo- cAMP.

RNA extraction and Northern blot hybridization: Total RNAs (20 μg) extracted from MDCK cells as previously reported (6) were electrophoresed through formaldehyde-1.2% agarose gels and transferred to nylon membranes and hybridized with [^{32}P]-labeled dog BMP7 cDNA (vide infra), or 1.2 kb PstI fragment of human GAPDH cDNA (American Tissue collection #57090) as probes. Hybridization was performed in a solution of 50% formamide, 6X SSPE, 0.5% SDS, 5X Denhardt's solution and 100μg/ml denatured salmon sperm DNA at 42°C for 20 h. Blot were washed two times in 2X SSC, 0.1% SDS at room temperature for 10 min each and twice in 1X SSC, 0.1% SDS at 37°C for 15 min each and 0.1X SCC, 0.1% SDS at 60°C for 15 min. Blots were exposed to X-ray films. The developed films were scanned with a densitometer to quantify each bolt.

[^{32}P]DNA probe for BMP7. The used primers from human BMP7 sequence (7) were 5'-GGCAAGCACAACTCGGCACC-3' (codons Gly^{74}-Pro^{80}, sense) and 5'-TTGGGGTTGATGCTCTGCCC-3' (codons Gly^{254}-Lys^{260}, antisense) avoiding sequence coding highly conserved mature peptide of BMPs. We detected the amplification products of expected size in renal epithelial cell lines, OK cells, LLCPK cells and MDCK cells by RT-PCR (not shown) with GeneAmp DNA amplification reagent kit and Program temperature control system PC-700 (ASTEC). As the band was strongest in MDCK cells, we selected MDCK cells for further study. The MDCK cDNA was made from total RNA using cDNA synthesis kit following the manufacturer's instruction. A 560bp PCR product from MDCK cDNA, using the above primers from human BMP7 cDNA sequence, was cloned into plasmid vector (pUC 18) using TA cloning kit. Sequencing was performed by the dideoxy chain termination method using Sequenase 2.0. The insert was labelled with [α-^{32}P]dCTP using the multiprimed labelling kit.

RESULTS

To establish the identity of BMP7 mRNA of MDCK cells, we sequenced the PCR product (Fig. 1). Comparison of this sequence with human BMP7 revealed 93.3% identity of nucleotide sequence and 98.8% identity of deduced amino-acid sequence excluding primers. In comparison with mouse BMP7 (3), 95.9% identity of deduced amino-acid sequence was noted. Given the highly homologous sequence of PCR product with human and mouse BMP7, we took this PCR product as a part of dog BMP7 cDNA. Thus, BMP7 mRNA or a closely related species was present in MDCK cells. Basal expression of BMP7 mRNA (4.1, 2.4, 2.2kb) in MDCK cells was high enough to be detected in Northern blot analysis of total RNA. We also detected this message in other kidney epithelial cell lines; OK cells and LLCPK cells (data not shown). Therefore, BMP7 mRNA in the kidney is most likely expressed in tubular cells.

Next we studied the regulation of BMP7 mRNA expression in MDCK cells after stimulations of PKA and PKC. After serum starved for 48h, forskolin (50μM) was added in serum free DMEM. Forskolin enhanced BMP7 mRNA at 6h to 24h (Fig. 2). 8-bromo-cAMP (1mM) also enhanced BMP7 mRNA at 6h (Fig. 3). Therefore, protein kinase A pathway may stimulate the level of BMP7 mRNA. On the other hand, PMA (10^{-7}M), a stimulator of protein kinase C, increased BMP7 mRNA at 6h but down-regulated it at 12h and 20h (Fig. 4). As

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  1 GGCAAGCACAACTCGGCACCCATGTTCATGCTGGACCTGTACAATGCCATGGCGGTGGAG
  1  G  K  H  N  S  A  P  M  F  M  L  D  L  Y  N  A  M  A  V  E

 61 GAGGGCGGCGGGCCAGCCGGCCAGGGCTTCTCCTACCCCTACAAGGCCGTCTTCAGCACC
 21  E  G  G  G  P  A  G  Q  G  F  S  Y  P  Y  K  A  V  F  S  T

121 CAGGGCCCCCCTCTAGCCAGCCTGCAAGACAGCCACTTCCTCACCGACGCCGACATGGTC
 41  Q  G  P  P  L  A  S  L  Q  D  S  H  F  L  T  D  A  D  M  V

181 ATGAGCTTCGTCAACCTCGTGGAGCATGACAAAGAGTTCTTCCACCCACGTTACCACCAC
 61  M  S  F  V  N  L  V  E  H  D  K  E  F  F  H  P  R  Y  H  H

241 CGGGAGTTCCGATTCGATCTCTCCAAGATCCCAGAAGGGGAAGCTGTGACTGCAGCCGAA
 81  R  E  F  R  F  D  L  S  K  I  P  E  G  E  A  V  T  A  A  E

301 TTCCGGATCTACAAGGACTACATCCGGGAACGCTTCGACAACGAAACGTTCCGGATCAGC
101  F  R  I  Y  K  D  Y  I  R  E  R  F  D  N  E  T  F  R  I  S

361 GTTTACCAGGTGCTGCAGGAGCACTTGGGCAGGGAGTCAGACCTGTTCCTGCTGGACAGC
121  V  Y  Q  V  L  Q  E  H  L  G  R  E  S  D  L  F  L  L  D  S

421 CGCACCCTCTGGGCCTCGGAGGAGGGCTGGCTGGTGTTCGACATCACAGCCACCAGCAAC
141  R  T  L  W  A  S  E  E  G  W  L  V  F  D  I  T  A  T  S  N

481 CACTGGGTGGTCAACCCACGACACAACCTGGGCCTGCAGCTCTGCGTGGAGACCTTGGAC
161  H  W  V  V  N  P  R  H  N  L  G  L  Q  L  C  V  E  T  L  D

541 GGGCAGAGCATCAACCCCAAA
181  G  Q  S  I  N  P  K
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Fig. 1. Nucleotide sequence and deduced amino-acid sequence of dog BMP7 partial cDNA clone. Only two amino acids were different between dog and human; Ala^{26}(dog) vs Gly(human); Cys^{174}(dog) vs Ser(human).

PMA stimulates PKC initially and later depletes PKC, the result may be best explained by the mechanism that the initial induction of BMP7 mRNA by PMA was due to the activation of PKC and the later down-regulation was due to the depletion of PKC. Summary of these studies was shown in Fig. 5.

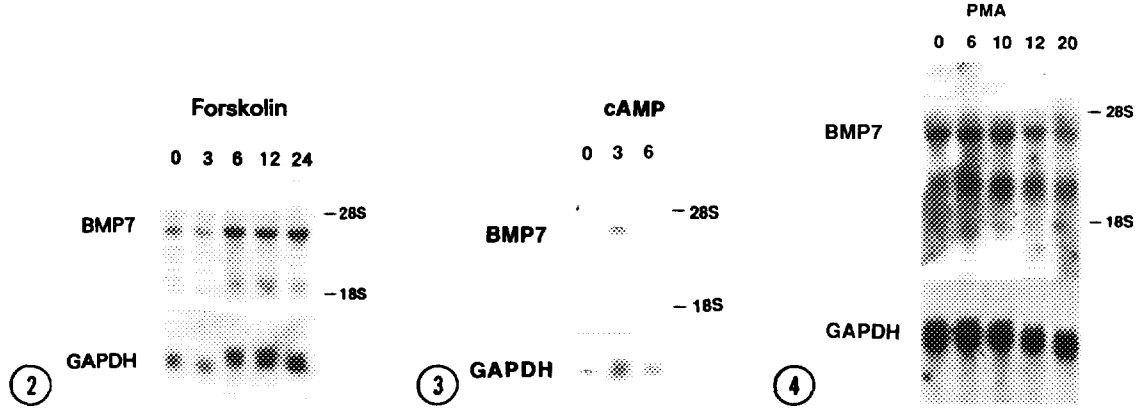

Fig. 2. Effect of forskolin (50 μM) on BMP7 mRNA expression in MDCK cells. The numbers represent hours after treatment. GAPDH seemed to increase after 6h in this particular experiment. However, forskolin treatment itself did not appreciably change GAPDH mRNA expression in other five experiments.

Fig. 3. Effect of 8-bromo-cAMP (1mM) on BMP7 mRNA expression in MDCK cells.

Fig. 4. Effect of PMA (0.1 μM) on BMP mRNA expression in MDCK cells.

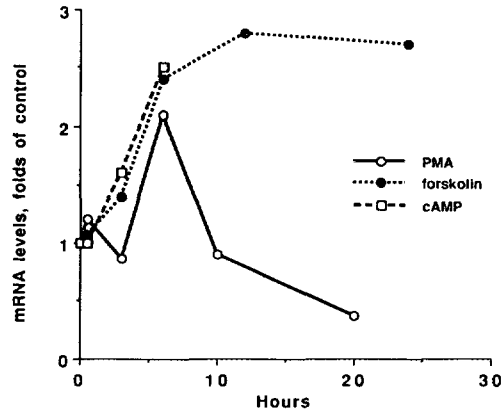

Fig. 5. Summary of changes of BMP7 mRNA levels by PMA, forskolin, and cAMP. Each point represents 2-4 experiments and corrected for GAPDH mRNA level.

DISCUSSION

We have shown the presence of BMP7 mRNA in a renal tubular epithelial cell line, MDCK cells. Cell lines expressing BMP7 have not been reported before. The identification of BMP7 expressing cell line would facilitate the studies of regulation of BMP7 expression. We also have shown the modulation of BMP7 mRNA expression by PKA and PKC. Other member of TGFβ family like activin or TGFβ1 has also been reported to be induced by cAMP (8) or PKC (9). The results suggest that hormones which work on the bone like PTH or calcitonin may induce BMP7 in the kidney through PKC or PKA pathway in the renal tubule which have receptors for PTH and calcitonin. Hormonal modulation of BMP7 expression in the kidney should be examined in the future studies. TGFβ family peptides work mostly as local factors. However, endocrine function of activin has been shown and its blood level has been reported to be 8.3 ng/ml in men (10). If the similar endocrine system is present with BMP7, the kidney may play a major role as mRNA of BMP7 is most abundant in the kidney (3, 4). The measurement of blood level of BMP7 may substantiate above hypothesis.

Other than its osteo-inductive property, BMPs are also active in mesodermal tissue induction. In situ hybridization studies suggest that BMP genes are expressed in many other tissues other than skeletal tissues (11). Recently BMP5 mutation has been detected in the mouse short ear genes (12). Interestingly, short ear mice have hydrotic kidneys, suggesting that BMPs may play an important role in nephrogenesis. BMP2 has been shown to inhibit limb growth in the early mouse limb-bud (13). BMP7 may also have some important function in the kidney such as tissue repair through influencing the metabolism of extracellular matrix components and affecting solute transport properties. In the brain BMP7 has been shown to induce the neural cell adhesion molecule in the neuroblastoma-glioma hybrid cell line and produces morphogenic change (14) and BMP2 affects p-glycoprotein-mediated multidrug transport of glioma cells (15). Further study of its local action in the kidney will be a fruitful area of study.

In conclusion, we identified BMP7 mRNA in MDCK cells, which was upregulated by PKA and PKC. This cell line can be used in further studies of BMP7 regulation.

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