

# Characterization of Acute-Phase Proteins (Apps)

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## 1. Introduction

Upon Injury, a complex biological response of tissues is initiated to protect the organism and remove the injurious stimuli then trigger the healing process. Inflammation is a part of this complex response. The local response to tissue injury or infection is acute inflammation. Without inflammation, wounds and infections would never heal. This response is called the acute-phase response. After the beginning of inflammation, a large number of changes in the physiological system occur and last for 1 or 2 days; the system then returns to normal for 4 to 7 days provided there is no further stimulation. This systemic response is called acute-phase reaction (APR), also called acute-phase response. APR is characterized by fever and by an increased number of peripheral white blood cells. At the same time, cellular and biochemical changes occur in liver or other cells. One of the important events in acute-phase response is the change of the protein molecules in the plasma, known as the acute-phase proteins (APPs) (1-6).

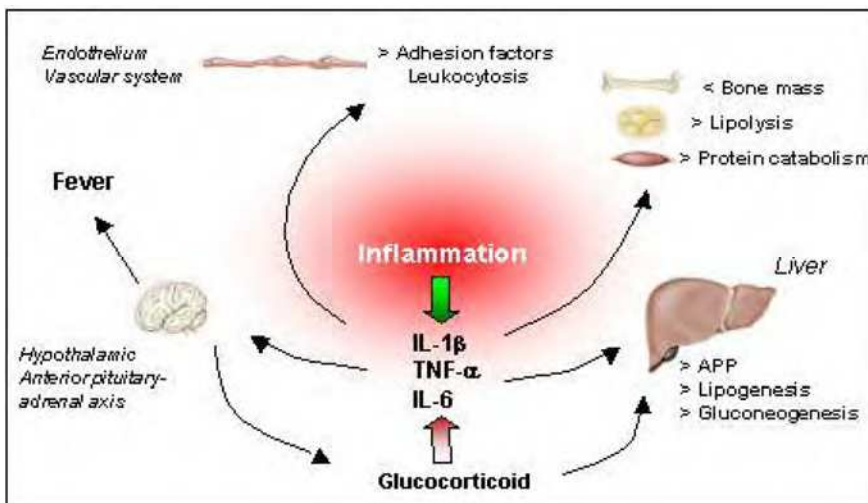


Fig. 1. The acute phase reaction. Green arrow indicates the promoting activity. Red arrow indicates the inhibitory activity.

The level of acute-phase proteins changes rapidly in response to inflammation, and these proteins serve as useful indicators of stress and disease. The APPs are mainly synthesized and secreted from the liver, under cytokine stimulation (Fig.1 is from ref.7). These cytokines can drive the production of anti-inflammatory glucocorticoids by regulating the hypothalamic-pituitary-adrenal (HPA).

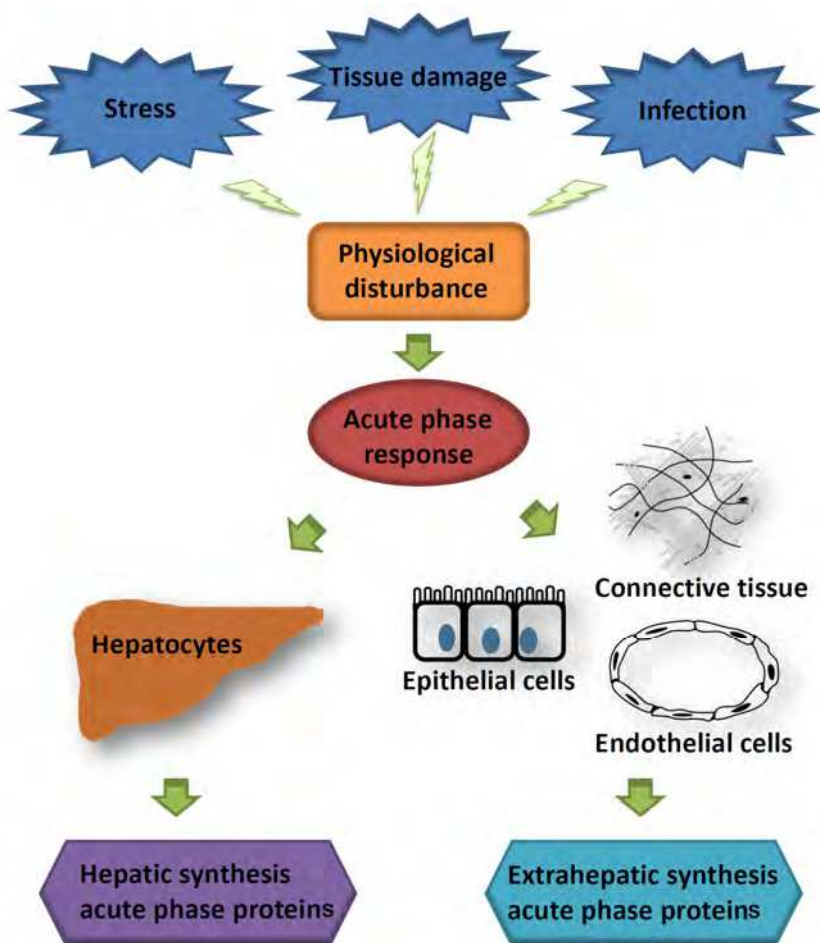


Fig. 2. The summary of acute phase protein production.

The three most common APPs are C-reactive protein (CRP), serum amyloid A (SAA) and serum amyloid P (SAP). Many other APPs have been found, and they all play important roles. According to the report (7), the APPs are stimulated by the release of cytokines such as IL-6 (Fig. 1), which is induced locally and systemically. APPs have showed a correlation with markers of oxidative stress (8). If the tissue damage stimulation has repeated pulses, the acute-phase reaction can become chronic. The chronic inflammation can continuously provide the increasing serum APPs. Some APPs would be expressed in the liver, stimulated

by injury; APP expression might also be a promoter of a benign or malignant tumor, such as SAA in ovarian tumors, and may trigger the tissue disorder. Acute-phase SAA is synthesized in the liver; extrahepatic production of SAA has been observed in several mammalian species (9). Based on these findings, the production of APPs can be summarized as in Fig. 2.

In addition to the production of acute-phase protein, other physical responses would happen during the acute-phase response. They are listed in Table 1 (Table is from ref.10). Acute-phase phenomena may be included in a large number of behavioral, physiological, biological and nutritional changes. APPs can be induced under all of these phenomena.

Neuroendocrine changes
Fever, somnolence, and anorexia
Increased secretion of corticotropin-releasing hormone, corticotropin, and cortisol
Increased secretion of arginine vasopressin
Decreased production of insulin-like growth factor I
Increased adrenal secretion of catecholamines
Hematopoietic changes
Anemia of chronic disease
Leukocytosis
Thrombocytosis
Metabolic changes
Loss of muscle and negative nitrogen balance
Decreased gluconeogenesis
Osteoporosis
Increased hepatic lipogenesis
Increased lipolysis in adipose tissue
Decreased lipoprotein lipase activity in muscle and adipose tissue
Cachexia
Hepatic changes
Increased metallothionein, inducible nitric oxide synthase, heme oxygenase, manganese superoxide dismutase, and tissue inhibitor of metalloproteinase-1
Decreased phosphoenolpyruvate carboxykinase activity
Changes in nonprotein plasma constituents
Hypozincemia, hypoferremia, and hypercupremia
Increased plasma retinol and glutathione concentrations

Table 1. Other acute phase phenomena

## 2. Acute-phase proteins (APPs)

Currently, numerous proteins are considered APPs, human APPs are listed in Table 2 (10). (Table 2 is from ref.10). The proteins can serve as inhibitors or mediators of the inflammatory processes. These proteins are a large and varied group of glycoproteins that would appear in the bloodstream and would be unrelated to immunoglobulin being responsive to inflammatory reaction (11).

**Proteins whose plasma concentrations increase**

- Complement system
  - C3
  - C4
  - C9
  - Factor B
  - C1 inhibitor
  - C4b-binding protein
  - Mannose-binding lectin
- Coagulation and fibrinolytic system
  - Fibrinogen
  - Plasminogen
  - Tissue plasminogen activator
  - Urokinase
  - Protein S
  - Vitronectin
  - Plasminogen-activator inhibitor 1
- Antiproteases
  - $\alpha_1$ -Protease inhibitor
  - $\alpha_1$ -Antichymotrypsin
  - Pancreatic secretory trypsin inhibitor
  - Inter- $\alpha$ -trypsin inhibitors
- Transport proteins
  - Ceruloplasmin
  - Haptoglobin
  - Hemopexin
- Participants in inflammatory responses
  - Secreted phospholipase A<sub>2</sub>
  - Lipopolysaccharide-binding protein
  - Interleukin-1-receptor antagonist
  - Granulocyte colony-stimulating factor
- Others
  - C-reactive protein
  - Serum amyloid A
  - $\alpha_1$ -Acid glycoprotein
  - Fibronectin
  - Ferritin
  - Angiotensinogen

**Proteins whose plasma concentrations decrease**

- Albumin
- Transferrin
- Transthyretin
- $\alpha_2$ -HS glycoprotein
- Alpha-fetoprotein
- Thyroxine-binding globulin
- Insulin-like growth factor I
- Factor XII

Table 2. Human acute phase proteins

## 2.1 Classification of acute-phase proteins (APPs)

Based on the protein concentration in plasma, APPs can be divided into two classes.

### 2.1.1 Negative acute-phase protein

APPs are produced by the liver and have an increased concentration in the serum. When APP concentration in the serum is decreased, the APPs are called negative APPs. Albumin, transferrin, transthyretin and retinol-binding protein (vitamin A binding protein, RBP) have been found as negative APPs (12). In chronic inflammation in humans, especially in developing countries, vitamin A deficiency is serious (13, 14). It is well-known to have a negative feedback effect on immunity. As this stress, nutrient deficiency, RBP would be reduced.

### 2.1.2 Positive acute-phase proteins

When APP concentration in the serum increases, the APPs are called positive APPs. They include such proteins as CRP, mannose-binding protein,  $\alpha$ -1 antitrypsin, etc, as listed in Table 3 (Table is from ref.5). The overall changed APP concentration in the serum includes negative and positive APPs. The APP pattern may vary from one species to another. Serum amyloid p-component (SAP) is an APP in mice but not in humans. Age may also be a factor. For example, some APPs exist in the infant stage normally, but they may not be found in adults. Furthermore, some APPs would be increased in some species but decreased in others. Transferrin is a negative APP in most mammalian species, but it is a positive APP in chickens (15). According to the report of González et al., (16), they mentioned that the serum albumin would be decreased significantly at 48h and a lot of APPs would be increased, such as fibrinogen, SAA and Hp etc. However, there has no evidence to conclude the correlation between albumin and induction of positive APPs.

Mammals	Birds
Positive reactants	
TNF- $\alpha$ , IL-1, IL-6, cortisol	TNF- $\alpha$ , IL-1, IL-6, cortisol
SAA, CRP, Hp, AGP, etc.	SAA, CRP, hemopexin, AGP, etc.
Fibrinogen, Ceruloplasmin Cu	Fibrinogen, Transferrin, Ceruloplasmin Cu, Ca
Negative reactants	
TTR, RBP	Hp
Albumin, Transferrin	Albumin
Fe, Zn, Ca	Unbound serum iron, Zn

TNF: Tumour necrosis factor; IL: Interleukin; SAA: Serum amyloid A; CRP: C-reactive protein; Hp: Haptoglobin; AGP:  $\alpha$ 1-acid glycoprotein; Cu: Copper; Ca: Calcium; TTR: Transthyretin; RBP: Retinol binding protein; Fe: Iron; Zn: Zinc

Table 3. Major positive and negative acute phase reactants in mammals and birds.

## 2.2 The function of APPs

The function of APPs has not been completely clarified. In general, the positive APPs serve different physiological functions in the immune system and in regulating and trapping

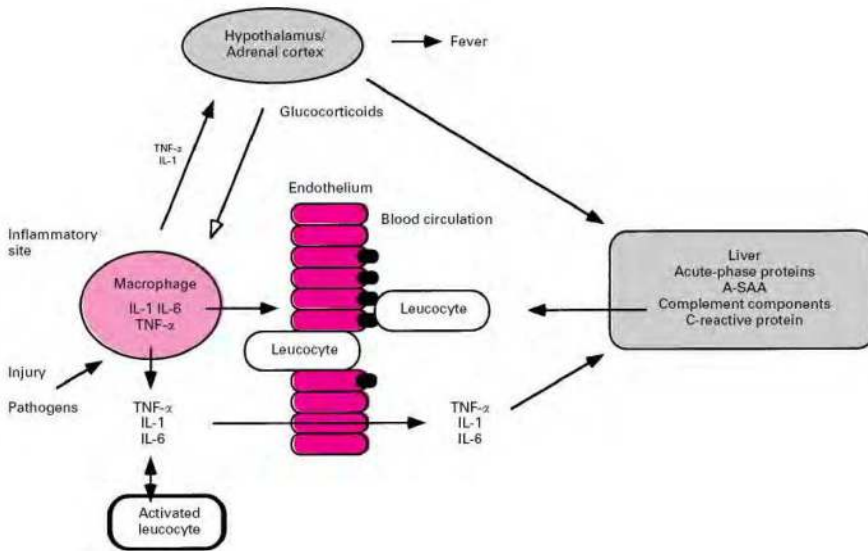
infected microorganisms and their products. In addition, the alteration of APP production can serve a useful purpose in inflammation, healing to injury or adaptation to infection. For example, the concentration of C-reactive protein (CRP) in serum rose significantly under acute-phase reaction; it is referred to as an acute-phase protein. It can participate in inflammatory response by inducing production of inflammatory cytokines and display anti-inflammatory effects (17). CRP, characterized as a calcium-dependent binding to various substrates, such as DNA or cellular proteins, increases dramatically in response to tissue-destructive processes (18). Currently, CRP has been found in direct stimulation of angiogenesis and may play a role in vessel formation (19). It indicates that APPs have multiple functions in the biosystem.

### 2.3 Regulation of APPs

Acute-phase reaction (APR) is a systemic response to injury and/or infection associated with endocrine and metabolic changes, including alteration of behavior, body temperature, production of cytokines and induction of APPs. Inflammatory cytokines, produced by inflammatory cells, are induced locally and systemically. The inflammatory mediators activate many cells and trigger a systemic release of cytokines (as pro-inflammatory cytokines). The increased serum-cytokines would result in the production of APPs. IL-1, TNF, INF- $\alpha$  are major and important cytokines for the expression of inflammatory mediators to induce the production of cytokines from the liver, such as IL-6. It is the major mediator of liver secretion of many APPs. In mammals, APR activities are enhanced indirectly by the activation of the pituitary (Fig. 1). As the APR occurring, increase of the glucocorticoids is a result of cytokine secretion from the pituitary. The human acute phase serum amyloid A (SAA), a positive acute phase protein, was up-regulated significantly after stimulation by glucocorticoid and cytokine in human hepatoma cells (20). Besides, many non-hepatic cell types including monocytes, endothelial cells and others have been shown to express SAA in high levels when stimulated with cytokines and glucocorticoids (21). This indicates that cytokines and glucocorticoids regulate the synthesis of APPs (22). Glucocorticoid can be a positive and negative regulator of APP synthesis during APR (Scheme 1 is from ref.22).

To our knowledge, cytokines are released from an injury or pathogen's infection site. The proinflammatory cytokines, TNF, IL-1 and IL-6, are considered the primary mediators of the APR. Upon injury or infection, macrophage or circulating monocytes are activated; they release cytokines locally and stimulate the liver to synthesize the APPs. At the same time, these early responsive pro-inflammatory cytokines can also activate the hypothalamus/adrenal cortex and induce fever and synthesis of glucocorticoids. Glucocorticoids have been proved to enhance the cytokine-dependent APPs' synthesis in the liver during APR. Numerous studies have provided evidences that glucocorticoids clearly show positive effects on APP synthesis during APR by way of a synergistic enhancement of pro-inflammatory cytokine effects (23–25).

APPs are almost glycosylated and change with their structure of side sugar chain during different inflammatory processes (11). Glycosylation changes in APPs could be markers of disease. As an example,  $\alpha$ -1-acid glycoprotein (AGP) is an APP and has expressed more sialyl Lewis X (SLe<sup>x</sup>) linkages in pancreatic cancer (PaC) tissue than in inflamed pancreatic tissue; however, the SLe<sup>x</sup> linkages were barely detected in healthy pancreatic tissue (26). This reveals that the glycosylation of APPs can regulate the function of APPs.



Scheme 1. Activation of the AP response

The cytokine-mediated network linking the inflammatory site and target organs/tissues is shown. Following exposure to the stimulating agent, cytokines produced by macrophages act on neighbouring cells, e.g. leucocytes (monocytes, neutrophils and lymphocytes) and endothelial cells, in the vicinity of the inflamed sites. These cells themselves become activated and express additional cytokines and receptors. Endothelial cells express selectins (R) which recruit circulating leucocytes and platelets from the bloodstream. Cytokines released to the circulation induce the hepatic AP response, which involves the increased synthesis of AP proteins e.g. A-SAA, C-reactive protein and complement components. Stimulation of the central nervous system by cytokines induces fever and the synthesis of glucocorticoids by the adrenal cortex. Glucocorticoids enhance the hepatic AP response and at the same time feed back to down-regulate the local inflammation (open arrow) and its systemic inflammatory consequences.

### 3. Acute phase index

During the APR, there is an increase in APPs (positive APPs) and there are decreases in some APPs (negative APPs); the quantification of these proteins provides valuable clinical information in diagnosis and treatment. Numerous researchers have developed the method in animals to assess-nonhealthy animals versus healthy ones, and the calculating index is called the “acute phase index” (6, 27).

$$\text{Nutritional and acute phase index (NAPI)} = \frac{\text{Value of a rapid positive APP} \times \text{Value of a slow positive APP}}{\text{Value of a rapid negative APP} \times \text{Value of a slow negative APP}}$$

(Eq. is from ref.6)

In general, the calculated indexes were significantly higher in nonhealthy animals than in normal ones, and decreased indexes were observed after treatment. For calculating the indexes, the ratio between positive and negative acute-phase proteins has to be determined. If the ratio is combined with those of rapid and slow changes in positive and negative APPs, the acute-phase signal can be enhanced. The index has been used as a prognostic inflammatory for human patients, who might also have a nutritional deficiency. Determination of the index with several APPs can help in monitoring the health of individual subjects.

Before calculating the index, the protein concentrations have been measured quantitatively. Many technologies, such as the protein chip, the protein microarray method or quantitative polymerase chain reaction (qPCR), can be used for APP measurement. These technological developments should have crucial importance in future diagnostics.

#### 4. Acute-phase proteins related to oxidative stress

The reactive oxygen species (ROS) in the environment indicates the stress status. It has been implicated in inflammation. Elevation of ROS induces gene expression, which is involved in inflammatory and acute-phase responses (28, 29). It indicates the stress will trigger the APR and the occurrence of APPs synthesis. For example, patients with pressure sores present a systemic inflammatory response associated with the decrease of ascorbic acid levels, suggesting that the patient may be nutritionally deficient (30). Nutritional deficiency is a kind of stress *in vivo* and triggers the elevation of the ROS level. The high ROS level *in vivo*, known as an acute-phase status, includes alternations of APPs and is related to cell apoptosis. These may contribute to the development of pressure sores in patients and may impede the wound-healing process.

Infections are also associated with elevating the intracellular oxidative stress via the reaction of proinflammatory cytokines. The bactericidal factors and the inflammatory response confer oxidative stress to the cells that may lead to cell apoptosis. This means APR was developed by infections and initiated an increase in oxidative stress and possibly triggered cell apoptosis via APPs (31). For example, LCN2 could induce the increase of intracellular ROS significantly within a short time, upon the protein interaction with the cells and suppression by the ROS inhibitor (Fig. 3) (32).

This protein has been described as being mainly expressed in tissues that may be exposed to microorganisms (33), or detected in acute inflammatory response (34) in keeping with these conditions' stress status. Cowland *et al.* (35) showed that during lung inflammation, the human LCN2 protein (also called Neutrophil Gelatinase Associated Lipocalin; NGAL or human 24p3 protein) synthesis increases in the bronchial epithelial cells, so it was considered as a disease activity marker (36, 37). All of these imply that the LCN2 protein correlates with environmental stress and tissue damage. In cultured cells, the LCN2 protein increment is observed in response to glucocorticoid stimulation, and also in other conditions such as serum deprivation. Elevating the glucocorticoid level in circulation during stress may trigger the LCN2 protein being highly expressed under this stress condition and exert an autocrine control (38) during stress, thus playing a role in cell death (39). Over the years, ROS has been perceived as a biological hazard, causing oxidative damage to the cellular components, and leading to cancer, cell degeneration and disorders related to aging (40).



Based on the descriptions, APPs may initiate the aberrant cell growth under stress conditions.

In general, we have known that cytokines are stimulators of most APPs' synthesis during APR. At the same time, APP is also a stimulator of cytokine production as cells are responsive to the APP. Exposure of endometrial carcinoma cell line (RL95-2) to LCN2 for >24 h reduced LCN2-induced cell apoptosis, changed the cell proliferation and up-regulated cytokine secretions, including: interleukin-8 (IL-8), inteleukin-6 (IL-6), monocyte chemotatic protein-1 (MCP-1) and growth-related oncogene (GRO) (Fig. 4) (41). These cytokines may change the growth of the cells.

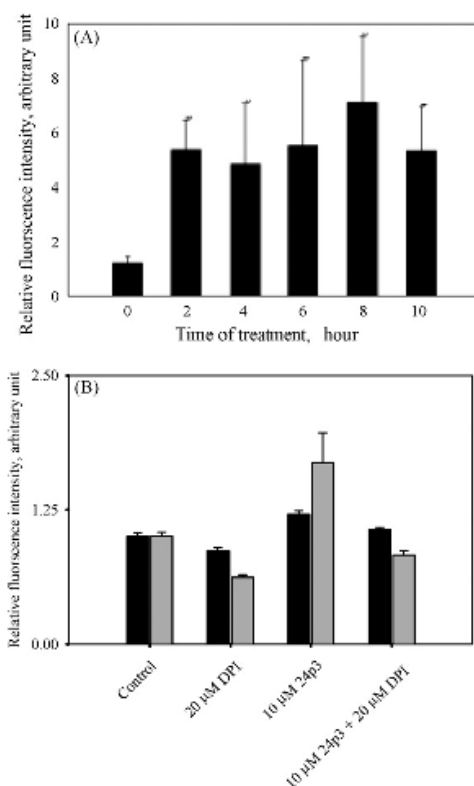


Fig. 3. Induction of ROS by 24p3 protein (also called mouse LCN2) treatment. RL95-2 cells were treated with 5 $\mu$ M 24p3 protein for various time intervals, followed by incubation with 20 $\mu$ M DCFH-DA for 15 min. The amount of intracellular ROS can be quantified by detection via a microfluorometer with excitation and emission wavelengths at 485 and 535 nm, respectively. The results were confirmed in multiple experiments and presented as the mean $\pm$ S.D. (\*\*) $p < 0.01$ , (\*\*\*) $p < 0.001$ ,  $n = 5$ . (A) The time course of 24p3 protein effect on RL95-2 cells. (B) The DPI prevents the 24p3 protein from inducing ROS in RL95-2 cells. The black bar indicates the 30-min incubation and the gray bar indicates 60-min incubation of 24p3 protein with the cells.

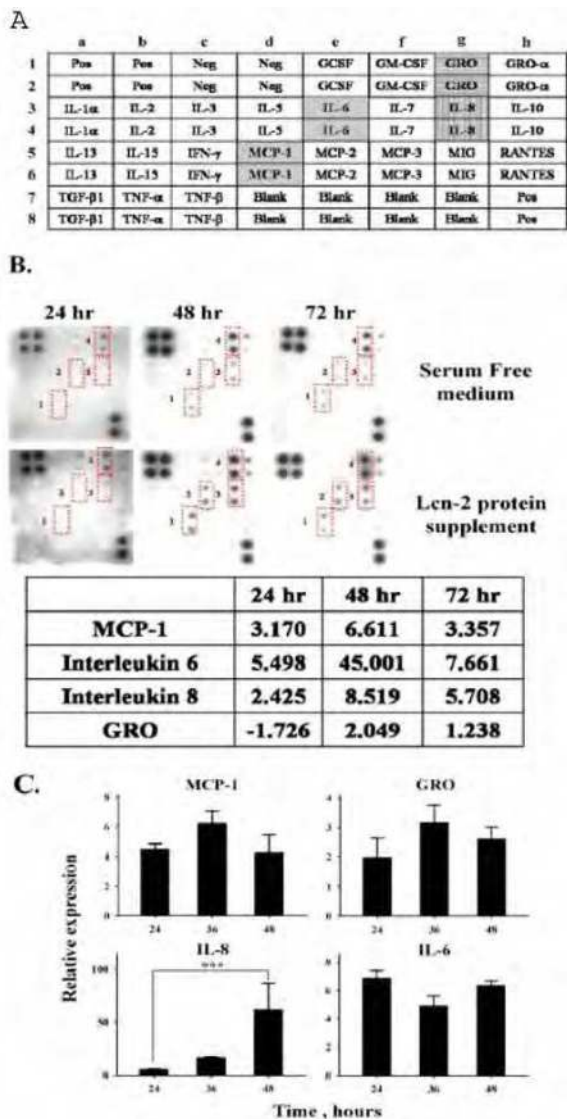


Fig. 4. Cytokine array analysis of conditioned medium from Lcn-2-treated RL95-2 cells.

(A) RayBio® Human Cytokine Antibody Array I Map. (B) The signals of cytokine concentrations in conditioned media; significant signals are labeled with Arabic numerals: 1, MCP-1; 2, IL-6; 3, IL-8; and 4, GRO. Relative intensities of these four cytokines are shown in the lower panels with the intensities of values from control serum-free media set as 1. (C) mRNA levels of cytokines in Lcn-2-treated cells. A total of  $4 \times 10^4$  cells were incubated with or without 10  $\mu$ M Lcn-2 in serum-free medium for 24, 36 and 48 h. After incubation, the total RNA isolated from cells was reverse transcribed and amplified by RT-PCR using primers for MCP-1, IL-6, IL-8 and GRO. Levels of mRNA were determined by semi-quantitative RT-PCR.

However, IL-8 mRNA and protein levels were dramatically increased in LCN2-treated RL95-2 cells. The major focus was to determine the IL-8 effect on LCN2-treated RL95-2 cells. Adding recombinant IL-8 (rIL-8) resulted in decreased caspase-3 activity in LCN2-treated cells, whereas the addition of IL-8 antibodies resulted in significantly increased caspase-3 activity and decreased cell migration. Data indicate that IL-8 plays a crucial role in the induction of cell migration (Fig. 5) (41).

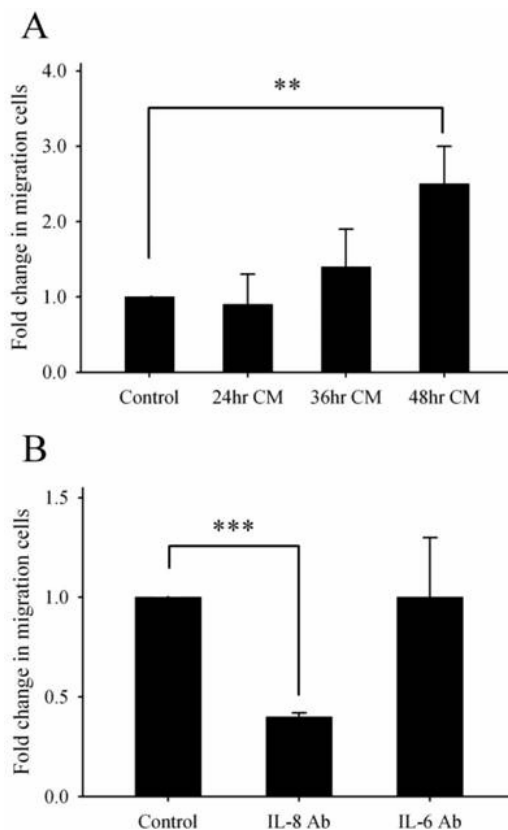


Fig. 5. Effects of LCN2-conditioned medium on RL95-2 cell migration. RL95-2 cells were stimulated with LCN2 for varying lengths of time, and then culture supernatants were collected from conditioned medium. Cytokine antibodies (anti-IL-8 or anti-IL-6) were added to clarify the effect of these cytokines on 48 h CM-induced cell migration. Cell migration was measured using the transwell assay in the absence or presence of conditioned medium. (A) After incubation for 24 h, RL95-2 cells were stained with 0.5% crystal violet, and cells were counted in ten random fields under a microscope at X20 magnification. (B) OD570 values of cells on the lower surface of the membrane extracted with 33% acetic acid after anti-IL-8 neutralization. These data are representative of three independent experiments. The cell number of RL95-2 incubation under 48 h CM for 24 h is as a control experiment. Values are the mean  $\pm$  SEM. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

Interestingly, LCN2-induced cytokines, secretion from RL95-2 cells, could not show the potent cell migration ability with the exception of IL-8. We concluded that LCN2 triggered cytokine secretions to prevent RL95-2 cells from undergoing apoptosis and subsequently increased cell migration. We hypothesize that LCN-2 increased cytokine secretion by RL95-2 cells, which in turn activated a cellular defense system. This means the APP would secrete from the cell under stress and then promote the secretion of cytokines and enhance cell growth.

## 5. Acute-phase proteins related to cytokines

Furthermore, tumorigenesis and the invasion capacity of tumor cells are mediated by growth factors, including cytokines, which promote cell proliferation, including the invasion of tumor cells. Cytokines are a part of homeostasis, stress response, inflammation and tumorigenesis (42). IL-6 is known to be a proinflammatory cytokine. The elevation of IL-6 levels in patients with a Ras-induced cancer can trigger the other secreting cytokine, IL-8, an important factor for tumor growth in HeLa cells. It provided the information linking cytokines to tumorigenesis and also hinted at the linkage of APPs to tumorigenesis (43–46). IL-8 can be induced in endometrial cells by an acute-phase protein, LCN2, also an angiogenic factor in some cancers (47). According to the report of Arenberg *et al.*, (37) inhibition of IL-8 expression would reduce the tumor development of human lung cancer in mice. It was announced that the IL-8 plays role in mediating angiogenesis during tumorigenesis of human cancer. IL-8 thereby offers the potential to promote the formation of tumor (48). Based on the result of Lin *et al.* (41), LCN2 could induce IL-8 expression and secretion and enhance cell migration and invasion. This suggests IL-8 as an LCN2-induced tumor factor. It indicates that the LCN2-induced IL-8 secretion from uterine endometrial cells and promotes the cell migration therefore the result provides evidence for APP playing a role in tumorigenesis.

## 6. Acute-phase proteins as tumorigenic factors or diseases inducers

APPs may initiate tumor formation indirectly via induced cytokines, or/and directly by themselves. To our knowledge, serum amyloid A (SAA) is an APP; it has been found that it may contribute the role in directing and enhancing the tumor process. Especially, numerous studies on SAA had been focused on the tumor progression.

The acute-phase serum amyloid A proteins (SAAs) are multifunctional proteins that would be up-regulated by proinflammatory cytokines during inflammation, infection, trauma or stress. Several biological effects of SAA have been described in relation to inflammation, including cell adhesion, cell migration, tissue infiltration of inflammatory cells, enhancing matrix metalloproteinases (MMPs) increasing expression of cytokines, or stimulating angiogenesis. The liver is the main site for SAA synthesis; however, extra hepatic expression has been found in many normal human tissues (20). In more recent studies, SAA in serum levels were found elevated in a wide range of cancers. SAA is expressed locally in colon carcinoma and also overexpression in endometrial carcinoma and ovarian tumors (49). Breast cancer is one of the most common cancers in women worldwide. Finding the potential biomarkers to identify the types of breast cancer is an important work in progress. LCN2 is a newly identified biomarker for breast cancer; clarification of the possible

mechanisms underlying its role in tumorigenesis is ongoing. LCN2, originally an inflammatory marker in both adipose and liver tissue (50), can be induced by lipopolysaccharides, suggesting that LCN2 is an acute-phase protein as in previous mentioned. However, increased systemic LCN2 levels in several diseases have been reported, such as chronic renal failure, chronic inflammation and some cancers. This may reveal that APPs play multi-functions in the biological system.

### 6.1 Endometrial hyperplasia

Human LCN2 (NGAL) is also found in human endometrial hyperplasia, a uterine disorder disease. Up-regulation of NGAL protein and mRNA was much higher in endometrial hyperplasia than in adenomyosis. Endometrial carcinoma is more often associated with endometrial hyperplasia (55%) than with endometrial adenomyosis (16%) (51). It seems to

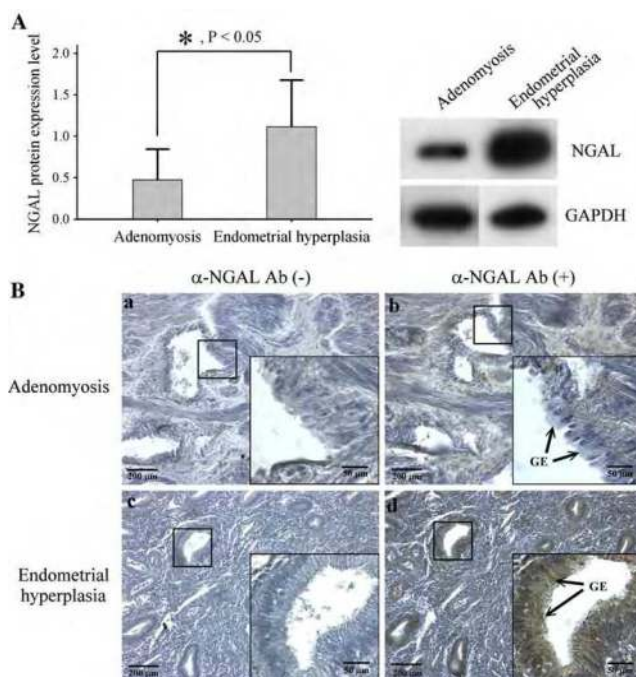


Fig. 6. Western blot analysis of NGAL in biopsy samples of endometrial disorders and immunohistochemical staining of endometrial adenomyosis and hyperplasia samples with anti-NGAL. A. Immunoblotting with anti-NGAL and anti-GAPDH. The data represent the mean  $\pm$  SEM from all biopsies (eight adenomyosis samples and 27 hyperplasia samples), and were calculated using the ratio of NGAL to the internal control (GAPDH). The right panel shows the signal after immunoblotting. B. NGAL immunoreactivity was not observed in adenomyosis in the presence of the NGAL antibody (b). Similarly, no NGAL expression was observed in either tissue in the absence of NGAL antibody (a, c). However, in the presence of NGAL antibody, strong NGAL expression was observed as a light brown color in the cytoplasm of glandular epithelia (GE) in endometrial hyperplasia (arrows). The nuclei were stained with hematoxylin (blue). Magnification x100 and x400 (inset).

indicate that endometrial hyperplasia itself presents a higher risk for progressing to endometrial carcinoma compared to adenomyosis. Therefore, we asked whether the cancer marker NGAL was responsive to tumorigenic transformation of endometrial hyperplasia and if it was expressed at high levels. Immunohistochemical analysis revealed that NGAL expression in the glandular epithelia was strongly elevated in endometrial hyperplasia compared to adenomyosis (Fig. 6) (52). Some studies have shown that NGAL can be a marker for ovarian, breast, bladder, and pancreatic cancers, and that it is a survival factor for thyroid neoplastic cells (53–55).

Consistent with our findings, a significant increase in NGAL expression in endometrial hyperplasia may be a part of the tumorigenic process. Therefore, we propose an autocrine function for NGAL, which may play a role in uterine disorders or carcinomas. Previous studies have suggested that NGAL overexpression may be required for tumorigenesis by promoting tissue invasion (56). Based on these studies, it seems likely that NGAL is related to the transition from endometrial hyperplasia to endometrial carcinoma. The data showed that NGAL expression was significantly increased in endometrial hyperplasia compared to adenomyosis and correlated positively with COX-2 expression ( $r = 0.42$ ). The increased COX-2 expression in hyperplasia may signify an early step in carcinogenesis. These uterine disorders may be inflammatory disorders and may trigger COX-2 gene expression. COX-2 is also important during tumorigenic transformation of hyperplasia (57, 58), where it decreases endometrial cell apoptosis and increases angiogenesis. It is the evidence for the correlation of APP with disease.

## 6.2 Endometriosis

Endometriosis, which usually develops in pelvic organs such as the ovaries and may contribute to infertility, is an estrogen-dependent disease characterized by the presence of endometrium-like tissue outside the uterine cavity (59). A relationship between ovarian endometriosis and certain types of ovarian cancer has been suggested, and endometriosis is believed to increase cancer risk. Endometriosis is similar to cancerous tumors in that it requires angiogenesis for expansion (60–62). Epithelial-mesenchymal transition (EMT) is a process whereby epithelial cells are converted to a mesenchymal phenotype and may be essential for the migration, invasion and relocalization of epithelial cells (59). EMT also can be induced by other signals, including the acute stress response (63). We hypothesized that EMT might be involved in the development of endometriosis. LCN2 is an oxidative stress factor that responds to environmental stress and triggers changes in cellular physiology. This signaling pathway may be activated under physiological as well as pathophysiological conditions (64). Cannito *et al.* found that intracellular ROS also are involved in the regulation of EMT (64), and Yanga *et al.* (65) found that LCN2 is associated with breast cancer progression *via* EMT. In addition, LCN2 also triggered cell migration and invasion (Fig. 7) (unpublished data), and this effect might contribute to the development of ectopic endometrial tissue implantation. Based on our evidence, we propose that LCN2 induces EMT in endometrial epithelial cells under nutrient-deprived conditions and thereby promotes the development of endometriosis.

In summary, during inflammation or stress, APPs can be induced by proinflammatory mediators and trigger the changes in cell physiological balance. Actually, cell apoptosis and cell proliferation are involved in the APPs triggered pro-inflammatory (cell apoptosis) or anti-inflammatory (wound-healing). According to Khatami's theory (66–68), acute phase

response is a highly regulated immune response to achieve a well-balance of cell death and cell growth in biological system; and indicates the APPs reaction is a kind of "Yin-Yang" doctrine. Therefore, the regulation of inflammatory response could initiate the challenge to the balance of tumoricidal versus tumorigenesis in immune system. The alteration of balance is considered as factor for causing the diseases; however, the regulatory pathways of APPs in disease formation remain unanswered. Future elucidation of the complex network of APPs, cytokines and pathological conditions is essential. The knowledge of APPs might be useful for providing an important method to monitor mammalian health.

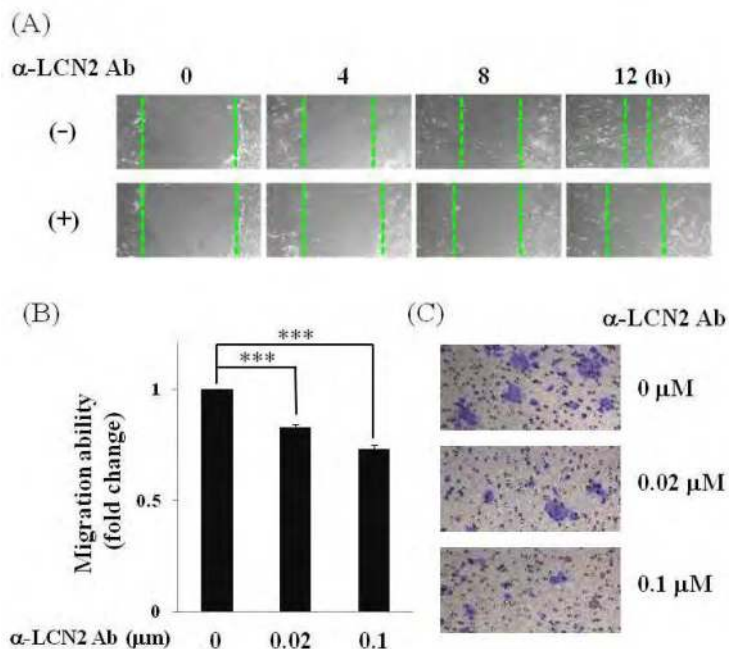


Fig. 7. Endometrial epithelial cell migration assays. Primary endometrial epithelial cells were harvested and then cultured in 1% FBS/DMEM/F12 medium for 48 h. The medium was collected and centrifuged to remove the suspended cells and cell debris and was used as a conditioned medium. Conditioned medium with or without 0.02  $\mu$ M LCN2 antibody was used for wound-healing experiments and the Transwell assay. A, Wound-healing assay. Endometrial epithelial cells ( $5 \times 10^4$ ) were cultured in 24-well plates for 48 h until near confluence ( $\sim 90\%$ ). A sterile 200- $\mu$ l pipette tip was used to scratch through the cells to simulate a wound. After conditioned medium was added, the scratches were observed microscopically over a 12-h period. The green lines indicate the edge of each side of the scratch to show cell migration. B, C, Transwell assay. Endometrial epithelial cells ( $1 \times 10^5$ ) in 100  $\mu$ l 1% FBS/DMEM/F12 medium were added to Transwell units. Conditioned medium (400  $\mu$ l) was then added, and cells were allowed to migrate for 24 h in a 37°C, 5% CO<sub>2</sub> incubator. After incubation, the cells in the membrane insert were stained as described in the text and visualized (C); cells were then counted (B).

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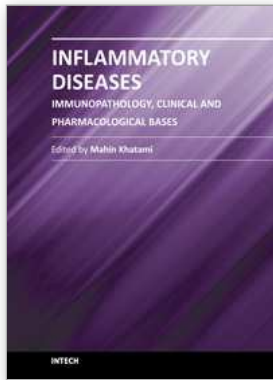


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