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Purification and Characterization of a Novel Inhibitor of the Proliferation of Hepatic Stellate Cells[1]

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An inhibitor of the proliferation of hepatic stellate cells (HSC) was purified from rat liver by a combination of gel filtration and ion exchange chromatography. The molecular mass of this non-arginase growth inhibitory factor (NAGIF) was determined to be 38 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel filtration. The proliferation of HSC was inhibited by NAGIF with a 50% inhibitory dose of 5 nmol/liter. The inhibitory activity of NAGIF was not limited to HSC but also affected the growth of bovine endothelial cells and 3T6 fibroblasts. However, the growth of B16 mouse melanoma was not inhibited by NAGIF. The NH_2-terminal sequence of NAGIF, AEPVEPWS, is identical to an internal sequence of rat Zn-α_2-glycoprotein. Although the action mode of this inhibitor remains to be investigated, it seems very likely that NAGIF is involved in the negative control mechanism of HSC growth.

Key words: arginase, growth inhibitor, hepatic stellate cells, Zn-α_2-glycoprotein.

Hepatic fibrosis is predominantly characterized by excessive accumulation of extracellular matrix (ECM) components in the liver, in which hepatocytes and non-parenchymal cells (hepatic stellate cells, Kupffer cells, and sinusoidal endothelial cells) are all involved. Of these, hepatic stellate cells (HSC, also referred to as fat-storing cells, Ito cells, or lipocytes) are considered to be the main cell types producing ECM (*1–3*). In this context, the search for an inhibitor of HSC proliferation is crucial for the development of anti-fibrotic agents as well as understanding fibrogenesis. In the liver, several factors that stimulate or inhibit cell proliferation have been characterized (*4, 5*). Among the inhibitors in liver, arginase has been considered to be a potent growth inhibitor of various cells including lymphocytes (*6–10*). Therefore, we used normal liver as a source of inhibitor and focused on the purification of a novel inhibitor other than arginase. In this report, we describe the isolation, purification, and partial characterization of a novel liver-derived inhibitory factor that inhibits the proliferation of HSC *in vitro*.

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Abbreviations: HSC, hepatic stellate cells; NAGIF, non-arginase growth inhibitory factor; ZAG, Zn-α_2-glycoprotein; ED_{50}, effective dose giving 50% inhibition of cell proliferation; DMEM, Dulbecco's modified Eagle medium; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling; ELISA, enzyme-linked immunoabsorbent assay; FITC, fluorescein isothiocyanate.

MATERIALS AND METHODS

Isolation of Hepatic Stellate Cells—HSC were prepared from 6-month-old male Sprague-Dawley rats (the body weight was approximately 400–500 g) which had had free access to standard laboratory chow diet. Non-parenchymal liver cells were isolated by the pronase-collagenase method (*11*). HSC were purified from the non-parenchymal cell suspension by a single step density gradient centrifugation (*12*). The HSC were identified by their typical light microscopic appearance and immunofluorescence staining for desmin (Sigma) (*13*). The mean purity of freshly isolated cells as analyzed by fluorescence-activated cell sorting (FACSCalibur, Becton Dickinson, San Jose, CA) was 85 ± 5%, cell viability as checked by Trypan blue exclusion was 90 ± 5%, and the yield ranged from 1.2 to 2.0 × 10^6 cells/liver. The cells were seeded with a density of 1 × 10^4 cells/cm^2, maintained on DMEM containing 10% fetal bovine serum, and cultured in a humidified atmosphere of 5% CO_2–95% air. The first change of the medium was made 16 h after seeding, after which the purity of HSC was greater than 95%. The medium was changed every 24 h.

HSC Proliferation Assay and Arginase Assay—Inhibitory activity was assayed using HSC as target cells. Experimental manipulations were performed with cells at passages 5–12. HSC were seeded in 24-well tissue culture plates with a density of 2 × 10^4 cells/well, and after 12 h, various concentrations of inhibitor preparation were added to the culture medium. Cells were pulse-labeled [^{3}H]thymidine 24 h before harvesting. All cultures were performed in triplicate, and the inhibition of proliferation was calculated as follows: Inhibition (%) = (1 – cpm of experimental culture/cpm of control culture) × 100. One unit of inhibitor activity was defined as the activity of the inhibitor that led to 50% inhibition of the cell proliferation with a density of 2 × 10^4 cells/

well under the conditions described above. Arginase activity was assayed by measuring the amount of urea cleaved from arginine (*14*). One unit of arginase activity is equivalent to 1 μmol of urea produced min^{-1} at 37°C.

Purification and Characterization of the Non-Arginase Growth Inhibitory Factor (NAGIF)—The rats were killed by means of cardiac puncture after anesthesia. The livers were promptly removed and cut into small pieces. All purification procedures were carried out at 4°C. Liver tissue (10 g) was homogenized with 50 ml of lysis buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% Nonidet P-40, 10 mM sodium fluoride, 2 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 2 mM EGTA). After centrifugation at 10,000 ×*g* for 30 min, 50 ml of supernatant was loaded onto a Sephacryl S-300 column (5 × 47 cm, Pharmacia, Uppsala, Sweden) equilibrated with buffer A (20 mM sodium phosphate pH 6.3, 5 mM EGTA, 0.02% NaN_3) containing 100 mM NaCl. The column was eluted with the same buffer at a flow rate of 20 ml/h. The inhibitory activity fractions of HSCs were pooled, diluted with two volumes of 20 mM sodium phosphate, pH 6.3, and loaded onto DEAE Sepharose column (2.6 × 10 cm, Pharmacia). The column was washed with buffer A and developed with a linear NaCl gradient from 0.1 to 0.5 M. Fractions were collected, and aliqouts were assayed for the inhibitory activity and arginase activity. The non-arginase growth inhibitory fractions were concentrated to 4 ml by use of YM 10 membrane (molecular cut-off; 10,000, Amicon, Beverly, MA) and applied to Sephacryl S-100 column (1.6 × 90 cm, Pharmacia) equilibrated with buffer A containing 100 mM NaCl. The column was eluted with buffer A containing 100 mM NaCl at a flow rate of 10 ml/h. The flow-through fractions were assayed for inhibitory activity, of HSC and the active fractions were applied to a Mono Q HR 5/5 column equipped with an FPLC system (Pharmacia). The charged column was eluted with a linear NaCl gradient from 0 to 0.5 M. The active NAGIF fractions were pooled and subjected to further characterization as described below.

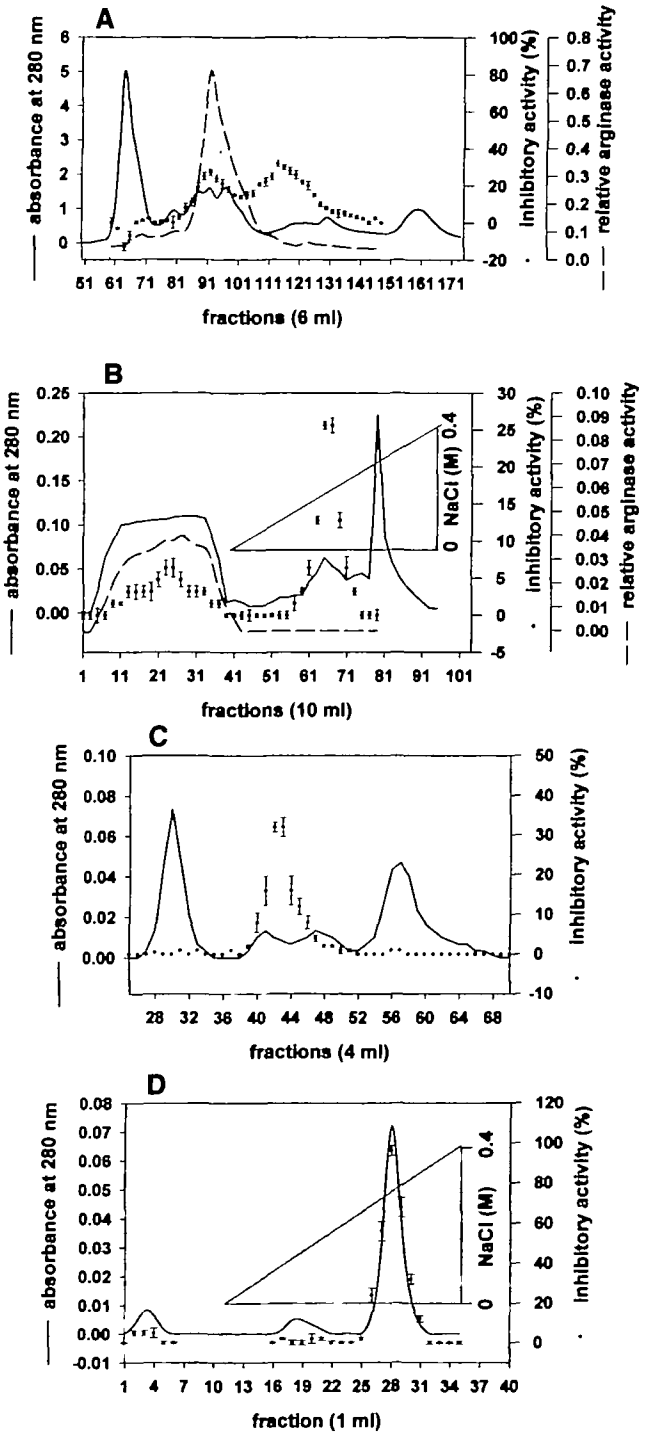
Physicochemical Properties of NAGIF—The denatured molecular weight of NAGIF was determined by SDS-PAGE and the native MW was estimated by HPLC gel filtration (Pharmacia-LKB model, Pharmacia). Proteins were stained with Coomassie Brilliant Blue R250, and the carbohydrate moieties in glycoproteins were detected with a GlycoTrack™ detection kit (Oxford GlycoSystem, Abingdon, UK). For the amino acid analysis of NAGIF, the purified NAGIF was electrophoresed on SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The corresponding protein band was excised with a scalpel, and the N-terminal amino acid sequence was determined with an automatic 447A Protein Sequencer/120A Analyzer (Applied Biosystems, Foster City, CA).

Apoptosis Analysis—Apoptosis in individual cells was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) techniques as described by Gavrieli *et al.* (*15*). Cytoplasmic histone-associated DNA fragments (mono and oligonucleosomes) were determined with a cell death detection ELISA kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. DNA fragmentation was determined to evaluate apoptosis (*16*).

Growth Inhibitory Activity of NAGIF on Various Cell Lines—The growth inhibitory effect of NAGIF was assayed against various mammalian cells. The cell lines used were as follows: 3T6 (mouse embryo fibroblasts), BCE (bovine capillary endothelial cells), B16 (mouse melanoma). BCE was isolated from bovine adrenal cortex (*17*). The cells were cultured in suitable standard media.

RESULTS

Purification of NAGIF—Liver extracts prepared from nor-



mal rats inhibited the proliferation of HSC in a dose-dependent manner (data not shown). Since arginase has previously been identified as a liver-derived proliferation inhibitor by depleting arginine, the inhibitory effects of liver extracts on HSC growth after arginine supplementation into culture media were investigated. If the inhibitory activity of liver extracts is due to arginase activity, additional arginine should be able to rescue cells from the inhibitory action. However, adding additional arginine to the starting medium suppressed inhibitory activity of liver extracts (100 μg/ml) to 40% of control values. This result suggested that not all of the inhibitory activity is ascribed to arginine depletion by arginase activity and that other inhibitors may exist in liver extracts. To exclude the possibility that arginase might be co-purified with our inhibitory activity, we monitored the arginase activity and inhibitory activity throughout all purification steps. Liver extracts were fractionated on a Sephacryl S-300 gel filtration column. As shown in Fig. 1A, the profile of arginase activity is similar to that of inhibitory activity on the Sephacryl S-300 column, but the elution profile of arginase was broad and shifted to the left of the inhibitory activity. To separate the inhibitory activity from arginase activity, we performed DEAE Sepharose chromatography. Figure 1B shows that arginase could be separated from the non-arginase growth inhibitory factor (NAGIF). The inhibitory activity due to arginase was eluted in the flow-through peak at pH 6.3 at low ionic strength, whereas the activity of NAGIF was eluted at 0.2 M NaCl by a linear gradient. Through Sephacryl S-100 gel filtration column, the non-arginase growth inhibitory activity was recovered in a region around MW 38 kDa (Fig. 1C). For the first time only after the active fraction was subjected to Mono Q, the growth inhibitory activity was recovered coincidentally with a protein peak (Fig. 1D). The purification procedure is summarized in Table I; 0.84 mg of the protein was isolated with overall purification of 245-fold and 10% recovery.

Characteristics of NAGIF—The growth of HSC was inhibited in a dose-dependent manner by NAGIF. Approximately 50% inhibition of cell growth was obtained at 0.19 μg protein of the inhibitor per ml of control medium after incubation for 24 h. At a concentration of 0.5 μg per ml, more than 80% of the cell growth was inhibited when compared with the control. To test whether the NAGIF affected cells other than HSC, we examined its effect on the prolif-

Fig. 1. **Purification of NAGIF.** (A) Gel filtration of liver extracts on a Sephacryl S-300 column. The liver extracts (50 ml) were applied to the column (5 × 47 cm) and eluates were collected in fractions of 6 ml. Each fraction was tested for its ability to inhibit HSC proliferation and for arginase activity. Fractions represented by straight line were collected and used for further purification of NAGIF. (B) DEAE Sepharose chromatography of growth-inhibitory fraction obtained from Sephacryl S-300 column. The dialyzed sample (120 ml) from the Sephacryl S-300 step was applied to a DEAE Sepharose column (2.6 × 10 cm) and developed with a linear NaCl gradient from 0 to 0.5 M. Fractions containing inhibitory activity for the proliferation of HSC and no arginase activity were combined and concentrated to 4 ml. (C) Sephacryl S-100 gel filtration of growth-inhibitory fraction obtained from DEAE Sepharose column. The active fractions obtained from the DEAE Sepharose column were applied to a Sephacryl S-300 column (1.6 × 90 cm), and the eluates were assayed for the inhibitory activity of HSC. (D) FPLC of NAGIF on Mono Q column. The active fractions of Sephacryl S-300 column were pooled, dialyzed, and applied to a Mono Q column (0.5 × 5 cm). The active fractions of Mono Q were used for further characterization. The results of inhibitory activity on HSC proliferation were expressed as mean ± SE of three separate experiments.

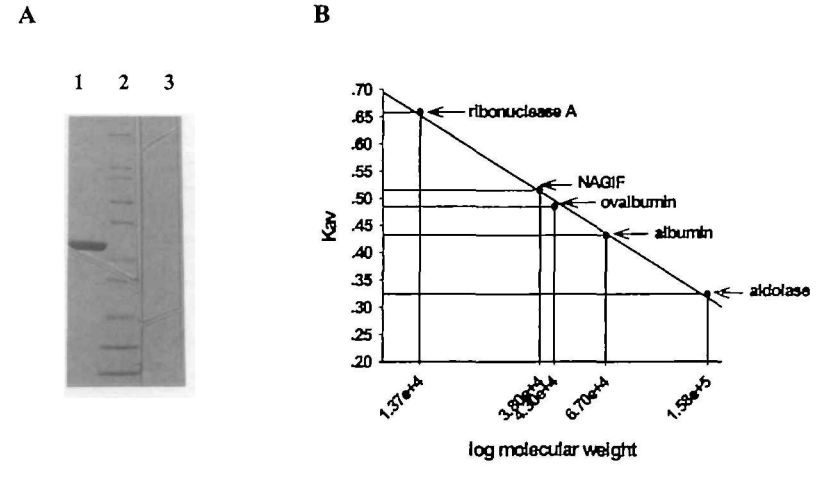


Fig. 2. **Biochemical properties of NAGIF.** (A) SDS-PAGE (4–20% gradient gel) of NAGIF: lane 1, purified NAGIF (protein stain); lane 2, wide-range protein markers (Novex, from top: myosin, 200 kDa; β-galactosidase, 116.3 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.3 kDa; glutamic dehydrogenase, 55.4 kDa; lactate dehydrogenase, 36.5 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; aprotinin, 6 kDa); lane 3, purified NAGIF (carbohydrate stain). (B) Molecular weight determination of NAGIF by HPLC system. It was carried out by measuring the elution volumes of several standard substances, calculating their corresponding K_{av} values [$K_{av}=(V_e - V_o/V_t - V_o)$: V_e, elution volume for the protein; V_o, column void volume; V_t, total bed volumn], and plotting their K_{av} values *versus* the logarithm of their M_r. (C) Comparison of the NH_2-terminal sequence of NAGIF and internal sequence of Zn-α_2-glycoprotein. Search for sequence homology was performed using the FASTA program.

TABLE I. **Purification of NAGIF.**

	Total protein (mg)	Total activity (unit)	Specific activity (U/mg)	Recovery (%)
Liver extracts	2,090	43,600	21	(100)
Sephacryl S-300	197	19,200	97	44
DEAE Sepharose	25.5	12,300	482	28
Sephacryl S-100	3.22	7,320	2,273	17
Mono Q	0.84	4,320	5,143	10

ED_{50} is the concentration required for 50% growth inhibition of FSC at 2×10^4 cells per well after incubation for 24 h in 10% FBS containing DMEM at 37°C.

TABLE II. **Growth inhibition of various mammalians cells in the presence of NAGIF.**

NAGIF (μg/ml)	HSC	3T6	BCE	B16
0.0	100	100	100	100
0.05	93.6±5.5	96.2±2.8	83.5±0.7	93.3±1.1
0.1	75.5±1.6	78.0±2.4	68.0±1.8	95.5±1.0
0.2	50.3±2.6	62.4±4.6	19.3±2.0	93.1±2.3
0.4	29.9±2.1	54.8±2.0	13.4±1.6	92.8±1.4

The various cells were cultured at 2×10^4 cells/ml in suitable media. The cell proliferation was determined by thymidine incorporation and the inhibition was calculated as follows: Inhibition (%) = (1 − cpm of experimental culture/cpm of control culture) × 100. The results were expressed as mean ± SE of three separate experiments.

eration of several cells (Table II). Interestingly, bovine capillary endothelial cells were most sensitive to NAGIF, but B16 mouse melanoma cells were completely resistant. When NAGIF was analyzed by SDS-PAGE under reducing conditions and by HPLC gel filtration, the purified inhibitor showed a single band with approximate M_r of 38 kDa (Fig. 2, A and B). The NH_2-terminal sequence of NAGIF could not be aligned with other proteins in the data banks (PIR, Swissprot, Genbank). However, as depicted in Fig. 2C, the sequence AEPVEPWS of NAGIF is identical to an internal portion of rat Zn-α_2-glycoprotein (ZAG), which was generated by carboxyl-terminal cleavage of Lys^{59} (*18*). NAGIF seems to be a C-terminal portion of rat ZAG. The difference between the apparent M_r of NAGIF (38 kDa) and the calculated M_r (31.4 kDa) of truncated ZAG may be due to glycosylation of Asn^{120} and Asn^{251} in ZAG. To investigate whether NAGIF treatment causes apoptosis in HSC, DNA fragmentation, a typical phenomenon of apoptosis, was examined by using TUNEL, ELISA, and an agarose gel method. The typical nucleosomal DNA ladders appeared 8 h after NAGIF treatment, and the intensity of fragmented DNA was increased consistently, indicating the progression of massive fragmentation of chromosomal DNA (Fig. 3A). In addition, a sandwich-enzyme-immunoassay was performed for the quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments after NAGIF-induced cell death. The amounts of mono- and oligonucleosomes in the cytoplasmic fraction of HSC lysates were increased in a dose-dependent manner by NAGIF (Fig. 3B). As shown in Fig. 3C, after treatment of PBS control for 12 h, staining by the TUNEL method appeared minimal and random, indicating a minimal level of apoptosis. In contrast, HSC treated with NAGIF showed a majority of the cells intensely staining with FITC.

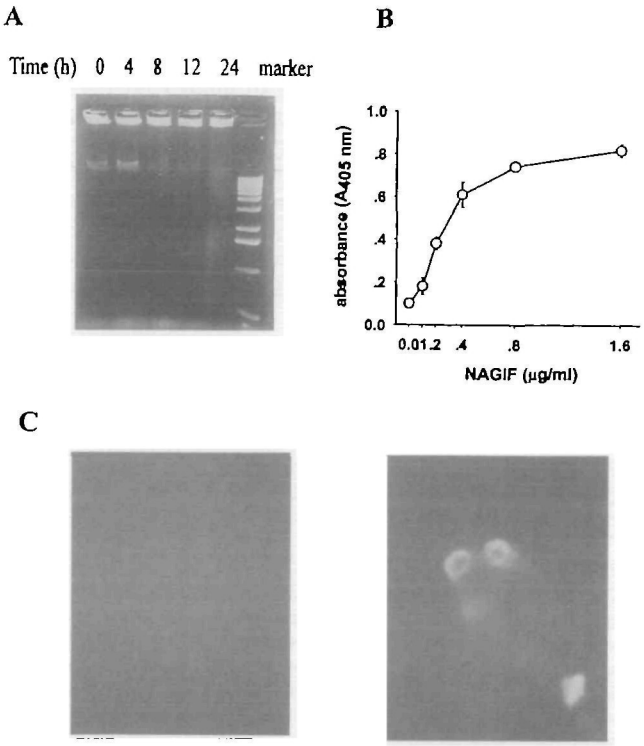


Fig. 3. **Induction of apoptosis in HSC by NAGIF.** (A) Time course of NAGIF-induced DNA fragmentation. HSC were plated on 60-mm dishes and were cultured to approximately 60% confluency. The cells were then exposed to 250 nM NAGIF in a 5% serum containing DMEM for various times. Soluble cytoplasmic DNA was isolated and run on 1.2% agarose gels. The DNA was stained with ethidium bromide. (B) ELISA assay of NAGIF-induced apoptosis. HSC of 60% confluency were exposed to different NAGIF concentrations for 12 h. After cell lysis and centrifugation, cytoplasmic histone associated DNA fragment was determined with anti-histone and anti-DNA-peroxidase antibody. The amount of peroxidase retained in the immunocomplex was determined photometrically with ABTS at 405 nm. (C) TUNEL analysis of NAGIF-induced apoptosis. HSC of 60% confluency were treated with 250 nM NAGIF for 12 h. Then the cells were fixed with ethanol/acetic acid for 1 min and washed with PBS. Apoptotic cells were identified *in situ* by using terminal deoxynucleotidyl transferase to transfer biotin-dUTP to the free 3′-OH of cleaved DNA. The biotin-labeled cleavage sites are then visualized by reaction with FITC conjugated avidin. The left panel shows FITC staining fluorescence in normal HSC and the right panel shows FITC staining fluorescence in NAGIF-treated HSC.

DISCUSSION

Growth inhibitors are considered to be of similar importance to growth factors in the regulation of cell proliferation *in vitro* and *in vivo* (*19*). The liver has been widely used as a source of growth inhibitor (*20*). Arginase was previously identified as an inhibitory substance present in liver homogenates (*6–9*). Besides arginase, several proliferation inhibitors with different physicochemical and biological properties were reported to be present in the liver (*20–26*). Here, it was demonstrated that inhibitory activity remained after the removal of arginase by ion exchange chromatography or compensation of arginine in culture media by amino acid supplement, suggesting the presence of another inhibitor. From the biochemical data presently

available, no components have been described with molecular properties similar to the inhibitor that was isolated (38 kDa, ED_{50} = 5.1 nM). NH_2-terminal sequence analysis raises the possibility that NAGIF is a truncated form of rat Zn-α_2-glycoprotein (ZAG). ZAG is a plasma protein with a high degree of sequence similarity to class-I major histocompatibility complex antigens, and Northern blot analysis showed that ZAG is expressed in the liver (*18*). The function of ZAG is unknown, but several studies have shown that it may participate in the development of mammary diseases including breast cysts and breast carcinomas (*27, 28*). A recent report suggested that tumor production of ZAG is responsible for the lipid catabolism (*29*). The NAGIF isolated in the present study could either be metabolically derived from rat ZAG or it could be derived from the proteolysis of rat ZAG during purification. Although ZAG or a fragment of ZAG has a yet to be reported to show growth inhibitory activity, there is increasing evidence that the RGDV region of human ZAG interacts with the cell surface integrins to regulate cell attachment and spreading (*30, 31*). Unfortunately, an amino acid sequence (RGD or LDVP) associated with cell adhesion was not found on the rat ZAG. The action mechanism and physiological significance of NAGIF remains to be explored. NAGIF had no significant inhibitory effect on the proliferation of tumor cell line B16 mouse melanoma, while in addition to inhibiting HSC proliferation, it also strongly suppressed the multiplication of bovine capillary endothelial cells. These results indicate that the antiproliferative effect of NAGIF depends on cell type.

In conclusion, NAGIF is a liver-derived protein that inhibits HSC proliferation *in vitro*. The novelty of NAGIF is supported by its apparent unique physicochemical properties and biological activity. The inhibitor may be intrinsically endogenous in nature. Although its biological function is not known, NAGIF is believed to be a negative effector in the regulation of HSC.

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