Vol. 183, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1992 Pages 83-92

EXPRESSION OF THE HEPARIN-BINDING GROWTH FACTOR RECEPTOR GENES IN HUMAN MEGAKARYOCYTIC LEUKEMIA CELLS

Osamu Katoh[1], Yutaka Hattori[1], Takeyuki Sato[2], Akiro Kimura[3], Atsushi Kuramoto[3], Takashi Sugimura[1] and Masaaki Terada[1]

[1]Genetics Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan

[2]Department of Pediatrics, School of Medicine, Chiba University, Inohana 1-8-1, Chiba-shi 280, Japan

[3]Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima-shi 734, Japan

Received January 6, 1992

K-*sam/bek*, N-*sam/flg* and FGFR3/*sam3* establish gene family of the receptors for heparin-binding growth factors (HBGFs) or FGFs. These mRNAs were detected in human leukemia cells, CMK, K562 and HEL, which have megakaryocytic phenotype or the potency to differentiate into megakaryocytic lineage. In CMK cells N-*sam/flg* transcript level was enhanced by the culture with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). cDNA-polymerase chain reaction identified K-*sam/bek* mRNA in human platelets, suggesting the involvement of HBGFs in megakaryocytopoiesis and functions of platelets.

The heparin-binding growth factor (HBGFs) or FGF family are multifunctional growth factors considered to play important roles in tumor development or progression, angiogenesis, wound healing, and embryonal development. This family includes acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), the products of *INT2*, *HST1*, *FGF5*, and *HST2/FGF6* genes and keratinocyte growth factor (KGF).[1-7] The genes of HBGF receptors also

0006-291X/92 $1.50

comprise a family. The K-*sam/bek*, N-*sam/flg*, FGFR3/*sam3* and FGFR4 cDNA have been isolated as the genes of this family whose products are receptor tyrosine kinase proteins.[8-14]

There are few reports which clarify the functional roles of the HBGFs or these receptors on regulation of hematopoiesis. It has been reported that bFGF augments the effects of inteleukin 3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin on the colony formation efficiency of human hematopoietic progenitors.[15] Effects of HBGFs on the cells committed to megakaryocytic lineage, however, are not yet known.

The human leukemia cells, CMK, with megakaryocytic characteristics were established from a patient with acute megakaryoblastic leukemia.[16] It has been reported that treatment of CMK cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) greatly enhanced the reactivity with anti platelet antibodies. The human leukemia cells, K562 and HEL also have the capacity to differentiate into the cells in megakaryocytic lineage by the induction with TPA.[17-18]

We describe here the presence of K-*sam/bek*, N-*sam/flg* and FGFR3/*sam3* mRNAs in CMK cells and HEL cells, and N-*sam/flg* in K562 cells detected by RNA blot hybridizaiton analysis. We also report here the expression of K-*sam/bek* gene in total RNA extracted from human platelets circulating in peripheral blood by using reverse transcriptase-polymerase chain reaction (RT-PCR) techniques.

MATERIALS AND METHODS

CELL LINES: THE CMK, K562 and HEL cells were grown in RPMI1640 medium supplemented with 10% fetal calf serum in a humid atmosphere with 5% CO_2 at 37°C. The CMK cells were

induced to differentiate by culture with $1x10^{-8}$M 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma Chemical Co., St. Louis). The cells were harvested after 0-12 hours cultured with TPA for RNA blot hybridization.

RNA extraction and RNA blot hybridization analysis: RNA extraction from cell lines and RNA blot hybridization analysis were performed essentially as described.[19] The filters were hybridized in a buffer containing 50% formamide, 5xSSC (1xSSC is 0.15M sodium chloride plus 0.015M sodium citrate) at 42°C for 12-16 hours followed by washing in a buffer consisting of 0.1xSSC and 0.1% sodium dodecyl sulfate at 65°C for 1 hour. Probes used were ApAp 0.5, a 0.5kbp *Apa*I-*Apa*I fragment of human N-*sam* gene cDNA; RA0.7, 0.7kbp fragment of human K-*sam* gene cDNA[10]; P31d, a fragment of mouse *sam3* cDNA (submitted) probe which has 88% homology to the nucleotide sequences of human counterpart, FGFR3; aFGF and bFGF cDNA, fragments of the open reading frame portion of the human bFGF and aFGF cDNAs,[20] and chicken ß-actin cDNA as a control of RNAs' condition.

Isolation of human platelets: Human platelets were isolated from whole blood as previously described.[21] Venous blood from a normal healthy volunteer anticoagulated with 1/5 volume of acid citrate dextrose (ACD) was collected and centrifuged at 110g for 20 min. The upper half of platelet-rich plasma obtained was further centrifuged at 1000g for 10 min. The precipitated platelets were then suspended in phosphate-bufferd saline (pH7.4) (PBS) containing 20% ACD and centrifuged at 110g for 20 min. This process was repeated twice to avoid the contamination of cells in other lineages. Contaminated cells in other lineages were not detected by microscopic observation.

Reverse transcriptase-polymerase chain reaction (RT-PCR): cDNA was synthesized from total RNA of human platelets using MuMLV reverse transcriptase (BRL). PCR was carried out for 30 cycles using upsteam primer AMP2; 5'-ATGGAGATGATGAAGATGAT-3' (corresponding to nucleotide positions 1927-1946 of K-*sam*7) and downstream primer RAMP3; 5'-TCCCTCATCATCATGTACAG-3' (2537-2518).[10,22] These primers were designed to be able to also detect the N-*sam*/*flg* and FGFR3/*sam3* transcripts. By digestion with *Ban*I or *Bal*I, it is understandable which of the PCR products are fragments of K-*sam*/*bek*, N-*sam*/*flg* or FGFR3/*sam3* gene. The 611bp products derived from K-*sam*/*bek* gene were divided into 382bp and 229bp fragments by *Bal*I, while the ones derived from N-*sam*/*flg* and FGFR3/*sam3* were divided into 408bp and 203bp fragments, 359bp, 200bp and 52bp fragments with *Ban*I, respectively. The PCR products and fragments digested by restriction enzymes were checked by 3.5% agarose gel elecrophoresis and ethidium bromide staining.

RESULTS

By RNA blot hybridization analysis, the K-*sam*/*bek*, N-*sam*/*flg* and FGFR3/*sam3* gene transcripts were detected in

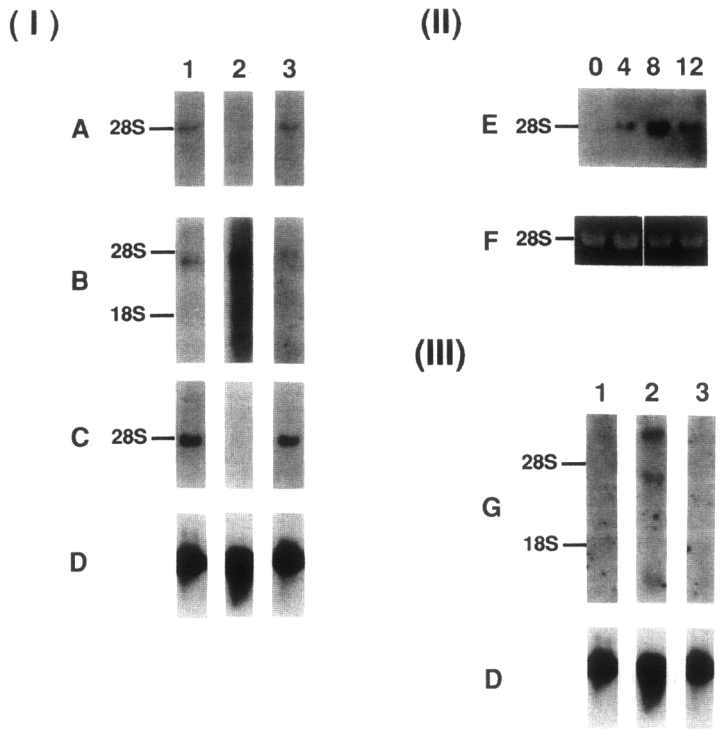

Figure 1. Transcripts of heparin-binding growth factor receptor genes or heparin-binding growth factor genes in human leukemia cells. **(I) A**, Two μg of poly(A)$^+$RNAs prepared from cell lines, CMK (lane 1), K562 (lane 2) and HEL (lane 3) were hybridized to K-*sam*-specific probe, RA0.7; **B**, N-*sam*-specific probe, ApAp0.5; **C**, mouse *sam3*-specific probe, P31d; and chicken ß-actin cDNA **D**. Transcripts of the N-*sam/flg* in the CMK cells treated with TPA for 0-12 hours of culture. **(II) E**, Twenty μg of total RNAs were hybridized to N-*sam*-specific probe, ApAp0.5. Lanes 0, 4, 8 and 12 indicate that RNAs were prepared from the cells cultured with $1x10^{-8}$M TPA for 0, 4, 8 and 12 hours, respectively. **F**, 28S Ribosomal RNA stained by ethidium bromide was shown in the bottom of each lane. **(III) G**, Two μg of poly(A)$^+$RNA from these cells were hybridized to bFGF cDNA.

poly(A)$^+$RNA of CMK cells. We used three probes which can specifically detect these three transcripts, respectively. Signals were observed at 4.5kb by the K-*sam*-specific probe, RA0.7, at 6.0 and 4.0kb by the N-*sam*-specific probe, ApAp0.5 and at 7.0 and 4.5kb by the FGFR3/*sam3*-specific probe, p31d, respectively (Fig.1-I). The amounts of the transcripts were highest for the FGFR3/*sam3* gene and lowest

for the N-*sam* gene in CMK cells. The transcripts of three genes were also detected in HEL, and the relative amounts of three transcripts in HEL cells were similar to those in CMK cells. In contrast, large amounts of N-*sam/flg* transcripts were detectable, but those of K-*sam/bek* or FGFR3/*sam3* were not detected in K562 cells.

The amounts of N-*sam/flg* transcripts in CMK cells increased after 4-12 hours of culture with $1x10^{-8}$M TPA. The maximum amount of transcripts was detected in the cells after 8 hours cultured with TPA (Fig.1-II). The amounts of K-*sam/bek* and FGFR3/*sam3* transcripts, however, did not increase with the TPA-treatment (data not shown).

We also examined for the presence of aFGF or bFGF mRNAs. Signals at 6.4, 3.7, 2.0 and 1.4kb of bFGF mRNA were detected only in K562 cells (Fig.1-III). Signals of aFGF mRNA, however, were not detected in any of the cells (data not shown). We examined the effects of bFGF on the incorporation of [^{3}H]thymidine into the CMK cells and on the colony formation of CMK cells in agar cultures. However, any effects of bFGF added exogenously on the CMK cells could not be detected.

We next studied whether or not the HBGF receptor genes' mRNAs are present in normal human platelets circulating in peripheral blood. RT-PCR was performed with total RNA extracted from platelets by the primers corresponding to the highly conserved region in tyrosine kinase domain of this gene family. To identify whether the RT-PCR products were derived from K-*sam/bek*, N-*sam/flg* or FGFR3/*sam3*, the products were digested with *Ban*I or *Bal*I (Fig.2-I, Fig.2-II). The 611bp PCR products which were the expected size corresponding to the fragment of three genes

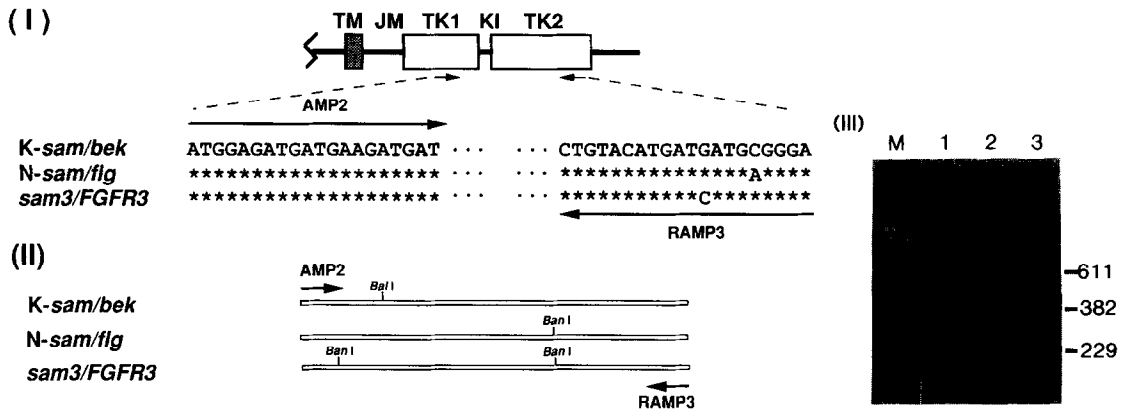

Figure 2. A schematic structure of the cytoplasmic region of HBGF receptor with the positions of the PCR primers and analysis on the presence of HBGF receptor mRNAs in human platelets by RT-PCR. The primer, AMP2, is located in tyrosine kinase domain 1 (TK1) and the antisense primer RAMP3 is tyrosine kinase domain 2 (TK2). **(I)** Nucleotide sequences of two primers are well conserved in three receptor genes. TM, JM and KI indicate transmembrane domain, juxtamembrane domain and kinase insert domain, respectively. **(II)** The 611bp-fragments of three HBGF receptor genes by PCR have different restriction enzyme recognition sites. **(III)** The presence of HBGF receptor mRNAs in RNAs from human platelets was analyzed by RT-PCR using primers AMP2 and RAMP3, followed by 3.5% agarose gel electrophoresis and ethidium bromide staining. Lane M, øX174/*Hae*III fragments as markers; Lane 1, PCR products undigested; Lane 2, PCR products digested with *Ban*I; Lane 3, PCR products digested with *Bal*I.

were detected by agarose gel electrophoresis and ethidium bromide staining (Fig.2-III). The PCR products were digested into the fragments of 382bp and 229bp with *Bal*I, but were not digested with *Ban*I. These data show that the PCR products were derived from the K-*sam/bek* mRNA, but not from the N-*sam/flg* or FGFR3/*sam3*.

DISCUSSION

We report here the presence of the K-*sam/bek*, N-*sam/flg* and FGFR3/*sam3* transcripts in human leukemia cells, CMK, K562 and HEL cells. The CMK cells have the markers characteristic for megakaryocytes, and the proportion of the cells containing megakaryocytic markers increases upon culture with TPA. The amounts of N-*sam/flg* transcripts in CMK cells increased after culture with TPA, indicating that

the N-*sam/flg* receptor increases on the CMK cells upon differentiating into megakaryocytic lineage. Significant homology was observed between N-*sam/flg* gene and chicken bFGF receptor gene, indicating that N-*sam/flg* is a human homologue of bFGF receptor gene.[12] We have previously reported that the *HST2/FGF6* transcripts were detected in CMK and HEL cells,[5] suggesting that HBGFs such as the *HST2/FGF6* protein in these two cell lines or bFGF in K562 cells may be involved in leukemogenesis or progression of human megakaryocytic leukemia in an autocrine or a paracrine manner. It should be noted that the *HST2/FGF6* gene whose deduced product contains a signal peptide has a transforming ability.[5] HBGF family consists of aFGF, bFGF, the *INT2*, *HST1*, *FGF5* and *HST2/FGF6* proteins and KGF.[1-7] HBGF receptor family, at present, consists of K-*sam/bek*, N-*sam/flg*, FGFR3/*sam3* and FGFR4 proteins. It is yet to be determined whether or not these growth factors have specific receptors or vice versa, and the biological significance of the presence of the three HBGF receptors in leukemia cells with megakaryocytic markers is not known.

A variety of growth factors, including colony-stimulating factors, stimulate hematopoiesis, and some factors induce committed progenitor cells into proliferation or differentiation. It has been reported that several growth factors such as GM-CSF, granulocyte colony-stimulating factor (G-CSF), IL-3 and IL-6 regulate megakaryocytopoiesis interacting synergistically or additively.[23] It has also been reported that bFGF acts on hematopoietic progenitors as a colony-stimulating factor.[15] However, any factor which acts specifically on the cells in megakaryocytic lineage has not yet been determined.

Heparin-like molecules are required for the binding of bFGF to its high affinity receptor,[24] and heparan sulphate is known to be one of the major components of extracellular matrix of bone marrow stromal cells.[25] In the microenvironment of bone marrow, bFGF or other HBGFs may be stored, being associated with the cell membranes of stromal cells, and may interact with its receptor of hematopoietic cells including progenitors committed to megakaryocytic lineage by direct cell-to-cell contact.

We also report here the detection of K-*sam/bek* transcripts in normal human circulating platelets. This result shows that HBGFs, especially ligands of the K-*sam/bek* protein, may be involved in the maturation of normal hematopoietic cells in megakaryocytic lineage or in the production of platelets. K-*sam/bek* was reported to be expressed in epithelial cells, and KGF, a growth factor specific for epithelial cells, was reproted to be a ligand for K-*sam/bek*.[26] However,the presented data indicated that target cells of KGF included megakaryocytes. bFGF works for wound healing or neovascularization on the damaged tissues[1], where circulating platelets aggregate in the damaged vessels not only for hemostasis but also for tissue repair by releasing such growth factors as platelet-derived growth factor (PDGF) or transforming growth factor-ß (TGF-ß).[27,28] The HBGFs may influence functions of platelet on damaged tissues or vessels through the K-*sam/bek* protein on platelets. The biological significance of the presence of K-*sam/bek* mRNAs in platelets has yet to be determined.

ACKNOWLEDGMENTS

We thank Drs. Teruhiko Yoshida and Yukishige Yamada, National Cancer Center Research Institute, for providing the PCR primers, and we also thank Dr. Koichi Igarashi,

Takeda Chemical Industries, Ltd., for the gift of the aFGF and bFGF cDNA probes. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare and from the Ministry of Education and by a Grant-in-Aid from the Ministry of Health and Welfare for a Comprehensive 10-year Strategy for Cancer Control, Japan.

REFERENCES

1. Burgess,W.H. & Maciag,T. (1989) Ann. Rev. Biochem. 58, 575-606.
2. Sakamoto,H., Mori,M., Taira,M., Yoshida,T., Matsukawa,S, Shimizu,K., Sekiguchi,M., Terada,M., Sugimura, T. (1986) Proc.Natl.Acad.Sci.USA. 83,3997-4001.
3. Yoshida,T., Miyagawa,K., Odagiri,H., Sakamoto,H., Little,P.F.R., Terada,M., & Sugimura,T. (1987) Proc. Natl.Acad.Sci.USA. 84,7305-7309.
4. Zhan,X.I., Bates,B., Hu,X. & Goldfarb,M. (1988) Mol. Cell. Biol. 8,3487-3495.
5. Iida,S., Yoshida,T., Naito,K., Sakamoto,H., Katoh,O., Hirohashi,S., Sato,T., Onda,M., Sugimura,T. & Terada,M. (Oncogene, in press)
6. Marics,I., Adelaide,J., Raybaud,F., Mattei,M., Coulier,F., Planche,J., Lapeyriere,O., Birnbaum,D. (1989) Oncogene 4,335-340.
7. Finch,P.W., Rubin,J.S., Miki,T., Ron,D. & Aaronson,S.A. (1989) Science 245,752-755.
8. Ruta,M., Howk,R., Ricca,G., Drohan,W., Zabelshansky,M., Laureys,G., Barton,D.E., Francke,U., Schlessinger,J. & Givol, D.(1988) Oncogene 3,9-15.
9. Kornbluth,S., Paulson,K.E., & Hanafusa,H.(1988) Mol. Cell. Biol. 8,5541-5544.
10. Hattori,Y., Odagiri,H., Nakatani,H., Miyagawa,K., Naito,K., Sakamoto,H., Katoh,O., Yoshida,T., Sugimura,T., & Terada,M.(1990) Proc.Natl.Acad.Sci.USA. 87,5983-5987.
11. Dionne,C.A., Crumley,G., Bellot,F., Kaplow,J.M., Searfoss,G., Ruta,M., Burgess, W.H., Jaye,M. & Schlessinger,J.(1990) EMBO J. 9,2685-2692.
12. Lee,P.L., Johnson,D.E., Cousens,L.S., Fried,V.A., & Williams,L.T.(1989) Science 245,57-60.
13. Keegen,K., Johnson,D.E., Williams,L.T. & Hayman,M.J., (1991) Proc.Natl.Acad.Sci.USA.88,1095-1099.
14. Paltanen,J., Makela,T.P., Eerola,E., Korhonen,J., Hirvonen,H., Claesson-Welsh,L. & Alitaro,K.(1991) EMBO J 10,1347-1354.
15. Gabbianelli,M., Sargiacomo,M., Pelosi,E., Testa,U., Isacchi,G. & Peschle,C.(1990) Science 249,1561-1564.
16. Sato,T., Fuse,A., Eguchi,M., Hayashi,Y., Ryo,R., Adachi,M., Kishimoto,Y., Teramura,M., Mizoguchi,H., Shima,Y., Komori,I., Sunami,S., Okimoto,Y. & Nakajima,H. (1989) Br.J.Haematol.72,184-190.
17. Colamonici,O.R., Trepel,J.B., Vidal,C.A. & Neckers,L.M. (1986) Mol.Cell.Biol.6,1847-1850.
18. Tabilio,A., Rosa,J.-P., Testa,U., Kieffer,N., Nurden,A.T., Del Canizo,M.C., Breston-Gorius,J. & Vainchenker,W.(1984) EMBO J.3,453-459.
19. Maniatis,T., Fritsch,E.F., Sambrook,J. (1989) In Molecular Cloning: A laboratory Manual, Cold Spring

Harbor Laboratory, Vol.I, pp.7.3-7.83. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
20. Kurosawa,T., Sasada,R., Iwane,M. & Igarashi,K. (1987) FEBS lett 213,189-194.
21. Katoh,O., Kimura,A. & Kuramoto,A. (1988) Am.J.Hematol. 27,276-280.
22. Saiki,R.K., Gelfand,D.H., Stofffel,S., Scarf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. & Erlich,H.A.(1988) Science 239,487-491.
23. Quesenberry,P.J., McGrath,H.E., Williams,M.E., Robinson,B.E., Deacon,D.H., Clark,S., Urdal,D. & McNiece,I.K.(1991) Exp.Hematol.19,35-41.
24. Yayon,A., Klagsbrun,M., Esko,J.D., Leder,P., & Ornitz,D.M.(1991) Cell 64,841-848.
25. Gordon,M.Y.(1991) Cancer Cells 3,127-133.
26. Miki,T., Fleming,T.P., Bottaro,D.P., Rubin, J.S., Ron,D. & Aaronson, S.A. (1991) Science 251,72-75.
27. Ross,R., Raines,E.W., Bowen-Pope,F.(1986) Cell 46,155-169.
28. Assoian,R.K., Komoriya,A., Meyers,C.A., Miller,D.M. & Sporn,M.B.(1983) J.Biol.Chem.258,7155-7160.