The Anti-inflammatory and Anti-viral Effects of an Ethnic Medicine: Glycyrrhizin

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Abstract

The extract of licorice (*Glycyrrhiza glabra* L.) has been widely used for many centuries in the traditional Chinese medicine as native anti-allergic agent. Glycyrrhizin (GL), a triterpenoid-saponin, extracted from the roots of licorice is the most effective compound for inflammation and allergic diseases in human body. The biological and pharmacological studies revealed that GL possesses many pharmacological effects, such as anti-inflammatory, anti-viral and liver protective effects, and the biological effects, such as induction of cytokines (interferon-γ and IL-12), chemokines as well as extrathymic T and anti-type 2 T cells. This review describes (i) the pharmacological property of GL as an effective anti-inflammatory and anti-viral drug; (ii) the biochemical characteristics of several GL-binding proteins (gbPs) involved in the anti-inflammatory and anti-viral effects of 68GL and the GL-induced selective inhibition of the phosphorylation of these gbPs by GL-binding protein kinases *in vitro*; and (iii) the mechanisms involved in the GL-induced inhibition of the replication of both RNA and DNA viruses. In addition, recent reports concerning the mechanical actions involved in the anti-inflammatory and anti-viral effects of GL *in vivo* and *in vitro* and its clinical effects on chronic active liver disease and viral infection are summarized.

Keywords: Glycyrrhizin, Anti-inflammatory, Anti-viral, Review

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Introduction

Glycyrrhizin (GL, 20β-carboxyl-11-oxo-30-norolean-12-en-3 β -yl-2-*O*- β -D-glucopyranuronosyl- β -D-glucopyranosiduronic acid, molecular weight = 822.92) is a triterpenoid saponin extracted from the roots of licorice (*Glycyrrhiza glabra* L.) and consists of one glycyrrhetic acid (GA, olean-11,13 (18)-diene-3 β, 30-diol-3 β, 30-di-*O*-hemiphthalate disodium salt) molecule as the aglycone connected to two glucuronic acid molecules through the hydroxyl group of C-3 by a glycosidic linkage (Fig. 1). During the last 30 years, the biological effects of GL have been extensively studied *in vitro* and *in vivo*. These studies include, among others, the metabolisms [1 – 4], pharmacokinetics [5 – 14], biochemical effects *in vitro* [15-20], clinical anti-viral effect [21-27] and anti-tumor effect of GL in human body and experimental animals [30, 31]. The pharmacological effect, anti-inflammatory effect, anti-viral effect as well as cancer and liver protecting effect of GL [32-34] are unique and outstanding among various anti-inflammatory natural compounds. These pharmacological properties of GL are verified clinically and mechanistically.

The pharmacological and biological effects of GL are explained on the basis of some logical backgrounds, because (i) the biochemical mechanisms involved in the GL-induced anti-inflammatory and anti-viral effects may be implicated with the GL-induced selective inhibition of the CK-II-mediated activation of the GL-binding functional cellular mediators [15-20]; and (ii) hepatitis C virus (HCV)-induced hepatic damage is due to the cytopathic effect of HCV and the inflammatory changes secondary to immune activation [25, 26]. Casein kinase CK-II could be copurified with its native phosphate acceptors (functional cellular proteins) from various cell sourses as GL-binding proteins (gbPs) by GL-affinity column chromatography. GL acts as a moderate anti-inflammatory medicine, because its potency is much less than the steroidal or non-steroidal clinical drugs, such as prednisolone, dexamethasone, indomethacin and diclofenac [32]. In addition, the *i.v.* administration with a high dose of GL exhibits the side effect of the steroidal drugs, such as salt retention and hypokalemia, but devoid of the gastrointestinal tract disturbing side effects of nonsteroidal anti-inflammatory drugs [33 - 35].

The anti-inflammatory and anti-viral effects in one molecule like GL are unavailable among anti-viral drugs. In addition, some viral infections, such as HCV, are complicated and followed by hepatocellular carcinoma (HCC; 30) and GL effectively inhibits HCC in combination with other anti-HCV drugs [26, 31]. This review describes mainly the anti-inflammatory and anti-viral effects of GL, and also summarizes the biochemical mechanisms involved in the GL-induced biological effects *in vitro* and *in vivo*.

Historical overview of GL

In 1959, the anti-inflammatory effect of GL in human body was originally reported by Finney [36]. In 1980, a pronounced anti-inflammatory effect of GL-derivatives was found [37], this effect was confirmed by other research groups [38, 39]. The anti-inflammatory effect of these GL derivatives, including oGA, remain to be unchanged under adrenalectomy. In 1979, the GL-induced anti-viral effect was reported by Pompei *et al.* [40]. As shown in Table 1, GL induces various biological effects, such as induction of interferon-γ (IFN-γ) production in mouse [41], augmentation of NK cells activity [42],

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Table 1- The GL-induced biological and clinical effects.

Year	Authors and remarked effects	References
1957	Finney *et al.* Ani-inflammatory effect	36
1979	Pompei *et al.* Anti-viral effect	40
1981	Ohuchi *et al.* Prostaglangin E2 production	38
1982	Abe *et al.* Induction of IFN-γ production	41
1983	Ito *et al.* Augmentation of NK cell	42
1987	Stewart *et al.* Induction of pseudoaldosteronism	33
1992	Kimura *et al.* Activation of extrathymic T cell	43
1996	Nakajima *et al.* Induction of anti-type 2 T cell	44
2000	Suzuki *et al.* Induction of β-chemokines	47
2001	Dai *et al.* (45); Utsunomiya *et al.* (46) Enhancement of IL-12 production	45, 46

extrathymic T cell in the liver of mouse [43] and anti-type 2 T cells in the thermally injured mice [44], the inducing ability of interleukin 12 (IL-12) in peritoneal macrophages [45], and in the thermally injured mice [46], induction of β-chemokines in the cultured peripheral blood mononuclear cells (PBMC) from HIV-1 positive patients, and indirect improvement of the resistance of host exposed to certain opportunistic pathogens [47]. Furthermore, the GL- and GA-induced biochemical effects (inhibition of the physiological activities of various enzymes and functional proteins *in vitro*) are summarized in Table 2.

The clinical effect of GL on acute and chronic viral hepatitis was demonstrated in 1984 [50]. The inhibitory effect of GL on the replication of varicella-zoster virus (VZV) *in vitro* [51] and HIV-1 *in vitro* [52, 53] and *in vivo* [54], respectively was reported. In 1990, the antigen expression of hepatitis A virus (HAV) and the reduction of its infectivity were inhibited dose-dependently by GL [23]. It was demonstrated that the GL-induced inhibition of the receptor-mediated endocytosis may be due to the prevention of viral penetration into the cells [23]. The inhibitory mechanisms of GL on the growth of hepatitis B virus (HBV; 24) and HIV-1 [18, 19, 21] in different experimental systems and aims were investigated. These reports show that GL is an effective clinical drug for HCV *in vivo* [50, 55]. The biochemical mechanisms involved in the anti-inflammatory and anti-viral effects of GL are more clearly explained through the identification and biochemical characterization of the novel gbPs, which are responsible for the cellular mediators involved in these GL-induced biological effects at the cellular level.

Pharmacokinetics, metabolisms and determination of GL

GL is hydrolyzed by human intestinal flora to the aglycone GA (Fig. 1), which is also an active compound in human body [4]. GL is

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Table 2- The GL-induced biochemical effects and the inhibition of the physiological activities of the targeting functional mediators by GL or GA *in vitro*

Year	Authors and remarked effects	References
1975	Ulmann *et al.* Glucocorticoid receptor	48
1978	Tamura *et al.* ⊿45α- and 5β-reductase	101
1981	Ohuchi *et al.* Prostaglandin E2	38
1986	Shiki *et al.* Phospholipase A2	49
1988	Ohtsuki and Ishida Casein kinase II (CK-II)	67, 68
1991	Shamsa *et al.* Protein kinase A	13
1993	Ohtsuki *et al.* Lipoxygenase	86
1994	Ohtsuki *et al.* Glucocorticoid receptor and Hsp-90	69
1995	Kato *et al.* 11β-Hydroxysteroid dehydrogenase	103
1996	Furuya *et al.* Hyaluronidase inhibitor	16
1997	Francischetti *et al*. Thrombin inhibitor	78
1998	Ohtsuki *et al.* *Habu* snake venom phospholipases A2	19
2000	Haneda *et al.* HIV-1 protease	18
2001	Sakamoto *et al.* DNA-binding ability of HMG1	65
2001	Shimoyama *et al.* Human type IIA phospholiase A2	72
2001	Tanigawa *et al.* RNase activity of angiogenin 1	74
2003	Kawakami *et al.* Complement C3 and C3a	75

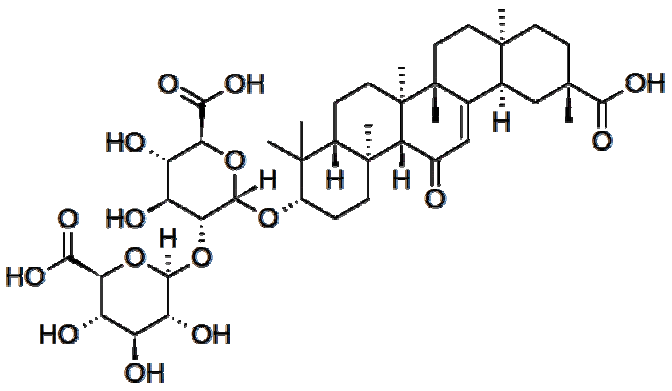

Fig. 1- Glycyrrhizin

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undetected in plasma at any time, but a considerable concentration of GA is detectable after oral administration of GL [6]. Meanwhile GA is undetectable in plasma of germ-free rats at 12 hrs after oral administration of GL. The hydrolysis of GL to GA by bacterial β-D-glucuronidase occurs slowly [2]. Actually, GL is administered by *i.v.* route to the patients with chronic viral hepatitis, but its intra-peritoneal administration has a better bioavailability over the *i.v.* and oral routes in rats [56 - 58].

Both GL and GA have enhancing activity on the intestinal absorption of co-administered drugs [59, 60]. This is estimated by changes in trans-epithelial electrical resistance and the permeation of sodium fluorescein in Caco-2 cell monolayers. It is further evaluated through the absorption of salmon calcitonin (sCT) in rat colon. The co-administration of sCT with GA in the rat colon induces the strongest plasma calcium-lowering effect and detects the highest plasma concentration of sCT. The absorption of amphotericin B lyophilized mixture with dipotassium glycyrrhizinate at a 1:9 molar ratio from a medium chain triglyceride base is significantly superior to that from the hydrophilic base of macrogol [59]. Co-administration of GL with prednisolone (PLS) results in an increase in the area under the curve (AUC), a decrease in total plasma clearance (CL) and an enhancement of the mean residence time (MRT) of PLS [61].

To determine the serum level of GL in humans, a simple and sensitive semi-micro high-performance liquid chromatography (HPLC) was established [62]. The detection limit of in serum is approx. 100 ng/ml (approx. 0.1 nM), which enable to determine the serum level of GL after administration of a therapeutic dose. In clinical practice, GL is used for its anti-viral and anti-inflammatory effects. This method is expected to aid in the safe and efficient use of the drug in clinical practice. Another useful detection method is also establised [63]: an enzyme-linked immunosorbent assay for glycyrrhizin using anti-glycyrrhizin monoclonal antibody and an eastern blotting technique for glucuronides of glycyrrhetic acid.

The binding site of GL on human serum albumin is determined by the competitive displacement experiments with GL and ibuprofen (IBU) (diazepam site), warfarin (WAR), salicylate (SAL) (digitoxin site) or deoxycholic acid (DCA) by means of the ultrafiltration technique [64]. The specific GL-binding site on human serum albumin may be located mostly within the low-affinity IBU-binding domain and partialy within the specific WAR-binding and the low-affinity SAL-binding domains. In contrast, at least two DNA-binding basic proteins [histone (14) and high mobility group 1 protein (HMG1; 65)] have a high affinity with GL and the binding of GL to them reduce their DNA-binding abilities *in vitro*.

GbPs involved in the GL-induced biological effects

Using a GL-affinity HPLC column, several GL-binding cellular proteins (gbPs) are selectively purified from various cell sources and their physiological activities are biochemically characterized *in vitro* [13 – 20]. Table 3 summarizes various gbPs purified and characterized from various cell sources, including synovial fluids of patients with rheumatoid arthritis and viral gene products (recombinants), by a GL-affinity column chromatography. To identify and characterize functional gbPs involved in the GL-induced biochemical effects, at least three PLA2s

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Table 3- The GL-induced anti-viral effects and the inhibition of viral infection by GL or GA.

Year	Authors and remarked effects	References
RNA viruses		
1984	Su *et al.*	
	Hepatitis C virus	50
1987	Ito *et al.*	
	Human immunodeficiency virus type 1	52
1990	Crance *et al.*	
	Hepatitis A virus	22
1996	Watanabe *et al.*	
	Murine retrovirus	117
1997	Badam	
	Japanese encephalitis virus	113
1997	Utsunomita *et al.*	
	Influenza A2 virus	114
2002	Tandon *et al.*	
	Hepatitis E virus	115
2003	Crance *et al.*	
	Flavivirus	118
2003	Cinatl *et al.*	
	Corona SARS virus	119
2003	Fujioka *et al.*	
	Hepatitis C virus	154
DNA viruses		
1987	Baba *et al.*	
	Varicella-zoster virus	51
1994	Numazaki *et al.*	
	Human cytomegalovirus	121
1995	Utsunomiya *et al.*	
	Herpes simplex virus	122
1996	Sato *et al.*	
	Hepatitis B virus	161

(PA2Y, PA21 and PA2B), metaloprotease and a 55 kDa gbP (gp55), an apoxin I-like protein are purified from *Habu* snake venom as gbPs [15]. The inhibitory effect of GL on the hemolytic activity of gp55 and its L-amino oxidase (LAO) with hemolytic activity is sensitive GL *in vitro* [20].

Furthermore, protein kinase A (PKA; 13, 66) and CK-II [18, 67-69] associated with their effective phosphate acceptors (p36 and gp100) are co-purified from different cell sources using a GL-affinity HPLC column [66, 69]. GL effectively inhibits the protein phosphorylation by these GL-binding protein kinases *in vitro*. Under similar experimental conditions, hyaluronidase (HAse) is purified from bovine testis as a gbP [16]. GL, but not GA, inhibits the activity of gbHAse in a dose-dependent manner [16]. Interestingly, GL inhibits the CK-II-mediated activation of HIV-1 enzymes [RNA-dependent DNA polymerase [reverse transcriptase (RT; 19)] and protease [18] *in vitro*. Finally, DNA-binding basic gbPs [histone [14], HMG1 [65], nuclear receptor [69, 70], and lactoferrins (LFs; 71) and other basic proteins, such as *Habu* snake venom PLA2s [15], human secretory type IIA PLA2 [72], LF-associated angiogenin and lactogenin [73, 74], and serum complement C3 and C3a (anaphylatoxin ; 75), are identified as gbPs. 60S acidic ribosomal P proteins are copurified with CK-II from the 0.6 M KCl extract of

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mammalian ribosome by GL-affinity column chromatography [77]. In addition, it has been shown that (i) GL directly binds to thrombin and acts as its potent inhibitor *in vitro* [78]; and (ii) GL has a binding affinity with some enzymes present in biological fluids, such as lysozyme and LF, which possess anti-bacterial and anti-viral activities [74, 76].

V. The anti-inflammatory effect of GL

GL is known in the traditional Chinese medicine for its anti-inflammatory effect, which is originally described by Finney in 1959 [36]. The mechanism of the GL-induced anti-inflammatory effect is based on different pathways of the GL-induced selective inhibition of the prostaglandin E2 production [38], the CK-II- mediated activation of both GL-binding lipoxygenase (gbLOX; 17) and PLA2 [15, 20, 72], an anti-thrombin action of GL [78] and production of the reactive oxygen species (ROS; 79). GL exerts liver protection properties by inhibiting PLA2 [80] or by the hydroxyl radical trapping action [81], leading to the lowering of serum alanine and aspartate transaminase levels [30, 82-84].

1. Inhibition of the arachidonic acid cascade pathway related enzymes

Some lipids that function as second messengers in cell signaling arise from the arachidonic acid pathway. Arachidonic acid (20-carbon unsaturated fatty acid) is a normal constituent of membrane phospholipid and an essential fatty acid of a precursor in the biosynthesis of prostaglandins, thromboxanes and leukotrienes. It is released from these phospholipids on the cell membrane by the action of PLA2. The selective hydrolysis of 2-acyl groups in sn-3-phosphoglycerides plays a central role in lipid metabolism in mammalian cells [85]. Biochemical studies revealed that (i) GL directly binds to the arachidonate cascade related enzymes, such as LOX [17, 86] and PLA2s [20, 72]; and (ii) GL effectively inhibits the CK-II-mediated stimulation of these enzymes activities *in vitro*. Since PLA2 is activated by Ca^{2+} and calmodulin [87], it may be inhibited by drugs, which reduce the availability of Ca^{2+} and calmodulin at the inflammatory site, because (i) GL decreases the intracellular Ca^{2+} in the stimulated diaphragm muscle of mouse [88]; and (ii) the CK-II phosphorylation sites of calmodulin are phosphorylated *in vivo* [89]. The phosphorylated form of calmodulin plays a role in the activation of PLA2, since the inhibition of this phosphorylation by GL results in a decreases of PLA2 activity. In another approach to clarify the physiological interaction between GL and PLA2s isoforms, at least three gbPLA2s (PA2Y, PA21 and PA2B) are purified from *habu* snake venom and PA2B (lysine-49 PLA2) is found to be a GL-sensitive PLA2. In addition, these three gbPLA2s function as phosphate acceptors for CK-II *in vitro* and the CK-II-mediated activation of PA2B is the most sensitive to GL *in vitro* [20].

LOX is another important factor involved in the inflammatory processes, which act on linoleic and arachidonic acids to produce chemical mediators, such as 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is converted to leukotriene A4 (LTA4). LTA4 is a precursor of LTB4, which induces inflammation by its chemotactic and degranulating actions on polymorphonuclear lymphocytes [90-93]. Therefore, direct inhibition of LOX or indirect inhibition of the CK-II-mediated activation of LOX by GL or a GA derivative (oGA) may involve a negative impact on the progress of inflammation. Therefore, the study has been carried out to evaluate the binding affinity of LOX with GL [17] and to explain another possible

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mechanism for the anti-inflammatory effect of GL. Thus, a gbLOX (LOX 3) in the partially purified soybean LOX-1 fraction is selectively purified as a gbP and the CK-II-mediated phosphorylation of the gbLOX results in a significant stimulation of its activity. gbLOX activity itself is further stimulated when the gbLOX is fully phosphorylated by CK-II in the presence of 1-10 μM GL, but significantly inhibited by 30 μM GL or 10 μM oGA [17, 86]. This biphasic effect of GL indicate the physiological correlation between the LOX activity and its phosphorylation by CK-II at cellular level. Based on these observations, the GL-induced selective inhibition of the CK-II-mediated activation of LOX by GL may be involved in a part of the anti-inflammatory effect of GL *in vivo*.

2. Biochemical characterization of GL-binding protein kinases

By GL-affinity column chromatography, at least three protein kinases (A-kinase, CK-I and CK-II) are selectively purified from the partially purified kinase fractions prepared from suitable cell sources [13, 18, 66 - 71]. It is well-known that CK-II, a cAMP-, cGMP- and Ca^{2+}/phospholipid-independent serine (Ser)/threonine (Thr)-protein kinase, plays important roles in the regulation of DNA replication, transcription and cell proliferation (94): it specifically modifies DNA-binding proteins [*e.g.* DNA-ligase, DNA topoisomerases (I and II)] and transcriptional factors [*e.g.* Ap-1, Sp1 and serum response factor (SRF)], oncogene products (*e.g.* erbAα, Myβ and Myc) and various viral gene products (see Table 2). Recently, we reported that (i) CK-II mediates the stimulation of the activities of several GL-binding enzymes, such as soybean LOX-3 [17], *Habu* snake venom PLA2s [20], and two HIV-1 enzymes [reverse transcriptase [19] and protease [18]] *in vitro*; and (ii) the CK-II-mediated extreme phosphorylation of a 98 kDa nucleolin-like DNA-binding protein (p98) is involved in cell activation induced by interleukin 2 (IL-2) [95], and in the fertilization of sea urchin eggs [96, 97].

On the other hand, CK-I is a ubiquitous and highly conserved second messenger-independent Ser/Thr-protein kinase with a molecular weight of 25~55 kDa and at least five isoforms (α, β, γ, δ and ε) are identified from a variety of cell sources [98, 99]. It is well-known that these CK-I isoforms are implicated in a diverse number of cellular functions, including DNA replication, DNA repair, nuclear shuttling of transcriptional factors, Wnt signaling, and circadian thythms [98]. However, the exact details of these CK-I-mediated regulatory mechanisms are unlcear at present. Recently, we reported that (i) CK-I preferentially phosphorylates Thr-residues on HMG1 in the presence of cholesterol-3-sulfate (CH-3S) *in vitro*; and (ii) CH-3S and GL directly induces drastic conformational changes of HMG1 [100]. These results show that HMG1 is a CH-3S-binding protein and that CH-3S acts as the sole effector for the phosphorylation of HMG1 by CK-I *in vitro* [99]. GL inhibits the phosphorylation of cellular proteins by two distinct casein kinases (CK-I and CK-II) and a GA derivative (oGA) effectively inhibits them at a one-tenth concentration of GL *in vitro* [69-72].

A-kinase, a cAMP-dependent protein kinase, plays an important role in the metabolic and tanscriptional regulation through specific phosphorylation of cellular regulatory mediators involved in the cell proliferation and differentiation. The biphasic effect of GL on the phosphorylation of histone or lipocortin by A-kinase is observed *in vitro*: a significant stimulation of A-kinase at low doses (1-3 μM) and the kinase activity is

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significantly inhibited by relatively high doses (over 30 μM) of GL in a dose-dependent manner [14, 66] in a similar manner observed with the GL-induced inhibition of the CK-II-mediated protein phosphorylation *in vitro* [69-72]. Interestingly, GL selectively inhibit the A-kinase-mediated phosphorylation of histones H2A and H2B [14] as well as lipocrtin *in vitro* [66]. This sugests that GL inhibits the A-kinase-mediated regulation of the physiological interaction between PLA2 and lipocrtin in the regulation of signal transduction at the cellular level.

3. Inhibition of the steroid metabolizing enzymes

Cortisol metabolism in rats is lowered by GA, because GA selectively inhibits the activities of both hepatic delta 4-5-reductase [101] and 11βhydroxysteroid dehydrogenase [11β-HSD1 (102) and 11β-HSD2 (102)] in a dose-dependent manner *in vitro*. Kato *et al.* reported that (i) 3-monoglucuronyl-glycyrrhetinic acid (3MGA) is a metabolic intermediate of GL administered orally; (ii) 3MGA highly inhibits the activity of 11β-HSD1 at the low dose rather than GA *in vitro*; and (iii) this intermediate is highly accumulated in the patients with psuedoaldosteronism [103]. Among GA derivatives, oGA is the most effective inhibitor (ID50 = approx. 0.1 μM) of 11β-HSD1 *in vitro* [104]. Therefore, the anti-inflammatory effect of GL could be partially attributed to the inhibition of this cortisol metabolic pathway. Both the GL-induced mineralocorticoid activity [103] and hypertension effect [104] are attributed to the GL-induced inhibition of the activity of 11β-HSD. Another evidence for the involvement of the steroids metabolizing enzymes involved in the anti-inflammatory effect of GL arises from the observation that GL affects the pharmacokinetics of other steroidal drugs. For example, the co-administration of GL with prednisolone results in an increase in the area under the curve (AUC), a decrease in total plasma clearance and enhancement of the mean residence time of PLS [61]. A similar behavior might occur for the endogenous cortisol, leading to a longer half-life and higher concentration of cortisol in serum.

4. Biphasic effect of GL *in vitro*

As mentioned above, GL can induce the augmentation of NK cell activity [42], differentiation of extrathymic T cell [43] and anti-type 2 T cell [44], and stimulation of the production of both cytokines [41, 46] and kemokines [47]. Although the biochemical mechanisms of these GL-induced biological effects are unclear at present, some unknown functional mediators, such as transcriptional factors, signal regulatory factors and cytokine receptors, may be involved in these GL-induced biological effects. It is possible to speculate that GL-binding protein kinases, such as PKA, PKC, CK-I and CK-II, may play as cellular mediators through specific phosphorylation of GL- and GA-binding proteins responsible for processing these biological events at the cellular level. As shown in Fig. 2, the phosphorylation of several functional cellular proteins, including human hC3a (anaphylatoxin), by two distinct protein kinases (PKA and CK-I) is significantly stimulated at a low dose (a: 0.1-1 μM) of GL, but inhibited by a high dose (b: over 10 μM) of GL *in vitro*. This biphasic effect of GL, but not by GA, on the protein phosphorylation is observed with other protein phosphorylation *in vitro*: (i) phosphorylation of lipocortin and histone by PKA [14, 66]; and phosphorylation of LOX, HIV-I enzymes (protease and RT),

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nuclear hormone receptor, PLA2 and ribosomal proteins by CK-II [17- 19, 69-72, 77]. In addition, GL enhance the activities of G-protein [109] and transcriptional factors, such as steroid hormone receptor [69, 70] and AP-1 [110] at the cellular level. Therefore, the GL-induced stimulatory effect of protein phosphorylation may be implicated in the main mechanism responsible for the GL-induced stimulation of the production of IFNγ, IL-12 and β-kemokinase, and augmentation of the activity of NK cells and induction of extrathymic T cells.

The binding kinetics of gbPs with either GL or GA and their CD spectrum analysis revealed that these gbPs contain two distinct GL-binding domains (low- and high-binding sites). The binding of GL to the low-binding site on PKA and CK-II results in the stimulation of their enzyme activities *in vitro*. In the GL-binding to the high-affinity domain on histone and HMG1 results in the reduction of their DNA-binding abilities *in vitro*. Finally, it is concluded that GL, at a low dose, induce the high stimulation of protein phosphorylation involved in the cell activation and viral replication at the cellular level.

5. Characteristics of GL-binding mediators

The gbPs involved in the GL-induced anti-inflammatory and anti-viral effects are identified and biochemically characterized (Tables 1 and 2). The gbPs are classified into two groups [GL-binding basic proteins, such as histones H2A and H2B, HMG1, nuclear receptor, LFcin, C3a (anaphylatoxin) and HIV-1 RT; and GA-binding proteins, such as 11β-HSD, HAse and LF-binding proteins (angiogenin and lactogenin)]. Determination of the GL-binding sites on DNA-binding basic gbPs using GL, GA, a derivative of GA (oGA) and a modified GL, the two glucuronic acid moiety in the GL molecule may be responsible for its binding to these gbPs *in vitro*. All these gbPs are characterized as functional mediators involved in inflammation and viral replication *in vivo*. The phosphorylation of these gbPs by Ser/Thr-protein kinases (PKA, PKC, CK-I and CK-II) is sensitive to GL and oGA *in vitro*. These suggest that the GL-induced selective inhibition of their activation mediated by Ser/Thr-protein kinases may be involved in the anti-viral effect of these two drugs (GL and GA) in virus-infected cells. These gbPs involved in the anti-inflammatory effect of GL are classified into four functional classes: (i) arachidonic cascade pathway related enzymes, such as PLA2 and LOX; (ii) intracellular signal regulatory mediators, such as protein kinases (PKA and CK-II) and G-proteins; (iii) transcriptional mediators, such as steroid hormone receptor and AP-1; and (iv) host defense signal factors, such as cytokines, kemokines and peptide hormones, and their specific receptors. In the case of the GL-induced anti-viral effect, gbPs are classified into two functional classes: (i) the GL-binding viral gene products, such as HIV-1 enzymes (protease and RT) and DNA-binding basic proteins; and (ii) the host mediators responsible for the viral replication, such as protein kinases, metabolic enzymes and regulatory factors of protein synthesis.

Furthermore, at inflammatory sites, macrophages produce several distinct inflammatory mediators, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) [105 – 109]. The inhibitory effect of GL on the physiological functions of both PKC and TNF-α had been reported [125, 127]. It has been demonstrated that (i) the serum level of HMG1 increases after the administration of endotoxin, and injection of HMG1 itself is lethal; (ii) delayed administration of antibodies to HMG1 attenuates endotoxin lethality; and (iii)

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proinflammatory cytokines, such as tumor necrosis factor (TNF) and IL-1, induce the release of HMG1 from pituicytes in time- and dose-dependent manners [111, 112]. HMG1 and C3a are basic proteins with similar biological and biochemical properties: (i) both are GL-binding proteins; (ii) the direct binding of GL to these proteins results in the reduction of their toxic abilities *in vivo*; and (iii) both proteins may participate in the physiological regulation of the inflammatory response as inflammatory mediators.

(Part II)

VI. The anti-viral effect of GL

In 1979, Pompei *et al.* originally reported the anti-viral effect of GL among anti-viral natural compounds [40]. As shown in Table 4, GL effectively inhibits the replication of RNA viruses, such as Japanese encephalitis virus [113], influenza A2 virus [114], hepatitis A virus (HAV; 22, 23), hepatitis C virus (HCV; 50, 154), hepatitis E virus (HEV; 115, 156), murine retrovirus [116], HIV-1 [52, 53, 117, 157], flavivirus [118] and corona SARS virus [119], and DNA viruses, such as varicella-zoster virus (VZV; 51), hepatitis B virus (HBV; 120, 162), human cytomegalovirus (HCV; 121) and herpes simplex virus (HSV; 122).

There are many different approaches to

Table 4- GL-binding proteins (gbPs) purified from various cell sourses by GL-affinity column chromatography. The CK-II-mediated phosphorylation of these gbPs is sensitive to GL *in vitro*

gbPs	Function and characteristics	References
Habu snake venom p25	Metalloprotease	15
p17	Phospholipase A2 (PA2Y)	20
p15-1, p15-2	PA 21, P A2B (PLA2)	15
p55	Apoxin I-like L-amino oxidase	20
Soybean p96	GL-sensitive lipoxygenase 3	17
HIV-1 p66, p51	Reverse transcriptase	19
p11	Protease	18
Bovine testis p55	GL-sensitive hyaluronidase	16
DNA-binding basicproteins*	Histones H2A, H2B	14
	High mobility group proteins 1 and 2	65
Lipocortin (p36)	Acute inflammatory factor	66
Nuclear p100	Glucocorticoid receptor	69, 48
Human serum p67	Albumin	64
p115, p9	Complement C3, C3a (anaphylatoxin)	75
Human fluid p14	Secretory type IIA PLA2	72
Human serum p80*	Lactoferrin (LF), an Fe^{3+}-binding glycoprotein with DNA-binding ability	73
LF-binding p15, p17	Angiogenin-1 (p15) and lactogenin (p17)	74
Ribosome p35, p15, p13	60S acidic ribosomal P proteins	77
Thrombin	GL-sensitive protease	78
Lysozyme	Anti-bacterial activity	82

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elucidate the replication mechanisms of RNA and DNA viruses. The molecular basic studies on the mechanisms of viral replication identified as several host mediators, including Ser/Thr-protein kinases (A-kinase, C-kinase and CK-II) and transcriptional factors, and the phosphorylation of viral gene products by these protein kinases in virus-infected cells. Particularly, CK-II has an important role in the replication of various RNA and DNA viruses through specific phosphorylation of viral gene products [123-151] (Table 4) and cellular mediators in virus-infected cells.

1. The inhibitory effect of GL on RNA viruses

(i) Clinical effect of GL on RNA virus infection

Chronic viral hepatitis is a major cause of liver disease world-wide. A GL preparation named Stronger neominophagen C (SNMC: Minophagen Pharmaceutical, Ltd., Tokyo, Japan), is available for *i.v.* administration that has been used for the treatment of chronic viral hepatitis in Japan during the past 60 years. When SNMC is administered *i.v.* for a period of more than one month and on 6 separate occasions to 3 hemophiliacs with AIDS (HIV-1 p24 is detectable at the beginning of 5 of the 6 treatment courses), the viral proteins are undetectable at the end of or during 3 of the 5 treatment courses and decreases to a lower level following the 2 other courses [21]. The prophylactic administration of a high-dose of SNMC to HIV positive hemophiliacs with impaired immunological ability and liver dysfunction is effective in preventing the development from asymptomatic carrier/AIDS related-complex to AIDS [54].

In the case of 8 patients with chronic hepatitis C (CHC), which did not respond to the initial IFN therapy, the efficacy of IFN combined with a high dose of SNMC is assessed [151]. Although the ALT level decreases approximately 70% in all patients (one became normal) compared to 50% in IFN therapy alone, a significant decrease of HCV RNA titers and HAI scores between these two therapies is not observed. Therefore, it is concluded that the treatment of IFN in combination with GL therapy is not more beneficial than IFN therapy alone in the treatment of patients with CHC resisting IFN therapy. In addition, SNMC has considered as a potential treatment for CHC [31] and a long term administration of SNMC in CHC patients is effective in preventing liver carcinogenesis [26]. Recently, three clinical research groups reported their clinical data of SNMC in Japan [152-154]: the usage of the newly developed suppository of SNMC can improve the quality of life for CHC patients, who do not respond with viral clearance to IFN therapy or following initial IFN therapy. In addition, SNMC is safe and efficacious in lowering or normalizing alanine aminotransferase (ALT) levels in the patients with chronic hepatitis B in the Netherlands [155], or Chinese patients with CHB [157] or patients with acute sporadic hepatitis E in India [115].

(ii) Anti-viral effect of GL in the *in vitro* experimental systems

The anti-viral effect of GL and its derivatives, including oGA (Fig. 1), on the replication of both HIV-1 and HSV-1 *in vitro* have been reported [52, 53]. Among several anti-viral compounds tested, a GA derivative (oGA) is the most effective compound on the replication of HIV-1 in MT-4 and MOLT-4 cells. The GA derivative completely inhibits HIV-1-induced cytopathogenicity. At a concentration of 160 nM oGA, an early stage of the replication of HAV in PLC/PRF/5 cells is effectively inhibited [22]. GL protects mice

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exposed to a lethal amount of influenza A2 virus through the stimulation of IFN-γ production in T cells [114]. However, the antiviral effect is undetected when GL is administered to infected mice in combination with anti-IFN-γ monoclonal antibody.

Recently, Suzuki research group reported that β-chemokines (CCL4 and CCL5), which inhibit the replication of a non-syncytium-inducing variant of HIV-1 (NSI-HIV), are induced in healthy peripheral blood mononuclear cells (PBMC) treated with GL [47]. As expected, GL possesses a potential inhibitory effect on NSI-HIV replication in the cultured peripheral blood mononuclear cells (PBMC) from HIV-1 positive patients by inducing the production of β-chemokines [117]. This report suggests that the induction of CCL4/CCL5 and the inhibition of CCL2 by GL may be involved in the anti-viral effect of GL on NSI-HIV replication.

In 2003, a new coronavirus has been identified in patients with severe acute respiratory syndrome (SARS) in China and other Far Eastern countries. Clinatl *et al.* reported that GL at a high dose (4 mg/ml: approx. 4.8 mM) acts as the most effective compound to inhibit the replication of SARS-CV in Vero cells [119]. Since infrequent side effects, such as psuedoaldosteronism [33, 34] and hypokalaemia [163, 164] are reported in some patients after several months of the treatment with GL at high doses, treatment of SARS should be considered for a short period. Indeed, the drug exhibits few toxic effects as compared with other regimens, such as ribavirin and 6-azauridine [119].

(iii) The inhibitory mechanism of GL on RNA viruses

The mechanisms involved in the anti-viral effect of GL have been investigated at different mechanical points. GL was found to induce the production of IFN-γ *in vivo* [41] and extrathymic T cells in mouse [43], and to inhibit the activities of several enzymes, such as virion-associated RNA-dependent DNA polymerase (RT) and HIV-1 protease [18, 19] as well as PKC [158]. Furthermore, GL prevents the penetration of HAV into the plasma membrane [23], the phosphorylation of some regulatory proteins [19, 145] and the suppression of the sialylation of surface antigen [24].

Recently, we reported that (i) CK-II, a host mediator responsible for the activation of functional cellular factors and viral gene products in virus-infected cells (see Table 2), phosphorylates several viral gene products (RT, Gag, Rev, Nef and integrase) in HIV-1-infected cells [159]; (ii) the phosphorylation of HIV-1RT and protease by CK-II *in vitro* results in the significant activation of these two GL-binding enzymes *in vitro*; and (iii) a GA derivative (oGA) at one-tenth dose of GL inhibits the CK-II-mediated activation of these two enzymes *in vitro* [17, 18]. Therefore, the anti-HIV-1 effect of GL is based on the GL-induced selective inhibition of the CK-II-mediated activation of HIV-1 enzymes (RT and protease) and the physiological abilities of other gbPs (Gag and Nef) at the cellular level [17, 18]. The inhibitory kinetics of oGA on the CK-II-mediated phosphorylation of RT and protease are similar to those observed with quercetin and epigallocatechin gallate (CK-II inhibitors) *in vitro* [17, 18]. Since the CK-II-mediated activation of RT and protease is an essential for the replication of HIV-1, the GL- and oGA-induced selective inhibition of the CK-II-mediated phosphorylation of HIV-1 gene products may be a major mechanism for the anti-HIV-1 effect of GL at the cellular level. This hypothesis is supported by other observation that the CK-II-mediated phosphorylation of the 63 kDa regulatory

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protein in HSV-1 is necessary for its replication [150]. In addition, quercetin as well as oGA [19] are characterized as potent CK-II inhibitors, which are in agreement with recent finding that other CK-II inhibitors, such as flavonoids and benzothiophenes, selectively inhibit HIV-1 transcription [160]. Therefore, the GL-induced suppression of the Rev-mediated stimulation of CK-II activity may be implicated in the anti-HIV-1 effect of GL in virus-infected cells.

A novel function of HIV-1Rev is characterized as a potent CK-II activator *in vitro* [159], as Rev is a gbP containing high level of Arg and Lys. The stimulatory effect of Rev on CK-II activity is disappeared when pre-incubated with GL *in vitro*. These observations suggest that the direct binding of GL to Rev may result in the suppression of the physiological functions, including CK-II activation, of Rev and in the reduction of its interaction with viral RNA in HIV-1-infected cells.

2. The inhibitory effect of GL on DNA viruses

There are few reports demonstrating the inhibitory effect of GL on the replication of DNA viruses, such as herpes simplex virus (HSV-1; 122, 139, 140), human cytomegalovirus (HCMV; 121), varicella-zoster virus (VZV; 51, 150) and hepatitis B virus (HBV; 157, 161, 162).

(i) Clinical effect of GL on DNA virus infection

When SNMC is administrated *i.v.* to the patients with chronic hepatitis B (CHB), their liver functions are improved with occasional complete recovery from HBV [158]. This observation supports the finding by Takahara *et al.* [24], who have demonstrated that GL suppress sialylation of hepatitis B surface antigen (HBsAg). When SNMC is administrated by *i.v.* for a period of more than a week to three infants with CMV infection, which exhibits abnormal liver function or hepatomegaly, liver function became normal at the end of the treatment course [27]. Therefore, this study is extensively conducted to compare the inhibitory effects of GL, cyclosporin A (CsA) and TNF-α on the DNA synthesis of HCMV and antigen expression of HCMV in U-937 and MRC-5 cells [121]. Although GL inhibits the viral antigen expression of HCMV in human monocytic cell line U-937 and human embryonic lung cell line MRC-5, as determined by flow cytometry and immunofluorescence assay, an early stage in the replication of HCMV is still detectable by the polymerase chain reaction. In this study, CsA and TNF-α lack the inhibitory effect on HCMV in U-937 cells. When thermally injured mice are treated *i.p.* with a 10 mg/kg dose of SNMC for 2 and 4 days after injection of HSV-1, the resistance of these mice to HSV-1 is improved to levels observed in normal mice [122].

(ii) The inhibitory mechanism of GL on DNA viruses

The regulatory protein (ICP27) of HSV-1 is required for the replication of this virus. Phosphoamino acid analysis shows that the Ser-residue at position 114 and Ser-residues at positions 16 and 18 are specifically phosphorylated by PKA and CK-II, respectively, during HSV-1 infection [139-142]. Therefore, the GL-induced selective inhibition of the PKA- and CK-II-mediated phosphorylation of ICP27 (63 kDa) may be considered as one of the mechanisms involved in the GL-induced inhibition of HSV-1. The effect of GL on the secretion of HBsAg has been examined *in vitro* [24]. According to this study, GL suppresses the secretion of HBsAg and dose-dependently accumulates it in

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PLC/PRF/5 cells. This action is further analyzed and determined in the HBsAg-expression system using VZV. GL effectively suppresses the secretion of HBsAg, resulting in its accumulation in the cytoplasmic vacuoles in the Golgi apparatus area.

VII. Clinical side effects of GL

Psuedoaldosteronism [33-35] and hypokalemia [163, 164] are known as the clinical side effects caused by a high dose and a long period of GL administration in some patients. In 1987, Stewart *et al.* originally reported that (i) GA hydrolyzed from GL is a potent inhibitor for the activity of 11β-HSD; and (ii) this inhibition may be involved in the licorice-induced psuedoaldosteronism [33]. Indeed, GA is the most sensitive to 11β-HSD, as compared with other GL-sensitive enzymes, such as LOX and PLA2, *in vitro* [102-104]. Kato *et al.* reported that (i) 3-monoglucuronyl-glycyrrhetinic acid (3MGA) is a metabolic intermediate of GL administrated orally; (ii) 3MGA highly inhibits 11β-HSD activity at a low dose rather than GA *in vitro*; and (iii) this intermediate is highly accumulated in the serum of patients with psuedoaldosteronism [103]. Other reports demonstrated that (i) the chronic high dose of GA suppresses mRNA and protein expression of 11β-HSD2 via indirect mechanisms [165]; and (ii) the prolonged symptoms is caused clinically after the cessation of GA administration in some psuedoaldosteronism patients [103].

VIII. Remarkable other GL-induced biological effects

It has been shown that the addition of GL (25-400 μg/ml) to cultured splenocytes and thymocytes from BALB/c mice definitely results in the induction of DNA fragmentation [166]. However, a single injection of GL (100 μg/mouse) into BALB/c mice did not cause DNA fragmentation, cell death of splenocytes and thymocytes. The repeated injections of GL (100 μg/mouse/day) into mice for 7 days actually results in the induction of low grade DNA fragmentation selectively in splenocytes. In the previous study, GL induces the reduction of DNA synthesis in human peripheral lymphocyte-macrophage cultures [166]. These reports suggest that (i) splenocyte and thymocyte are sensitive to GL in mice; and (iii) GL induces apoptosis of these GL-sensitive cells *in vivo*.

Interestingly, the preventing property of GL on carcinogen-induced DNA damage and the GL-induced apoptosis in cancer cells are reported [28, 29, 167]. GL and some analogues induce growth of primary cultured adult rat hepatocytes via epidermal growth factor receptors [168] and also induce the significant stimulation of melanogenesis by glycyrrhizin in B16 melanoma cells [169]. In contrast, a long-term treatment of chronic hepatitis C with SNMC results in the prevention of liver cirrhosis and hepatocellular carcinoma [170], and in the inhibition of experimental pulmonary metastasis in mice inoculated with B16 melanoma through the regulation of tumor-associated Th2 cells [171]. However, the mechanisms involved in these GL-induced stimulatory and inhibitory biological effects, including apoptosis, remain to be elucidated.

Conclusion

There are many functional cellular mediators involved in the GL-induced anti-inflammatory and anti-viral effects. Several mediators are characterized as gbPs, such as LOX, type IIA PLA2 and HIV-1 enzymes (RT and protease). As expected, the CK-II-mediated activation of these gbPs is sensitive to GL *in vitro*. The GL-induced inhibition of

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the physiological activities of these gbPs may be implicated in these two GL-induced biological effects *in vivo*. Therefore, it is concluded that the GL-induced selective inhibition of the arachidonic cascade pathway may be one of the major routes involved in the GL-induced anti-inflammatory effect *in vivo*. The anti-inflammatory effect of GL is superior to those of aspirin and indomethacin in abolishing the production of inflammation-mediating substances, because GL selectively inhibits the production of arachidontate at an earlier step catalyzing by PLA2 and LOX, whereas both aspirin and indomethacin selectively inhibit only COX in any tissues.

The replication of RNA and DNA viruses requires various functional cellular mediators, including protein kinases (PKA, PKC and CK-II), which may be responsible for the activation of viral gene products in virus-infected cells. The physiological activities of various viral proteins (Table 4) are activated through their specific phosphorylation by these GL-binding Ser/Thr-protein kinases at the cellular level. The gbPs are classified into two groups [GL-binding basic proteins, such as histones H2A and H2B, HMG1, nuclear receptor, LFs and HIV-1 RT; and GA-binding proteins, such as 11β-HSD, HAse, LF-binding proteins (angiogenin and lactogenin) and anaphylatoxins (C3a and C4a)]. The experimental observations that (i) all these gbPs are characterized as functional mediators involved in inflammation and viral replication *in vivo*; and (ii) the phosphorylation of these gbPs by Ser/Thr-protein kinases (A-kinase, CK-I and CK-II) is sensitive to GL and oGA *in vitro*, suggest that the GL-induced selective inhibition of their activation mediated by Ser/Thr-protein kinases may be involved in the anti-viral effect of these two drugs in virus-infected cells.

For a clear understanding of the detail mechanisms of the GL-induced anti-inflammatory and anti-viral effects, further analytical studies are required (i) to characterize other novel GL-binding cellular proteins and viral gene products responsible for the replication of RNA and DNA viruses; (ii) to identify the GL-binding targeting factors specifically phosphorylated by GL-sensitive protein kinases during inflammation; (iii) to detect novel gbPs in the serum of patients with viral infection and immunological diseases; and (iv) to determine the inhibitory effects of GL and GA on the physiological interaction of the GL-binding cytokines and their receptors containing GL-binding domains.

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