

Adiponectin does not bind to gelatin: a new and easy way to purify high-molecular-weight adiponectin from human plasma

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Abstract Human plasma contains three forms of adiponectin, a trimer, a hexamer, and a high-molecular-weight (HMW) multimer. We previously reported HMW adiponectin was a gelatin-binding protein of 28 kDa (GBP28), it having been purified due to its affinity to gelatin-Cellulofine (Nakano, Y., et al. Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. *J. Biochem.* 1996. 120: 803–12). Although HMW adiponectin binds to gelatin-Cellulofine, it cannot bind to gelatin-Sepharose. Gelatin-Cellulofine was made of formyl-Cellulofine and gelatin, and we found that HMW adiponectin binds to reduced formyl-Cellulofine with similar affinity as to gelatin-Cellulofine. **■** Through only two steps using reduced formyl-Cellulofine and DEAE-Sepharose, HMW adiponectin can be effectively purified from human plasma.—Nakano, Y., A. Shoji, A. Arakawa, Y. Iizuka, Y. Kikuchi, M. Kobayashi, and T. Tobe. Adiponectin does not bind to gelatin: a new and easy way to purify high-molecular-weight adiponectin from human plasma. *J. Lipid Res.* 2010. 51: 210–215.

Supplementary key words reduced formyl-Cellulofine • gelatin-Cellulofine • osteoclastogenesis

Adiponectin is secreted into the plasma from adipocytes and was found to circulate as a trimer, a hexamer, and a high-molecular-weight (HMW) multimer (1–3). We previously purified HMW adiponectin of 420 kDa from human plasma using gelatin-Cellulofine and reported it was a gelatin-binding protein of 28 kDa, GBP28, in 1996 (4). Adiponectin was also reported to bind to collagen I, III, and V *in vitro* and was detected on the walls of catheter-injured vessels (5). Recently, the sale of gelatin-Cellulofine was discontinued and so we tried gelatin-Sepharose. Unexpectedly, HMW adiponectin did not bind to gelatin-Sepharose. Because gelatin-Cellulofine was made of formyl-Cellulofine and gelatin, we made gelatin-Cellulofine from formyl-Cellulofine and gelatin and HMW adiponectin bound to

it. At the same time, we prepared a control resin with formyl-Cellulofine without gelatin and, surprisingly, HMW adiponectin bound to this reduced formyl-Cellulofine. Using the reduced formyl-Cellulofine, we established a new, easy, and effective purification method for human HMW adiponectin.

MATERIALS AND METHODS

Materials

Formyl-Cellulofine was purchased from Seikagaku Kogyo (Tokyo, Japan). DEAE-Sepharose, gelatin-Sepharose, N-hydroxysuccinimide (NHS)-activated Sepharose and EAH-Sepharose were from GE Healthcare (Buckinghamshire, UK), and the HiLoad 16/60 Superdex 200 prep-grade and Superdex 200 HR 10/30 columns and HMW gel filtration Calibration Kit were from Amersham Biosciences (Uppsala, Sweden). Hyaluronic acid sodium salt from streptococcus equi, naphthol AS-MX phosphate, Fast red violet LB salt, and BSA were from Sigma-Aldrich (Gillingham, UK). Precision Plus Protein Standards Dual Color was from Bio-Rad (Hercules, CA). Immobilon Western HRP Substrate was from Millipore (Bedford, MA). Gelatin and other chemicals were from Wako Pure Chemical (Osaka, Japan). A sandwich ELISA specific for HMW adiponectin was performed as described before using the mouse monoclonal anti-human HMW adiponectin antibody, IH7 (6). Rabbit anti-serum against the C-terminal 20 amino acids of adiponectin, anti-C, was described previously (4). Human plasma was kindly provided by the Japan Red Cross.

Preparation of reduced formyl-Cellulofine and other resins

Formyl-Cellulofine in 0.2 M NaHCO₃, pH8.5, was treated with NaBH₄ 7 mg/g wet gel at 4°C overnight according to the manufacturer's instructions. To obtain gelatin-Cellulofine, formyl-Cellulofine was incubated with gelatin and then reduced with NaBH₄ as described above. NHS-activated Sepharose was hydrolyzed in

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0.2 M NaHCO₃, pH8.5, at 4°C for 72 h. Hyaluronan-Sepharose was prepared as described before (7). Briefly, EAH-Sepharose (1 ml), hyaluronic acid (9 mg), and N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (75 mg) were mixed in 3 ml distilled water and then the pH was adjusted to 4.5 with 0.1 N HCl. The mixture was rotated at room temperature overnight.

Purification of HMW adiponectin

NaCl and PMSF were added to human plasma to final concentrations of 0.1 M and 0.1 mM, respectively, and then applied to reduced formyl-Cellulofine equilibrated with 10 mM Tris-HCl, pH7.4, and 0.1 M NaCl. After thorough washing, HMW adiponectin was eluted with a NaCl gradient from 0.1 to 2.0 M. The HMW adiponectin fractions were pooled and equilibrated against 10 mM Tris-HCl, pH8.0, 0.3 M NaCl, and 1 mM CaCl₂ by dialysis. Then, the HMW adiponectin pool was applied to DEAE-Sepharose equilibrated with 10 mM Tris-HCl, pH8.0, and 0.3 M NaCl, and after thorough washing with the same buffer, HMW adiponectin was eluted with a NaCl gradient from 0.3 to 2.0 M NaCl. If needed for further purification, HMW adiponectin fractions were pooled and applied to a HiLoad 16/60 Superdex 200 prep-grade column after concentration and equilibration against 10 mM Tris-HCl, pH7.4, and 0.15 M NaCl. To determine its purity and size, an aliquot of the HMW adiponectin pool was applied to a Superdex 200 HR 10/30 column.

Osteoclast formation assay

RAW cells were kept in serum-free medium, CHO-S-SFM II containing 1% penicillin/streptomycin, and for subculturing, 0.25% (w/v) Trypsin-0.53 mM EDTA was used. For osteoclast formation assay, RAW cells were plated at a density of 2.5×10^3 cells/well in 96-well plate in α -MEM containing 10% FBS and 1% penicillin/streptomycin, and cultured for 2 days. Then, medium was changed to fresh medium containing RANKL (70 ng/ml) with or without HMW adiponectin (2.5 or 5.0 μ g/ml). After 2 days, the medium was changed to fresh medium containing RANKL (70 ng/ml) and the next day, tartrate-resistant acid phosphatase (TRAP) staining was performed.

TRAP staining

After cells were fixed with 1% formaldehyde for 5 min and treated with acetone-ethanol (1:1) for 30 s, TRAP staining was performed at pH 5.0 in the presence of L(+)-tartaric acid using naphthol AS-MX phosphate in N, N-dimethyl formamide and Fast red violet LB salt.

General procedures

SDS-PAGE was performed by the method of Laemmli, followed by Coomassie staining or Western blotting. After treatment with anti-C and peroxidase-conjugated second antibody of a nitrocellulose membrane, bands were detected with Immobilon Western HRP Substrate according to the manufacturer's instructions. For protein size analysis, a prestained SDS-PAGE standard, Precision Plus Protein Standards Dual Color, was used.

RESULTS

HMW adiponectin binds to reduced formyl-Cellulofine

Recently, the sale of gelatin-Cellulofine was discontinued and so we purchased gelatin-Sepharose. Unexpectedly, HMW adiponectin did not bind to gelatin-Sepharose (Fig. 1B). Because gelatin-Cellulofine was made of formyl-Cellulofine and gelatin, we made gelatin-Cellulofine from

formyl-Cellulofine and gelatin and HMW adiponectin bound to it (Fig. 1C). At the same time, we prepared a control resin with formyl-Cellulofine without gelatin and, surprisingly, HMW adiponectin bound to this reduced formyl-Cellulofine (Fig. 1D). Next, we tried hydrolyzed NHS-activated Sepharose, which has a similar structure to reduced formyl-Cellulofine except that the resin is made of agarose. HMW adiponectin did not bind to the hydrolyzed NHS-activated Sepharose and the binding of HMW adiponectin to reduced formyl-Cellulofine was not inhibited by 0.1 M Gal (data not shown). We also checked Cellulofine itself but no binding was observed. We found that a major HMW adiponectin could be precipitated with 12% polyethylene glycol (PEG) 4000 from human plasma and the redissolved precipitate did not bind to gelatin-Cellulofine before depletion of PEG 4000. The binding of HMW adiponectin to reduced formyl-Cellulofine was also inhibited by PEG 4000 (Fig. 1E). As reported before, HMW adiponectin does not bind to sulfate-Cellulofine (6). There remains at least one possibility that HMW adiponectin binds to hyaluronan, which is a nonsulfated glycosaminoglycan. We made hyaluronan-Sepharose from hyaluronic acid and EAH-Sepharose but HMW adiponectin did not bind to it (Fig. 1F). Although the characteristics of the affinity to reduced formyl-Cellulofine are not clear, HMW adiponectin binds to it very firmly and can be eluted with NaCl.

Purification of HMW adiponectin

Human plasma containing 0.1 M NaCl and 0.1 mM PMSF was applied to reduced formyl-Cellulofine and eluted with a NaCl gradient (Fig. 2A). In this step, HMW adiponectin was separated from other major proteins effectively and after equilibration against 10 mM Tris-HCl, pH8.0, 0.3M NaCl, and 1 mM CaCl₂, the HMW adiponectin was applied to DEAE-Sepharose (Fig. 2B). When bound HMW adiponectin was eluted with NaCl, the resulting peak was almost only HMW adiponectin. Each aliquot of the original plasma, the flow-through pool, and the pool of HMW adiponectin fractions from reduced formyl-Cellulofine and the HMW adiponectin pool from DEAE-Sepharose was analyzed on a Superdex 200 HR 10/30 column. As shown in Fig. 2C, the original plasma contained HMW adiponectin, which was detected as a single peak by an ELISA specific for HMW adiponectin in fractions from 15 to 23 and as a molecule of about 420 kDa. By immunoblotting, three peaks corresponding to HMW, the hexamer and trimer adiponectins of apparent molecular weights of 420, 280, and 180 kDa were detected in fractions 19, 21, and 23, as described before (6). The flow-through pool contained HMW adiponectin of about 1/10 of the original plasma in fractions from 16 to 20 and the hexamer adiponectin of about 280 kDa in fractions from 20 to 23 became apparent by an ELISA specific for HMW adiponectin, which could detect the hexamer adiponectin with much lower efficiency than HMW adiponectin (Fig. 2D). When the pool of HMW adiponectin fractions eluted from reduced formyl-Cellulofine was analyzed, HMW adiponectin was shown to be separated from other major proteins effectively (Fig. 2E). And as shown in Fig. 2F, an analysis of

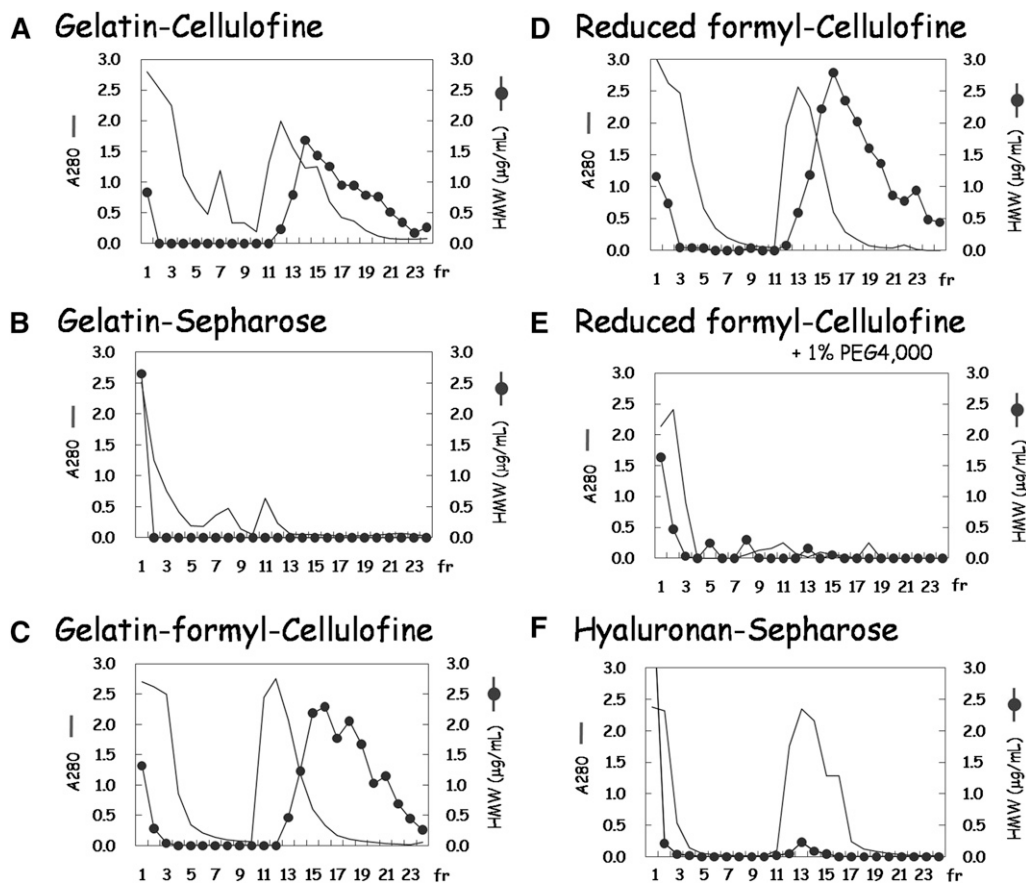


Fig. 1. Binding analysis of HMW adiponectin to various affinity resins. Human plasma, 10 ml, was applied to each affinity resin (1 ml) and then washed with 10 ml of 10 mM Tris-HCl, pH7.4 (fr. 1–10). Then, stepwise elution of bound proteins was performed with 1 ml each of 10 mM Tris-HCl, pH7.4, containing 0.1 to 1.0 M NaCl (fr.11–20) and 1.0 M NaCl (fr.21–24). The HMW adiponectin concentration was measured by an ELISA specific for HMW adiponectin. A: Gelatin-Cellulofine purchased from Seikagaku Kogyo. B: Gelatin-Sepharose. C: Gelatin-Cellulofine made from formyl-Cellulofine and gelatin. D: Reduced formyl-Cellulofine. E: Human plasma containing 1% PEG 4000 was applied to reduced formyl-Cellulofine. F: Hyaluronan-Sepharose.

HMW adiponectin pool from DEAE-Sepharose revealed that HMW adiponectin of about 420 kDa was successfully purified. In the case, a little contamination is observed in HMW adiponectin pool; for further purification a HiLoad 16/60 Superdex 200 prep-grade column can be used effectively.

The purified HMW adiponectin was analyzed by SDS-PAGE (Fig. 2G). As reported before (4), characteristic bands were seen, i.e., when HMW adiponectin was boiled in Laemmli's loading buffer, it gave a single band corresponding to 28 kDa under reducing conditions, and without boiling, under reducing conditions it gave a band corresponding to 65 kDa, which represents the adiponectin trimer, with minor bands of 150 kDa and bigger at almost the top of the gel, which represent the adiponectin hexamer and HMW multimer, respectively.

From 2.6 l of human plasma that contained 1.85 mg/L HMW adiponectin, using reduced formyl-Cellulofine, 50 ml, about 2.1 mg of HMW adiponectin could be effectively purified and the yield was about 43%. The protein concentration was determined to be 1 mg/ml when the absorbance at 280nm was 1.34 (8). Because HMW adiponectin is easily adsorbed to surfaces of glassware and so on, fewer

purification steps are better and the concentration step should be avoided if possible.

The activity of HMW adiponectin purified by formyl-Cellulofine

Adiponectin inhibits osteoclastogenesis (9) and HMW adiponectin is most potent inhibitor among adiponectin molecules containing globular adiponectin by our experiments (data not shown). Using HMW adiponectin purified by gelatin-Cellulofine and formyl-Cellulofine, their activities were assayed. HMW adiponectin purified by formyl-Cellulofine inhibited osteoclastogenesis as shown in Fig. 3C and D, and the inhibition activity was similar, but slightly stronger than HMW adiponectin purified by gelatin-Cellulofine (Fig. 3E, F).

DISCUSSION

Although we reported the purification of HMW adiponectin (GBP28) using gelatin-Cellulofine (4), and adiponectin was also reported to bind to collagen I, III, and

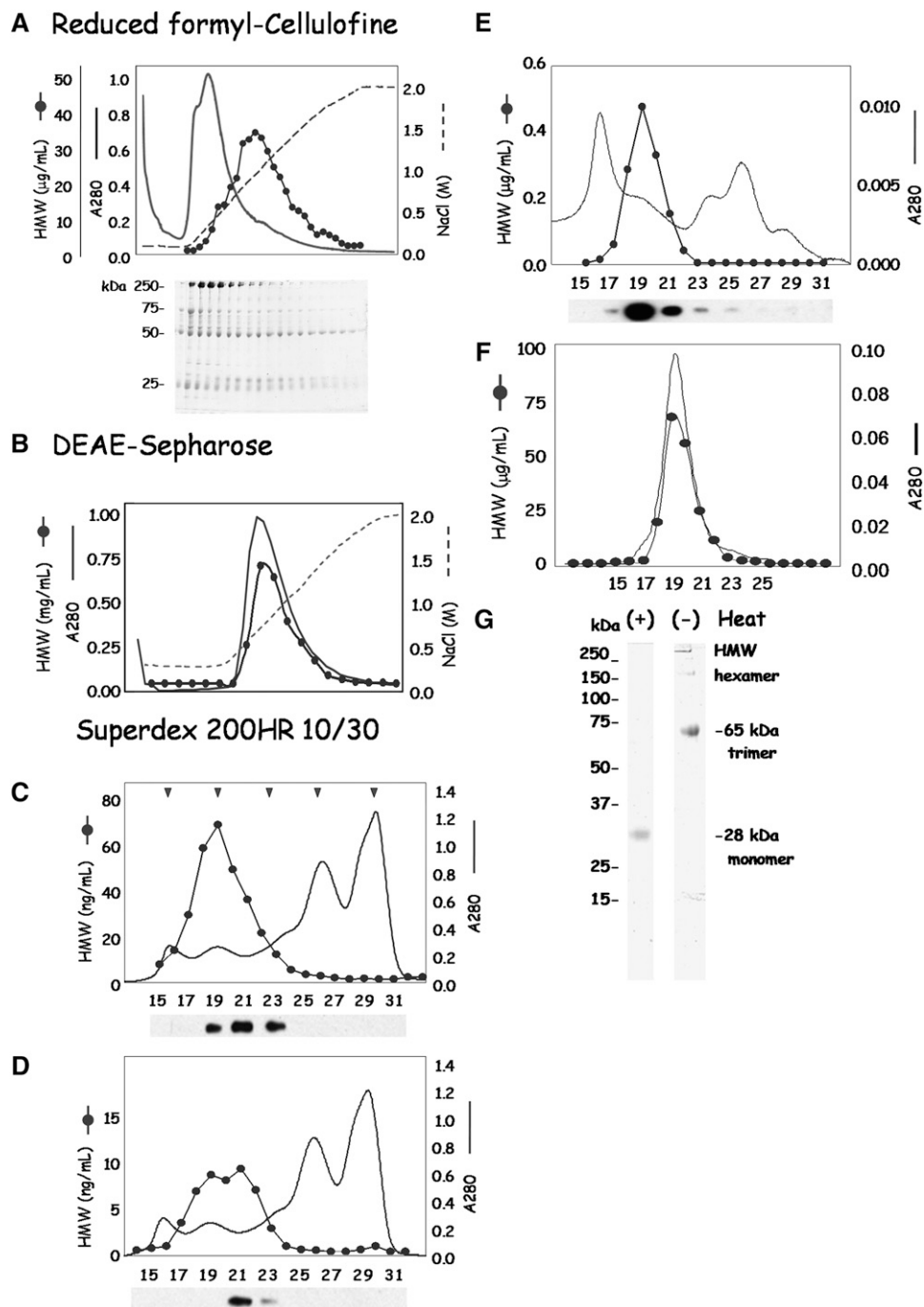


Fig. 2. Purification of HMW adiponectin. **A:** Human plasma of 2.6L containing 0.1 M NaCl and 0.1 mM PMSF was applied to reduced formyl-Cellulofine, 50 ml. The HMW adiponectin concentration was measured as Fig. 1, and each fraction was also analyzed by 12.5% Laemmli's SDS-PAGE after being heat-denatured at 100°C for 3 min, under reducing conditions, followed by Coomassie blue staining. **B:** The HMW adiponectin pool from reduced formyl-Cellulofine in 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl and 1 mM CaCl₂ was applied to DEAE-Sephrose, 10 ml. Each aliquot of the original human plasma (**C**), the flow-through pool from reduced formyl-Cellulofine (**D**), the pool of HMW adiponectin fractions eluted from reduced formyl-Cellulofine (**E**), and the HMW adiponectin pool from the DEAE-Sephrose (**F**) was applied to a Superdex 200 HR 10/30 column. Each fraction was analyzed by 12.5% Laemmli's SDS-PAGE after being heat-denatured at 100°C for 3 min, under reducing conditions, followed by immunoblotting with anti-C. Small triangles indicate elution peaks of thyroglobulin (669 kDa), ferritin (416 kDa), catalase (219 kDa), adolase (176 kDa), and BSA (67 kDa), respectively. **G:** 12.5% Laemmli's SDS-PAGE analysis of the purified HMW adiponectin. Under reducing conditions: +, heat-denatured at 100°C for 3 min; and -, not heat-denatured. Protein bands were stained with Coomassie blue.

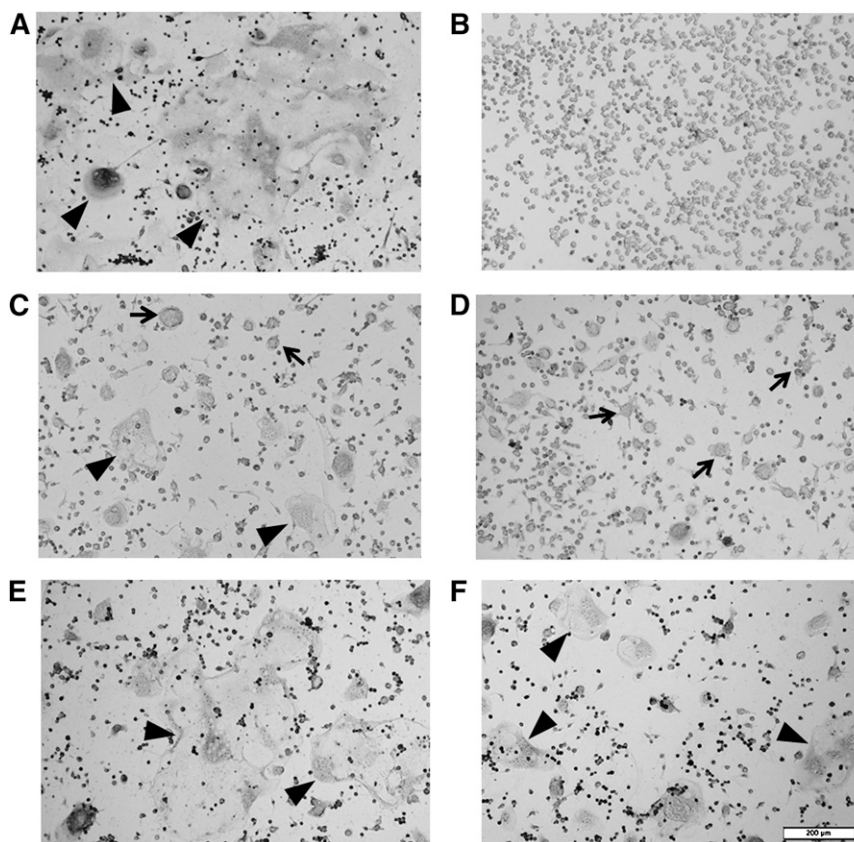


Fig. 3. The activity of HMW adiponectin purified by formyl-Cellulofine. RAW cells were treated with medium containing RANKL (70 ng/ml) with or without HMW adiponectin for 2 days and with RANKL for one more day, and then stained for TRAP. Arrowheads and arrows indicate large and small osteoclasts, respectively. A: RANKL. B: Control. C: 2.5 µg/ml, and D: 5.0 µg/ml of HMW adiponectin purified by formyl-Cellulofine. E: 2.5 µg/ml and F: 5.0 µg/ml of HMW adiponectin purified by gelatin-Cellulofine.

V (5), we found it does not bind to gelatin. Moreover, surprisingly, it binds to reduced formyl-cellulofine but not to hydrolyzed NHS-activated Sepharose, whose structures should be very similar to each other. The structures of formyl-Cellulofine and NHS-activated Sepharose are shown in **Fig. 4**. Because adiponectin has a similar structure to complement factor C1q and mannose-binding protein (MBP), there is a possibility that adiponectin recognizes a certain sugar structure or structures. As shown in Fig. 1F, HMW adiponectin does not bind to hyaluronan-Sepharose and, as reported before, it does not bind to sulfate-Cellulofine (4). Moreover, analysis involving glycoconjugate microarrays (10) did not reveal any interaction with many glycoconjugates containing heparin sulfate, chondroitin sulfate, hyaluronic acid, mannan, LPS, and so on (data not shown). Adiponectin was reported to bind to LPS at pH 5 to 6 and this binding was inhibited by many sugars like lactose, fucose, and N-acetyl glucosamine (11). In fact, with HMW adiponectin, we could detect a weak interaction with LPS. There remains a possibility that under certain conditions, HMW adiponectin might bind to a certain sugar chain or chains specifically. However, this cannot be the case for HMW adiponectin and reduced formyl-Cellulofine because HMW adiponectin binds to reduced formyl-Cellulofine

very firmly and needs a NaCl concentration of more than 0.3 M to be released.

After all, we could establish a very simple and effective purification method for HMW adiponectin involving reduced formyl-Cellulofine because most proteins do not show such strong affinity for reduced formyl-Cellulofine, flowing through it. Also, this short two-step method results in not only a good yield of the protein but also a protein exhibiting high activity. Until this method was developed, many steps were needed to purify HMW adiponectin and often resulted in a low yield and low activity, which is correlated with the

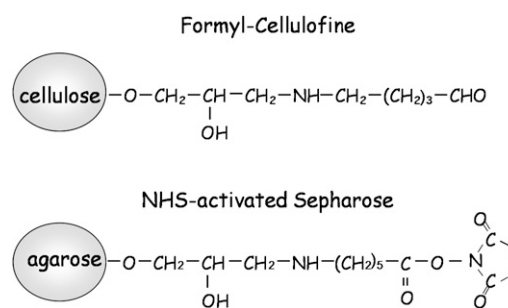



Fig. 4. Structures of formyl-Cellulofine and NHS-activated Sepharose.

low reactivity to the mouse monoclonal anti-human HMW adiponectin antibody, IH7. We believe that our report of an easy purification protocol for human native HMW adiponectin will lead to great advances in future research in many fields, especially that on metabolic syndromes. 

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