HDL/Apolipoprotein A-I Binds to Macrophage-Derived Progranulin and Suppresses its Conversion into Proinflammatory Granulins

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Aim: HDL has anti-inflammatory effects on macrophages, although the mechanism of action remains unclear. We hypothesized that HDL suppresses the conversion of macrophage-secreted factors into proinflammatory factors via binding, and tried to identify the factor that could form a complex with HDL and/or apolipoprotein (apo) A-I.

Methods and Results: In conditioned media obtained from human monocyte-derived macrophages, we found an apo A-I binding protein and identified the protein as progranulin/proepithelin/acrogranin/PCDGF. Co-immunoprecipitation analysis showing that progranulin binds and forms a complex with apo A-I and the presence of progranulin in the HDL fraction in the sera indicated that progranulin is a novel apolipoprotein. Conditioned media of HEK293 cells transfected with progranulin augmented the expression of TNF-alpha and IL-1-beta on macrophages, but these effects of progranulin were inhibited by co-incubation with HDL or apo A-I. Anti-progranulin antibodies also reduced the expression of TNF-alpha and IL-1-beta on macrophages. Granulins as conversion products derived from progranulin increased TNF-alpha and IL-1-beta expression and the effects were not suppressed by HDL.

Conclusions: Our results suggest that the anti-inflammatory effects of HDL on macrophages might be due to suppression of the conversion of progranulin into proinflammatory granulins by forming a complex.


Key words: HDL, Apolipoprotein A-I, Progranulin, Proepithelin, Acrogranin, PCDGF, Macrophage

Introduction

Several pathological studies have shown that low high-density lipoprotein (HDL) levels are associated with plaque instability in patients with acute coronary syndrome¹. Accordingly, the reverse cholesterol trans-
ence patients with acute coronary syndrome whose HDL levels are reduced before the occurrence of plaque instability.\(^9\)

Infiltration and accumulation of foam cells (macrophages) is a characteristic feature of atheromatous plaques.\(^7\) Once activated, macrophages secrete various pro-inflammatory cytokines and proteases, which could result in plaque instability and rupture.\(^7\) Are the anti-inflammatory effects of HDL mediated through suppression of the secretion of such cytokines from macrophages? The main theme of our research is to determine the mechanisms underlying the reduction of serum HDL during the acute phase of coronary events. In this study, we hypothesized that HDL modulates the expression levels of pro-inflammatory cytokines secreted by macrophages. Progranulin is here described as a macrophage-derived secretory factor, which is a pluripotent protein and a precursor of its proteolytic peptides, granulins, whose functional properties were different from their intact precursor in some cases,\(^9\) and whose pro-inflammatory properties were suppressed via binding to HDL.

**Materials and Methods**

**Lipoprotein Isolation**

Apo A-I was purchased from Sigma Aldrich (St. Louis, MO). HDL\(^3\) were isolated from human serum by ultracentrifugation at a density of 1.125-1.210 g/mL biotinylated-apo A-I. After incubation with peroxidase-conjugated streptavidin, the blots were visualized with an ECL kit (Amersham Pharmacia Bioscience, Uppsala, Sweden).

**Purification of Apo A-I Binding Protein from Macrophage-Conditioned Medium**

The conditioned medium (total volume, 20 L) was collected and then treated with 80% ammonium sulfate. The precipitate was dissolved in 2.5 mL, and then desalted and equilibrated into Tris-buffered saline (20 mmol/L Tris HCl, pH 7.4, and 135 mmol/L NaCl) using a PD-10 column (Amersham Pharmacia Biotech). The eluate was added to an apolipoprotein A-I-affinity column (Amino Link; Amersham Pharmacia Biotech), and allowed to stand overnight at 4°C. After vigorous washing with Tris buffer with 1 mol/L NaCl (20 mmol/L Tris/HCl, pH 7.4, and 1 mol/L NaCl), the binding proteins were eluted with 8 mol/L urea. The eluate was concentrated with Amicon Ultra-15 50,000 MWCO (Millipore, Bedford, MA) to ensure purity, and subjected to SDS-PAGE.

**Amino Acid Sequencing**

The purified apo A-I-binding protein was applied for in-gel digestion with V8 endopeptidase, transferred to a polyvinylidene fluoride (PVDF) membrane, and stained with Coomassie brilliant blue (CBB) R-250. The three apparent fragmented bands were subjected to amino acid sequencing in a sequencer (Perkin Elmer-Cetus, Foster City, CA).

**Construction of Progranulin, Granulin A and Granulin B Expression Vector**

The expression vectors of progranulin (aa 1-593, see RESULTS), myc- His-tagged progranulin and granulin were constructed from pcDNA3.1, as described previously.\(^10\) In short, cDNA obtained from human monocyte-derived macrophages underwent PCR using primer pairs 5’-agagcggcggaagccggagccg-3’ and 5’-tcggagtgaggtccggagggctg-3’, and for nested PCR, primer pairs 5’-gttcatggctccggacgctgtc-3’ and 5’-agggctcgagagtcttcagtactgtccctc-3’. The nested PCR product was digested with Bam HI and Xho I and then ligated into pcDNA3.1 pretreated with Bam HI and Xho I. The construct was used as a progranulin expression vector. To obtain myc- His tagged progranulin expression vector, cDNA obtained from human monocyte-derived macrophages underwent PCR using primer pairs 5’-agagcggcggaagccggagccg-3’ and 5’-tcggagtggaggtccggagggctg-3’, and for nested PCR, primer pairs 5’-gttcatggctccggacgctgtc-3’ and 5’-tcggagtggaggtccggagggctg-3’. The three apparent fragmented bands were subjected to amino acid sequencing in a sequencer (Perkin Elmer-Cetus, Foster City, CA).
tcactctagacgcactgctcaagc-3', and the nested PCR product was digested with Bam HI and Xba I and the digested product was ligated into pcDNA3.1/myc-His vector pretreated with Bam HI and Xba I. Human granulin A (aa 281–337) and B (aa 206–261) vectors were constructed with secretion being driven by the human progranulin signal peptide (aa 1–17).

**Immunoprecipitation**

The vectors were transiently expressed in HEK293 cells using a Calcium Phosphate Transfection Kit (Invitrogen Corp., Carlsbad, CA, USA). Three days after transfection, HEK293 cells were incubated with conditioned medium containing apo A-I (final concentration 5 µg/mL) at 37°C for 30 min. The apo A-I-containing media were collected and immunoprecipitated with anti-progranulin antibody (clone N19; Santa Cruz Biotechnology, Santa Cruz, CA), pulled-down with protein G (Amersham Pharmacia Biotech), separated by reducing SDS-PAGE, and Western blotted with anti-apo A-I antibody, or the reverse. In vitro translated progranulin was produced using an in vitro translation system (Duo, Tokyo, Japan), and mixed with apo A-I at a final concentration of 5 µg/mL. The mixture was then subjected to co-immunoprecipitation analysis.

**Immunoblotting Analysis**

The conditioned medium was subjected to immunoblotting analysis with anti-progranulin monoclonal antibody (clone N-19; Santa Cruz Biotechnology). The blots were visualized after incubation with peroxidase-conjugated anti-mouse IgG antibody (DAKO, Denmark).

**Quantitative Real-Time PCR**

The constructed expression vectors of progranulin, granulin A and granulin B, were transiently expressed in HEK293 cells using a Calcium Phosphate Transfection Kit (Invitrogen Corp.). Three days after transfection, the conditioned media were obtained and macrophages were incubated in conditioned media with or without HDL (10 µg/mL) or apo A-I (5 µg/mL) for 24 h. Total RNA was then isolated using RNeasy MINI kits (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. For cDNA synthesis, 600 ng total RNA was reverse transcribed using SuperScript III RTase (Invitrogen, San Diego, CA). TaqMan probe and primers for progranulin, CD14, CD36, CD68, TNF-alpha, IL-1beta and GAPDH were purchased from Applied Biosystems (Assay ID: Hs00173570_m1, Hs02621496_S1, Hs01567186_m1, Hs00154355_m1, Hs99999043_m1 and Hs00266705_g1, respectively). Quantitative real-time PCR was performed using the ABI Prism 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). The cDNA samples (10 ng in a total volume of 10 µL) were mixed with primers, probe and TaqMan Universal PCR Master Mix as described in the accompanying sheet supplied by the manufacturer (Applied Biosystems). PCR was conducted using the following settings: 50°C for 2 min, 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min.

**Results**

**Purification of Progranulin as Apo A-I Binding Protein**

We first tested whether HDL suppresses the pro-inflammatory cytokines secreted by macrophages by forming complexes with them, and thus searched for hypothetical proteins. The conditioned media obtained from 7-day-cultured human macrophages were concentrated with the ammonium sulfate precipitation method, desalted and subjected to ligand blotting analysis using biotinylated-apo A-I as a ligand. After separating the media on SDS-PAGE under non-reducing conditions, we detected an apo A-I-binding protein with a MW of 130 kDa (Fig. 1A). To purify the protein, the concentrated medium was subjected to an affinity column with immobilized human apo A-I and we obtained a concentrated eluate. To ensure purity, 10 µg proteins of the conditioned medium and 0.5 µg of the eluted protein were subjected to SDS-PAGE. Silver staining showed a single band of 80 kDa protein (Fig. 1B).

In the next step, we obtained three polypeptide fragments after in-gel-digestion with V8 proteases. Next, the amino-terminal sequence of these polypeptides was determined. The amino acid sequences were “avacgdgh”, “nartdllt” and “kapahsl”, respectively. All amino acid sequences were identical to those of progranulin (aa 89–96, 265–272 and 347–354, respectively).

To confirm that progranulin could bind to HDL, HDL fractions (0.5 µg) were applied for immunoblotting with anti-progranulin antibody (clone N19). As shown in Fig. 1C, progranulin was observed in the HDL fraction, and a small amount was observed in serum. As indicated in Fig. 1A, HDL binding protein had approximately 130 kDa molecular weight in non-reducing conditions; however, MW of progranulin was approximately 80 kDa, as shown in Fig. 1B. To clarify these discrepancies, purified proteins by affinity column with immobilized human apo A-I were...
applied for immunoblotting in non-reducing conditions. Progranulin-like immunoreactive bands was observed in 130 kDa and 80 kDa (Fig. 1D), indicating that progranulin could form a homo-dimer.

**Formation of Progranulin-Apo A-I Complex**

To confirm the formation of progranulin-apo A-I complex, we performed co-immunoprecipitation analysis (Fig. 2). Progranulin-expressing conditioned medium treated with apo A-I was subjected immunoprecipitated with anti-apo A-I antibody to bring down apo A-I and then Western blotted with anti-progranulin antibody or the reverse (Fig. 2A, B). The two proteins were recovered simultaneously, indicating that progranulin binds apo A-I. To further confirm the formation of the protein complex, progranulin was translated in vitro, co-incubated with apo A-I and then subjected to co-immunoprecipitation analysis (Fig. 2C, D). Progranulin, in vitro translated under non-reducing conditions (Fig. 2C, D), was detected in apo A-I immunoprecipitates while apo A-I was identified in progranulin immunoprecipitates or the reverse. These results indicate that progranulin and apo A-I could bind each other.

**Progranulin-Expressing Macrophages**

Although we purified and identified progranulin as an apo A-I binding protein from conditioned media derived from macrophages, it is important to confirm that macrophages express and produce progranulin. As shown in Fig. 3A, macrophages expressed progranulin and the expression level was dependent on maco-
Defense of progranulin expression was higher after 5-day culture. Next, we examined progranulin protein production by macrophages (Fig. 3B). After cultivation for the indicated time, macrophages were incubated without serum for 24 h and their conditioned media were blotted with anti-progranulin antibody. The production of progranulin protein by macrophages increased in a macrophage differentiation-dependent manner, similar to the gene expression. To examine whether progranulin could augment the expression of progranulin itself, TNF-alpha and IL-1-beta, 7-day-cultured human macrophages were cultured for 24 hours with progranulin. As shown in Fig. 3C, progranulin increased the expression of pro-
granulin itself, TNF-alpha and IL-1-beta (3.9-, 7.1- and 12.2-fold expressions, respectively).

Progranulin Activates Macrophages and HDL/Apo A-I Suppress as Such Activation

The production of cytokines by macrophages is often regulated in a paracrine or autocrine manner. Next, we examined whether progranulin had effects on macrophages and whether HDL and apo A-I suppress such effects of progranulin. TNF-beta and IL-1-alpha were selected in the present study as representative pro-inflammatory cytokines. Seven-day-cultured macrophages were incubated with progranulin-expressing conditioned medium for 24 h, and TNF-alpha and IL-1-beta gene expression levels were examined by quantitative PCR using a TaqMan Probe (Fig. 4A). Progranulin augmented the expression levels of TNF-alpha and IL-1beta, indicating that progranulin affects macrophages. To confirm the binding of progranulin and HDL/apo A-I, the supernatants of macrophages with progranulin-expressing conditioned medium, and apo A-I or HDL under the same conditions were applied for immunoprecipitation with anti-progranulin. The precipitated samples were resolved

Fig. 3. Human monocyte-derived macrophages express and secrete progranulin into conditioned media.

(A) Expression of progranulin on macrophages is macrophage maturation-dependent. Human monocyte-derived macrophages expressed progranulin and the expression level was dependent on macrophage differentiation by quantitative PCR. Maturation of monocytes-macrophages was associated with over-expression of CD36 and CD68 and under-expression of CD14. Data are the mean ± SE of 5 experiments.

(B) The amount of progranulin secreted by macrophages is macrophage maturation-dependent. Human monocyte-derived macrophages secreted progranulin into the conditioned media and the amount of progranulin protein increased with the differentiation of these cells.

(C) Autocrine regulation of progranulin production. Progranulin could augment the expression of progranulin itself, TNF-alpha (10 ng/mL) and IL-1-beta (10 ng/mL) on macrophages cultured with progranulin. Up-regulation of progranulin expression by progranulin itself, TNF-alpha, or IL-1-beta was observed. Data are the mean ± SE of 5 experiments.
Fig. 4. Effects of progranulin on the expression levels of TNF-alpha and IL-1-beta in macrophages.

(A) Progranulin activated macrophages and HDL/apo A-I suppressed the effects. Seven-day-cultured macrophages were incubated with progranulin-expressing conditioned medium for 24 h and the expression levels of TNF-alpha (a) and IL-1-beta (b) were examined with quantitative PCR using TaqMan Probe. Progranulin augmented the expressions of TNF-alpha and IL-1-beta. These properties of progranulin were suppressed by co-incubation with apo A-I (5 µg/mL) or HDL (10 µg/mL). Data are the mean ± SE of 5 experiments. The supernatants of macrophages with progranulin-expressing conditioned medium, and apo A-I or HDL under the same conditions were applied for immunoprecipitation with anti-progranulin. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized (c).

(B) Anti-progranulin antibodies reduced the expression of TNF-alpha and IL-1-beta. Macrophages were cultured in the conditioned medium with anti-progranulin antibody for 24 h. Compared to incubation with control IgG (100 µg/mL), anti-progranulin antibody (clone M12, N9 and S15, 100 µg/mL each) suppressed the expressions of TNF-alpha (a) and IL-1-beta (b). Data are the mean ± SE of 5 experiments.
by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized (c). These results indicated that the effects of progranulin were suppressed by co-incubation with HDL or apo A-I via binding.

**Anti-Progranulin Antibodies Reduce the Expression of TNF-Alpha and IL-1Beta**

To examine whether the effect of progranulin on macrophages is autocrine in nature, fully differentiated macrophages were cultured with anti-progranulin antibody for 24 h. Compared to incubation with control IgG, anti-progranulin antibody (clone M12, N9 and S15) suppressed the expression of TNF-alpha and IL-1-beta (Fig. 4B). These results suggest that progranulin is secreted by macrophages and its effect is autocrine in nature, indicating that progranulin is an autoactivating molecule.

**Progranulin Exerts its Properties Via Conversion into Proinflammatory Granulins**

We examine whether progranulin could be converted into fragments by macrophages and exerted its properties via conversion into proinflammatory granulins. C-terminal-myc-His-tagged progranulin construct was transfected into HEK293T, and the conditioned media were incubated with or without human macrophages for 24 hr. Probond beads were added to the media to capture His-tagged proteins. Next, the incubated probond beads were applied for immunoblotting with anti-myc antibody. C-terminal-myc-His-tagged progranulin was degraded by macrophages and small degraded products were observed but not with HDL incubation (Fig. 5A), indicating that progranulin could be converted by macrophages.

Next, we examined whether granulin could increased the expressions of TNF-alpha and IL-1-beta, and the increment could be suppressed by HDL (Fig. 5B). Progranulin could increase the expressions of pro-inflammatory cytokines, TNF-alpha and IL-1-beta, and this augmentation was suppressed by incubation with HDL. On the other hand, the augmentation effect on the expression of TNF-alpha and IL-1-beta of granulin was not blocked by incubation with HDL (Fig. 5B, left and right panels, respectively). These results suggested that progranulin could exert its pro-inflammatory properties via conversion into granulins, and that HDL could suppress the pro-inflammatory properties of progranulin by inhibiting the conversion into granulins.

**Discussion**

Progranulin, a PC-cell-derived growth factor (PCDGF), or acrogranin, was purified from the conditioned media of transformed cell lines as an auto-
crine growth factor\textsuperscript{11}. It is reported to be involved in cancer progression\textsuperscript{8}, development\textsuperscript{12}, wound healing\textsuperscript{13}, and myeloid cell proliferation\textsuperscript{14}, whereas mutation of progranulin causes frontotemporal dementia\textsuperscript{15, 16}. In the present study, we demonstrated that the macrophage-secreted factor was approximately 130 kDa, while the purified protein identified as progranulin was 80 kDa. The 130-kDa HDL binding protein might be a homodimer or heteromer that includes progranulin, which is known to be glycosylated and to have disulfide bonds\textsuperscript{11}.

Ong and colleagues\textsuperscript{10} used myeloid cell lines and reported the overexpression of progranulin in macrophages and monocyte-derived dendritic cells, and that the level of expression was dependent on cell differentiation. Our results also demonstrated that the differentiation of human monocyte-derived macrophages was associated with increased expression levels of progranulin and that progranulin expression was regulated in an autocrine fashion in human monocyte-derived macrophages. We also demonstrated that apo A-I, the major component of HDL, suppressed the conversion of progranulin into pro-inflammatory granulins on human peripheral monocyte-derived macrophages. This is in agreement with others, who indicated that the protein precursor (progranulin) and its processed fragments (granulins) are both bioactive and pro-inflammatory\textsuperscript{8}.

The role of progranulin in the inflammatory process was initially explored in research on the functions of secretory leukocyte protease inhibitor (SLPI) in wounds\textsuperscript{10}. Using a yeast two-hybrid approach, with SLPI as the bait, Zhu and colleagues\textsuperscript{10} demonstrated that progranulin is associated with SLPI\textsuperscript{10}. This interaction was confirmed by immunoprecipitation experiments demonstrating that progranulin regulates inflammation through a tripartite loop with SLPI, which protects progranulin from proteolysis, and elastase, which digests progranulin between granulin/epithelin domains, generating smaller granulin/epithelin peptides. SLPI blocks this proteolysis, by inhibiting both elastase activity directly and by binding progranulin and sequestering it from the enzyme\textsuperscript{10}. Intact progranulin is anti-inflammatory by the inhibition of certain actions of TNF-alpha, while proteolytic peptides may stimulate the production of pro-inflammatory cytokines, such as IL-8\textsuperscript{8}. We suppose that HDL/apo A-I have anti-inflammatory effects on macrophages through the formation of a complex with progranulin and prevent the conversion of progranulin into granulins by elastase secreted by macrophages such as SLPI, which is reported as a neutrophil-derived anti-inflammatory factor\textsuperscript{10}. In this study, the possibility could be not rejected that progranulin binds HDL particles itself but free apoA-I is dissociated from HDL, which is just a reserve of apoA-I \textit{in vivo} according to the limitations of the experimental conditions. In the near future, our colleagues will demonstrate which of apo A-I, lipid-free apo A-I dissociated from HDL, apo A-I in pre-beta HDL or apo A-I in HDL will bind to progranulin \textit{in vivo}.

An unstable and subsequently ruptured atherosclerotic coronary plaque superimposed on thrombosis constitutes the most common pathological background of acute coronary syndrome\textsuperscript{7}. High levels pro-inflammatory cytokines have been found in unstable angina, possibly supporting their role in acute coronary syndrome\textsuperscript{7}. Cytokines induce their own expression in an autocrine fashion and also the expression of various adhesion molecules via the cellular transcription factor NF-kappaB\textsuperscript{7}. Monocytes adhering to the endothelium and penetrating the plaque (macrophages) are activated by several paracrine/autocrine pro-inflammatory mediators. At this crucial stage, activated macrophages then synthesize and secrete pro-inflammatory cytokines TNF-alpha and IL-1-beta\textsuperscript{7}. The human and murine progranulin promoter contains potential inflammation-related promoter elements\textsuperscript{18, 19}. Furthermore, TNF-alpha and IL-1-beta activate progranulin gene expression through the NF-kappaB system\textsuperscript{18, 19}. The progranulin-granulin loop might play a role in autoactivation of macrophages. Given its actions in atherosclerosis, progranulin may prove a useful clinical target, both for prognosis and therapy.

In summary, we identified progranulin/proepithelin/acrogranin/PCDGF as a macrophage-derived factor whose properties were suppressed by binding to HDL/apo A-I. The normal function of progranulin is complex; the full-length form of the protein has both trophic and anti-inflammatory activities, whereas proteolytic cleavage generates granulin peptide that promotes inflammatory activity. Based on our results, we propose that HDL prevents the conversion of progranulin into its degraded proinflammatory products, granulins; however, the mechanisms involved in HDL-induced suppression of the pro-inflammatory effects of progranulin have not fully elucidated. Further studies are necessary to identify these mechanisms.

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References