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PURIFICATION OF ACIDIC FIBROBLAST GROWTH FACTOR FROM BOVINE OMENTUM

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SUMMARY: Two heparin binding growth factors with different molecular weight, 16.6 kD and 18.6 kD polypeptide,were purified from bovine omentum. The two factors have almost the same affinity to heparin; they were eluted with 1.0 M NaCl from the affinity column. The 16.6 kD polypeptide was found to be acidic fibroblast growth factor by amino acid sequence analysis. The 18.6 kD polypeptide was an N-terminus blocked polypeptide and was suggested to be β-endothelial cell growth factor. These molecular species may play significant roles in maintaining vascularized structure in omentum and be related to the angiogenic activity of the tissue.

Omentum is a highly vascularized adipose tissue and is known to have potent angiogenic activity. For example, omental tissue graft has been successfully utilized to revascuralize ischemic brain (1) or myocardium (2). Goldsmith et al. have reported that gangliosides in chloroform/methanol extract are the major angiogenic principle in omentum (3). However, we found considerable angiogenic activity also in proteinaceous fraction in our preliminary chick chorioallantoic membrane assay. This observation prompted us to identify FGF in omentum and its purification since FGF is known to have potent angiogenic activity (4,5).

Abbreviations FGF;fibroblast growth factor, ECGF;endothelial cell growth factor, CHAPS;3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, PMSF;phenylmethylsulfonyl fluoride, TFA;trifluoroacetic acid, CS;calf serum, FCS;foetal calf serum, MEM; minimum essential medium, BSA;bovine serum albumin, PBS;phosphate buffered saline.

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Acidic FGF was originally purified from brain (6) and retina (7). However purification of acidic FGF from non-neural tissues has been not successful, whereas basic FGF has been purified from various tissues (8-10). This led to misunderstanding that distribution of acidic FGF is restricted to neural tissues. But now there are accumulating evidences that show distribution of acidic FGF in several non-neural tissues although in less amount compared to basic FGF (11-15). Here we demonstarate that omentum has acidic FGF as a dominant mitogenic activity for fibroblasts.

Methods

Omentum Extraction: All the procedure was carried out at 4 ^{o}C. Forty five kg of bovine omentum was minced and stored at -80 ^{o}C until use. Each 15 kg of the frozen omentum was homogenized with a Waring blender (5000 rpm, 2 min, twice) in 30 l of PBS supplemented with 0.1 mM PMSF, 0.1 μg/ml pepstatin, 10 μg/ml leupeptin, 0.02% NaN_3 and 0.1 mM EDTA. The homogenate was filtered through cheese cloth and treated with 750 g of Cell Debris Remover (CDR, Whatman) for five minutes. Clear extract was obtained after removal of CDR by filtration. The CDR was washed with 2 l of PBS with additional 0.5 M NaCl and the resultant filtrate was combined with the extract. Total protein in the extract was precipitated with 80% saturation of ammonium sulfate. The precipitated protein was stored at -80 ^{o}C until further purification.

Ion Exchange Chromatography: The stored protein precipitate from 45 kg of omentum was dissolved in 3 l of PBS containing protease inhibitors and dialyzed first against 40 l of the same buffer and twice against 50 mM NaCl, 20 mM bistrispropane buffer (pH 7.0) containing protease inhibitors. The dialyzate was centrifuged and the supernatant was loaded onto a Q-Sepharose column (5 x 30 cm, Pharmacia) equilibrated with 50 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3, 20 mM bistrispropane (pH 7.0). The column was eluted subsequently with 150 mM, 400 mM, and 1 M NaCl at a flow rate of 8 ml/min. The flow through fractions were then applied to an S-Sepharose column (2.6 x 40 cm, Pharmacia) equilibrated with 20 mM phosphate buffer (pH 7.0) containing 0.1% CHAPS and protease inhibitors. The column was eluted stepwise with 200 mM, 500 mM, 1 M NaCl at a flow rate of 4 ml/min .

Heparin Affinity Chromatography: The 0.5 M NaCl eluate from the S-Sepharose column was directly applied to a Heparin-5PW HPLC column (0.75 x 7.5 cm, Toso, Tokyo, Japan) equilibrated with 0.5 M NaCl, 0.1 mM EDTA, 0.02% NaN_3, 0.1% CHAPS, 20 mM bistrispropane (pH 7.0). The column was eluted stepwise with 0.6 M, 0.8 M, 1.0 M, 1.2 M, and 2.0 M NaCl at a flow rate of 0.5 ml/min. Rechromatography was performed by linear gradient elution from 0.8 M to 1.4 M NaCl over 60 min.

Reversed phase HPLC: Pooled fractions of the heparin affinity chromatography were diluted with the same volume of 0.2 N acetic acid and injected to a C_4 reversed phase HPLC column (0.46 x 15 cm, 5 μm particle, 300 Å pore size, YMC, Kyoto, Japan) equilibrated with 20% acetonitrile/0.1% TFA. Elution was carried out

with a linear gradient from 20 to 50% acetonitrile/ 0.1% TFA over 50 min at a flow rate of 1 ml/min at 24 ^{o}C. Aliquots of each fraction were immediately diluted to 50 folds with 0.4% CS, 0.1% BSA/MEM and used for assays.

Assay for Mitogenic Activity: Balb c/3T3 cells were plated on 96 well microplate (Corning) at a cell density of 5,000 cells per well in 10% FCS/MEM. The media was replaced with 0.4% CS, 0.1% BSA/MEM on the next day and the cells were starved for 2 days. The starved cells were challenged with samples and incubated for 18 hours. Forty nCi of [methyl-^{3}H]-thymidine (6.7 Ci/mmol, NEN) was added to each well. After 4 h incubation, the cells were harvested onto a glassfiber filter (Whatman GF/B) using a cell harvester (Brandel) and free thymidine was washed out. After drying, the glassfiber filters were dipped into toluene-based scintilator and incorporated thymidine were counted in a liquid scintilation counter (Beckman).

Miscellaneous: Protein concentration was determined by Coomassie Protein Assay Reagent (Pierce) using BSA as a standard. SDS polyacrylamide slab gel electrophoresis was carried out in a Phast-system (Pharmacia). Sequence analysis was performed in ABI model 477A sequencer by standard procedures.

Results and Discussion

Omentum extract prepared by our procedures exhibited fairly high specific activity (Table 1) in DNA synthesis assay using Balb c/3T3 cells. This extract was first applied to a Q-Sepharose column. As shown in Table 1, most of the activity was not retained on the column. The flow through fractions were then chromatographed on an S-Sepharose column by stepwise elution. Major activity was recovered in 0.5 M NaCl eluate. This eluate was subjected to Heparin-5PW affinity HPLC. Elution profile (Fig. 1A) shows several peaks of the activity, the major peak eluted at 1.0 M NaCl and minor peaks at 1.2 M, 2.0 M NaCl. It is similar to a typical elution profile of brain FGFs (6), suggest-

Table 1

	Protein Recovery (mg)	ED_{50} (μg/ml)	Total Activity (Units)	Specific Activity (Units/mg)	Activity Recovery (%)
Extract	24800	1	24.8×10^{6}	1×10^{3}	100
Q-Sph FT	10800	0.5	21.6×10^{6}	2×10^{3}	87
S-Sph 0.5 M	230	2.5×10^{-2}	9.2×10^{6}	4×10^{4}	37
1st Hep-5PW 1.0 M	0.92	2.5×10^{-4}	3.7×10^{6}	4×10^{6}	15
2nd Hep-5PW 18.6 kD	0.075	1.1×10^{-4}	6.8×10^{5}	9×10^{6}	2.7
16.6 kD	0.089	6.3×10^{-5}	1.4×10^{6}	16×10^{6}	5.7

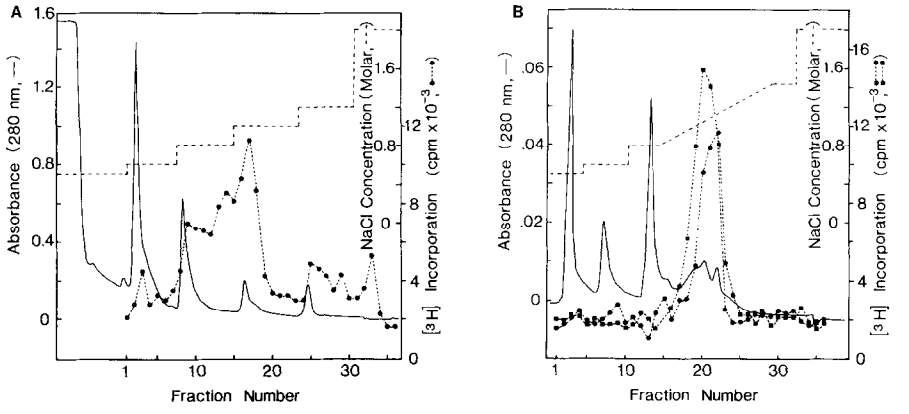

Fig. 1 (A)Elution Profile of the 1st Heparin-5PW Chromatography. The pooled fractions of the S-Sepharose chromatography were applied to the column. (B)Elution Profile of the 2nd Heparin-5PW Chromatography. The pooled fractions (no. 12 to 20) of the 1st Heparin-5PW chromatography were applied to the column. Absorbance at 280 nm; ——— , incorporation of tritiated thymidine; ●---● , NaCl concentration; - - - .

ing that the major mitogenic activity is aidic FGF or related polypeptide and that the activity at 2.0 M NaCl is basic FGF. Identification of basic FGF in the 2.0 M eluate was confirmed by neutralization of the mitogenic activity by anti-basic FGF antibody (a generous gift from Dr. Kato, Takeda Chemical Industries. Ltd. (16)).

However, such an elution pattern is rather surprising because acidic FGF has rarely been isolated from non-neural tissues. To determine the chemical structure, putative acidic FGF was further purified. As shown in Fig. 1B, rechromatography of the 1.0 M NaCl eluate (fraction no.12 through no.20) gave a rather broad peak of the activity over two UV peaks. However assays with different dilutions indicated that the peak is composed of two peaks of the activity, which correspond to two UV peaks. The first peak fractions (no.19 and 20) gave a 18.6 kD major band in SDS polyacrylamide gel electrophoresis and the second peak fractions (no.21 and 22) gave a 16.6 kD major band (Fig. 2). Both combined fractions have fully strong activity (Table 1). Half of the each pool was applied to a C_4 reversed phase HPLC column.

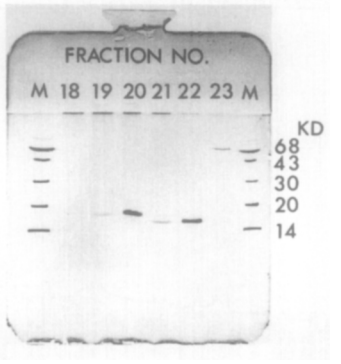

Fig.2 SDS polyacrylamide gel electrophoresis. Fractions of the 2nd Heparin-5PW chromatography were analyzed on a 20 % polyacrylamide ready-made gel in Phast-system. The gel was stained by silver stain method.

SharpUV peaks with the mitogenic activity were obtained in each run. Elution profile obtained for the 16.6 kD polypeptide fractions was shown in Fig. 3. The peak designated as A is 16.6 kD polypeptide peak and the peak B has the same retention time as 18.6 kD polypeptide peak obtained in another run. Bigger peaks at earlier retension time were derived from buffer ingredients. The whole amount of each peak fraction was subjected to ABI gas-phase protein sequencer. Sequence of twenty N-terminal amino acids of the 16.6 kD polypeptide was Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys-Lys-Pro-Lys-Leu-Leu-Tyr-X-Ser-Asn-Gly-Gly. The obtained sequence coincided with that of acidic FGF isolated from brain (17). Thus, affinity to heparin, molecular weight, and N-terminal amino acid sequence indicated that the 16.6 kD mitogen is acidic FGF. Analysis of the 18.6 kD polypeptide did not give any amino acid peaks, however, suggesting it is an N-terminus blocked polypeptide. The 18.6 kD growth factor seemed to be β-ECGF which is N-terminus blocked, 14 amino acid residues elongated form of acidic FGF (18).

In this study, it is revealed that omentum has potent mitogenic activity which was ascribed to acidic FGF and its related growth factor. Basic FGF was also found in omentum extract but

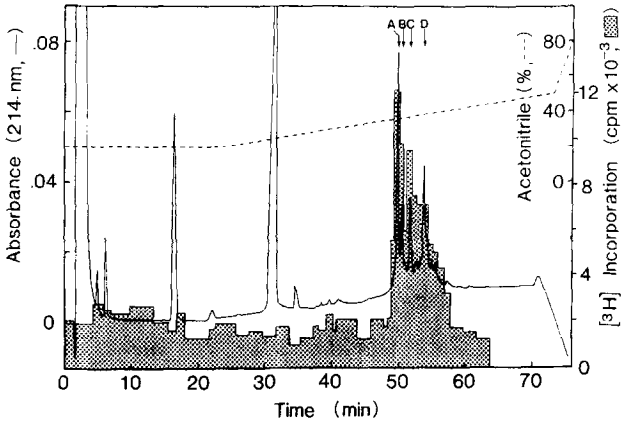

Fig.3 Reversed Phase HPLC. The pooled fractions (no. 21 and 22) of the 2nd Heparin-5PW chromatography were subjected to C_4 reversed phase HPLC. Absorbance at 214 nm;——— , acetonitrile concentration; - - - , incorporation of tritiated thymidine; ▨ .

in less amount. This proportion is similar to that in brain. Although acidic FGF has been found in other non-neural tissues such as kidney (11), bone (12), smooth muscle (13), heart (14) or vascular extracellular matrix (15), its content was lower than that of basic FGF. The acid treatment usually employed for purification of FGFs was omitted in the present purification, however, inclusion of the treatment (in another set of experiment) did not enhance total mitogenic activity (data not shown). Thus, the lower content of basic FGF in omentum is not due to the difference in the method to prepare the extract. This high relative content of acidic FGF as seen in brain may serve to maintain highly vascularized structure of omentum and be related to strong angiogenic activity of the tissue.

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