Volume 328, number 1,2, 17–20 FEBS 12789 August 1993

Spleen-derived growth factor, SDGF-3, is identified as keratinocyte growth factor (KGF)

Masashi Suzuki[a], Takehiro Itoh[a,b], Hiroyuki Osada[c], Jeffrey S. Rubin[d], Stuart A. Aaronson[d], Tohru Suzuki[e], Nobumitsu Koga[b,f], Takao Saito[f] and Youji Mitsui[a]

[a]*Cell Biology Laboratory, National Institute of Bioscience and Human-Technology, Higashi 1-1 Tsukuba Science City, Ibaraki 305, Japan,* [b]*Advanced Technology Laboratory, Mayekawa MFG. Co., Ltd, Moriya-machi, Ibaraki 302-01, Japan,* [c]*Antibiotics Laboratory, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 305-01, Japan,* [d]*Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD 20892, USA,* [e]*Department of Biotechnology, Faculty of Agriculture, Gifu University, Yanago, Gifu 501-11, Japan* and [f]*Material Chemistry Division, Government Industrial Research Institute, Nagoya, Hirate 1, kita, Nagoya 462, Japan*

Received 31 May 1993

A heparin-binding mitogen rat for rat hepatocytes was partially purified from bovine spleen by a combination of heparin-affinity, cation-exchange and gel-filtration chromatography Besides stimulating rat hepatocytes, this factor, which was designated spleen-derived growth factor-3 (SDGF-3), exhibited mitogenic activity for mouse epidermal keratinocytes but not mouse fibroblasts. Its apparent epithelial specificity and heparin-binding properties corresponded to those of keratinocyte growth factor (KGF). These findings, together with the fact that the mitogenic activity of SDGF-3 was abolished by a neutralizing monoclonal antibody specific for KGF, identify this bovine spleen-derived hepatocyte mitogen as KGF.

SDGF-3; Keratinocyte growth factor, Bovine spleen; Primary cultured hepatocyte, Heparin binding

1. INTRODUCTION

Control of growth in regenerating liver is one of the most attractive models for studying the mechanism of organ formation and repair [1,2]. Based on work with primary cultures of hepatocytes, EGF [3], TGF-α [4], FGF-1 [5,6]; T. Tanahashi et al., submitted) and HGF/SF [7–9] have been identified as growth factors for hepatocytes. In the course of exploring novel hepatocyte mitogens, we demonstrated that bovine spleen contained at least three heparin-binding proteins that stimulated hepatocyte proliferation, two of which were FGF-1 and FGF-2 and the third was a novel growth factor that (1) eluted from a heparin-Sepharose column at 0.75 M NaCl, (2) dit not react with anti-FGF-2 antibodies, and (3) had mitogenic activity which was additive with that of HGF [10,11]. We identify these growth factors as KGF, a member of the FGF family (FGF-7) with epithelial target-cell specificity.

Correspondence address: Y. Mitsui, National Institute of Bioscience and Human-Technology, Higashi 1-1 Tsukuba Science City, Ibaraki 305, Japan. Fax: (81) (298) 54 6009.

Abbreviations. KGF, keratinocyte growth factor (also designated FGF-7); SDGF-3, spleen derived growth factor-3; EGF, epidermal growth factor; TGF-a, transforming growth factor-a; FGF-1, fibroblast growth factor-1 (acidic FGF); FGF-2, fibroblast growth factor-2 (basic FGF); HGF/SF, hepatocyte growth factor/scatter factor; CHAPS, 3-[3-cholamidopropyl)dimethylammonio]1-propanesulfonate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant human KGF was expressed in *Escherichia coli* and purified as described elsewhere [12]. Mouse monoclonal antibody (designated 1G4) was obtained using recombinant KGF as antigen. It specifically neutralizes the mitogenic activity of KGF (J. Rubin, unpublished observations)

2.2. *Cell Culture*

Isolation and primary culture of rat hepatocytes were performed as previously described [11]. BALB/MK epidermal keratinocytes [13] were cultured in minimal essential medium (MEM) supplemented with 5 ng/ml of EGF (Collaborative Research Inc.) and 10% fetal bovine serum (FBS, Gibco BRL). BALB/3T3 cells were cultured in MEM supplemented with 10% FBS.

2.3. *Assay of DNA synthesis*

DNA synthesis of hepatocytes was determined as described previously [11]. DNA synthesis of BALB/MK cells was assayed with cultures that had been seeded at a density of 2×10^5 cells/0.4 ml/well in 48-well plates (Sumitomo Bakelite Inc., Tokyo) precoated with fibronectin (Collaborative Research Inc.). At confluence, the medium was changed to serum-free MEM supplemented with 30 nM Na_2SeO_3 and 5 μg/ml transferrin. EGF or test samples were added to the culture 2 days after the switch to serum-free medium. Incorporation of [^{3}H]thymidine (ICN, 74 kBq/ml, 2.1 TBq/mmol) was monitored for 6 h beginning 17 h after addition of samples. The cells were washed with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS(−)) once and with 5% trichloroacetic acid twice. They were solubilized with 0.25 M NaOH and the radioactivity was counted with a liquid scintillation counter

(Beckman). DNA synthesis of BALB/3T3 cells was determined as described for BALB/MK cells except that cells were seeded in a fibronectin-coated 96-well microtiter plate (Sumitomo Bakelite Inc., Tokyo) at a density of 10^5 cells/0 2 ml/well

2 4. *Partial purification of SDGF-3*

All procedures were performed at 4°C or on ice. Preparation of a soluble matrix fraction from bovine spleen was performed as described previously [11] After this step, all buffers contained 0.01% CHAPS to avoid the non-specific absorption to resin or tubes. The soluble matrix fraction was applied on a heparin-Sepharose column (5 × 15 cm) (Pharmacia) that had been equilibrated with PBS(−). After washing with PBS(−), bound proteins were eluted with a linear gradient of NaCl in PBS(−) Hepatocyte mitogenic activity was resolved into three peaks by this step: Peak 1, Peak 2, and Peak 3 eluted at 1.8 M NaCl (PBS–1.65 M NaCl), 1.35M NaCl (PBS–1.2 M NaCl), and 0 75M NaCl (PBS–0.6 M NaCl), respectively [11]. Peak 3 was dialyzed against PBS(−) and applied on an S-Sepharose column (1×10 cm) (Pharmacia) equilibrated with PBS(−). After washing the column, activity was eluted with a linear gradient of NaCl in PBS(−). Fractions of 2 4 ml were collected. Most of the activity was eluted at 0 85 M NaCl (PBS–0.7 M NaCl). This major peak was then loaded on a Superdex 75 gel filtration column (2.6 × 60 cm) (Pharmacia), equilibrated with 0 15 M NaCl in PBS(−), and eluted with the same buffer Fractions of 5ml were collected Active fractions were pooled and concentrated by ultrafiltration (Centricon-3, molecular mass, 3,000 Da cutoff, Amicon).

3. RESULTS

3.1. *Partial purification of SDGF-3*

Hepatocyte growth-promoting activity in the soluble matrix fraction from bovine spleen was separated into three fractions by heparin-Sepharose column chromatography [11]. Among them, Peak 1 and Peak 2 were identified as FGF-2 and FGF-1 or closely related molecules, respectively [11]. Peak 3, an unidentified spleen-derived growth factor for hepatocytes, eluted at 0.75 M NaCl. Its activity was further purified by sequential column chromatography using S-Sepharose and Superdex 75. Most of the activity was recovered from the S-Sepharose resin at 0.85 M NaCl, while a minor peak eluted at 0.5 M NaCl (Fig. 1). The major peak was then

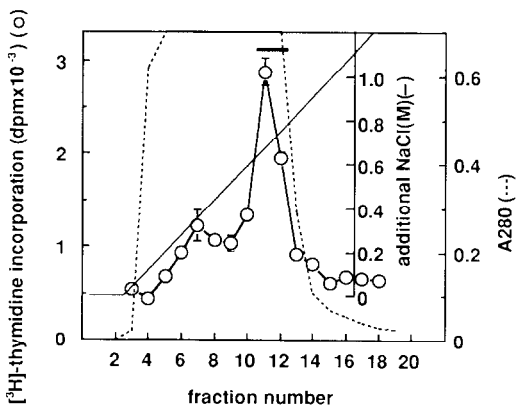

Fig. 1 S-Sepharose column chromatography of a hepatocyte mitogen. Chromatography was performed as described in section 2 Open circles: DNA synthesis. Values are means ± S.E. (number of wells = 3). The horizontal bars indicate the pooled fractions.

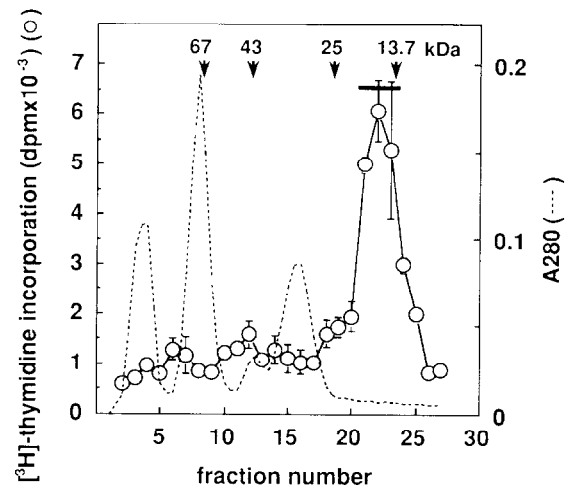

Fig. 2 Superdex 75 gel filtration chromatography of the active fraction of the S-Sepharose column chromatography Chromatography was performed as described in section 2. Open circles: DNA synthesis Values are means ± S E. (number of wells = 3). The elution position of molecular mass markers were indicated by the arrows with mass numbers The horizontal bars indicate the pooled fractions

applied onto a Superdex 75 gel filtration column. Hepatocyte mitogenic activity was separated from most of the protein and eluted at a position corresponding to 17 kDa (Fig. 2). When the active fractions were analyzed by SDS-PAGE, mutliple bands were detected. However, only a 17-kDa silver-stained band corresponded in intensity to the observed activity (data not shown). This active peak containing SDGF-3 was concentrated by ultrafiltration for further analysis.

3.2. *Target cell specificity of SDGF-3*

The conditions for elution of SDGF-3 from heparin-Sepharose suggested its possible relationship to keratinocyte growth factor (KGF), a member of the FGF family (FGF-7) with epithelial target-cell specificity [14,15]. To determine whether SDGF-3 had a similar epithelial specificity, we examined its growth promoting activity on BALB/MK cells (mouse epithelial cell line) and BALB/3T3 cells (a mouse fibroblast line). EGF, a known mitogen for both BALB/MK and BALB/3T3 cells, was used as a positive control. As shown in Fig. 3A,B, SDGF-3 stimulates DNA synthesis of BALB/MK cells more potently than EGF. However, it did not stimulate DNA synthesis of BALB/3T3 cells (Fig. 3C,D).

3.3. *Effects of SDGF-3 or recombinant KGF on DNA synthesis of hepatocytes in the presence or absence of anti-KGF neutralizing antibody*

Because the heparin elution and target-cell properties of SDGF-3 matched those of KGF, we tested the ability of recombinant KGF to stimulate DNA synthesis in rat hepatocytes, an epithelial cell type on which its activity had not been analyzed. As shown in Fig. 4A, KGF

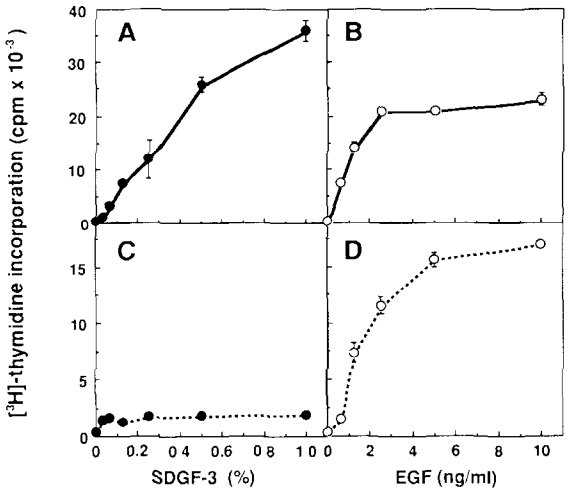

Fig 3 Target cells specificity of SDGF-3. Activities of SDGF-3 or EGF for the growth of BALB/MK cells and BALB/3T3 cells were assayed as described in section 2. Closed circles, SDGF-3 (A,C); open circles, EGF (B,D); solid line, BALB/MK cells (A,B); broken line; BALB/3T3 cells (C,D). Concentrations of SDGF-3 were expressed as percent of medium volume. Values are means ± S.E. (number of wells = 2).

stimulated DNA synthesis in rat hepatocytes and the activity was abolished by an anti-KGF monoclonal antibody specific for KGF. When the same KGF-neutralizing antibody was tested against SDGF-3 in the rat hepatocyte bioassay, a similar pattern of inhibition was observed (Fig. 4B).

4. DISCUSSION

We previously reported that the bovine spleen contained at least three heparin-binding hepatocyte growth factors. Two major factors were FGF-1 and FGF-2 or closely related molecules. The present study demonstrates that the third factor, SDGF-3, is KGF based on their indistinguishable heparin elution profiles, target-cell specificity and immuno-crossreactivity with a specific KGF-neutralizing monoclonal antibody.

KGF was first isolated from conditioned medium of a human embryonic lung fibroblast cell line as a heparin-binding growth factor for BALB/MK cells. It does not stimulate the growth of fibroblasts or endothelial cells suggesting that mitogenic activity of KGF is specific for epithelial cells [14]. An analysis of cloned cDNA revealed that kGF is a 194 amino acid polypeptide with calculated mass of 22,512 Da and structurally related to the fibroblast growth factors [15]. Purified native KGF lacked the amino-terminal 31 amino acids, which included a putative signal peptide sequence. The calculated mass of native KGF, 18,328 Da differs from the observed by SDS-PAGE (26–28 kDa), presumably due to N-linked glycosylation at the putative site of Asp_{45}-Cys-Ser [15]. In the present study, the mass of SDGF-3 was judged to be about 17 kDa by both gel filtration and SDS-PAGE. The difference in size between SDGF-3 and the previously characterized native human KGF may be due to the absence of glycosylation and/or N-terminal truncation is SDGF-3. A mitogenically active, N-terminally truncated ~ kDa KGF molecule also has been isolated from embryonic lung fibroblast conditioned medium (J. Rubin, unpublished observations). Similarly, recombinant expression of N-terminally truncated KGF molecules produced in *Escherichia coli* retained mitogenic activity for BALB/MK cells [12]. For the definitive explanation of this difference in molecular mass, amino acid sequence analysis of SDGF-3 will be required.

The ability of KGF to stimulate DNA synthesis in rat hepatocytes broadens the array of epithelial targets for this growth factor. Other epithelial cells responsive to KGF include: keratinocytes, mammary and bronchial epithelial cells [14]; type II pneumocytes [16]; prostatic epithelial cells ([17] and manuscript in preparation). There also is indirect evidence that this growth factor stimulates the proliferation and/or differentiation of other epithelial cells in the male and female reproductive tracts (manuscripts submitted or in preparation). In all of these contexts, the data is consistent with a paracrine mode of action. By contrast, a recent study indicated undetectable levels of KGF transcripts in normal liver or after partial hepateclomy [18]. Thus, our present demonstration of KGF mitogenic activity for hepatocytes and the recovery of KGF form bovine spleen raise the possibility that KGF might have an endocrine mode of action, reaching the liver via the portal circulation. In this regard, it might resemble HGF/SF, another

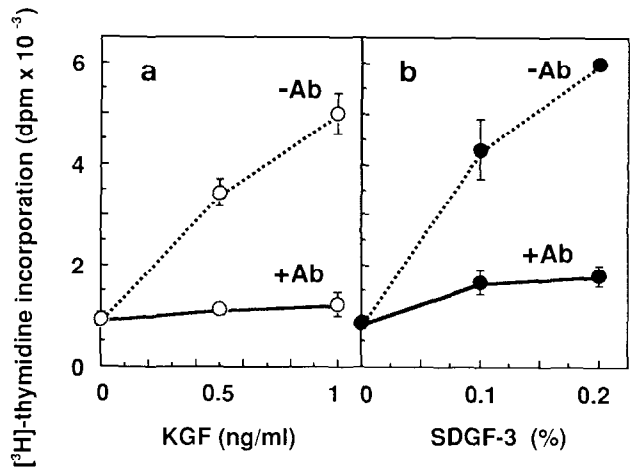

Fig. 4. Effects of SDGF-3 or recombinant KGF on DNA synthesis of hepatocytes in the presence or absence of anti-KGF neutralizing antibody. Each amount of SDGF-3 or recombinant KGF were incubated with or without 2.5 μg of anti-KGF monoclonal antibody (designated as 1G4) in a total volume of 12.5 μl with PBS(−) containing 10 mg/ml BSA at room temperature for 2 h. Then, they were added to cultures. Open circles, recombinant KGF (A); closed circles, SDGF-3 (B); solid line, with anti-KGF antibody; broken line, without antibody. Values are means ± S.E. (number of wells = 3).

mesenchymally-derived hepatocyte mitogen which is thought to function as a paracrine factor in some settings, including liver regeneration (for reviews see [1,19]). However, HGF/SD also appears to be recruited from distant sites such as the spleen and lung following liver injury [20]. Therefore, it will be of interest to determine whether KGF expression increases in spleen after liver injury and whether its secretion into the bloodstream rises accordingly.

S-Sepharose column chromatography revealed a minor hepatocyte mitogenic activity in addition to SDGF-3 (KGF) (Fig. 1). This minor activity was eluted at 0.75 M NaCl from a heparin-Sepharose column, and at 0.35 M from an S-Sepharose column. Thus, there remains a possibility that this activity may reflect a novel heparin-binding hepatocyte growth factor.

Acknowledgements: We thank Drs Sunil C Kaul and Renu Wadhwa and Mr. Naoto Okamoto (National Institute of Bioscience and Human-Technology) for their help This work was supported by the grant project of R & D for the Elucidation of Biological Function of AIST (Agency of Industrial Science & Technology).

REFERENCES

[1] Michalopoulos, G.K (1990) FASEB J. 4, 176–187.
[2] Bucher, N.L R (1991) J Gastroenterol. Hepatol. 6, 615–624.
[3] Richman, R.A., Claus, T.H , Pilkis, S J. and Friedman, D.L. (1976) Proc. Natl. Acad. Sci. USA 73, 3589–3593
[4] Mead, J.E. and Fausto, N (1989) Proc. Natl. Acad. Sci. USA 86, 1558–1562.
[5] Kan, M., Huan, J., Mansson, P., Yasumitsu, H., Carr, B and McKeehan, W (1989) Proc. Natl Acad. Sci. USA 86, 7432–7436.
[6] Houck, A K., Zarnegar, R., Muga, S J and Michalopoulos, G.K. (1990) J. Cell. Physiol 143, 129–132
[7] Nakamura, T , Nawa, K , Ichihara, A., Kaise, N. and Nishino, T. (1987) FEBS Lett 224, 311–316.
[8] Gohda, E , Tsubouchi, H., Nakayama, H., Hirono, S., Sakiyama, O , Takahashi, K., Miyazaki, H., Hashimoto, S and Daikuhara, Y. (1988) J. Clin Invest. 81, 414–419.
[9] Zarnegar, R. and Michalopoulos, G (1989) Cancer Res 49, 3314–3320.
[10] Suzuki, T., Koga, N., Imamura, T. and Mitsui, Y. (1988) Biochem Biophys. Res. Commun. 153, 1123–1128
[11] Suzuki, M., Itoh, T., Suzuki, T., Koga, N., Kato, K., Saito, T. and Youji, M. (1992) Biochem. Biophys. Res. Commun. 186, 1192–1200.
[12] Ron, D., Bottaro, D P., Finch, P.W., Morris, D., Rubin, J.S and Aaronson, S.A. (1993) J. Biol Chem. 268, 2984–2988
[13] Weissman, B E and Aaronson, S.A (1983) Cell 32, 599–606.
[14] Rubin, J.S., Osada, H , Finch, P.W., Taylor, W G., Rudikoff, S. and Aaronson, S A. (1989) Proc. Natl. Acad. Sci. USA 86, 802–806.
[15] Finch, P.W., Rubin, J.S., Miki, T., Ton, D. and Aaronson, S.A. (1989) Science 245, 752–755.
[16] Panos, R.J., Rubin, J.S., Aaronson, S.A and Mason, R.J. (1993) J. Clin. Invest , in press
[17] Yan, G., Fukabori, Y , Nikolaropoulos, S., Wang, F. and McKeehan, W L (1992) Mol. Endocrinol. 6, 2123–2128.
[18] Kan, M., Yan, G.-C , Xu, J. Nakahata, M and Hou, J (1992) In Vitro Cell. Dev. Biol 28A, 515–520.
[19] Matsumoto, K. and Nakamura, T (1992) Critical Rev. Oncogenesis 3, 27–54.
[20] Yanagita, K., Nagaike, M , Ishibashi, H., Niho, Y , Matsumoto, K. and Nakamura, T (1992) Biochem. Biophys. Res. Commun 182, 802–809.