C1 inhibitor, a multi-functional serine protease inhibitor

Alvin E. Davis III; Fengxin Lu; Pedro Mejia
Immune Disease Institute, Program in Cellular and Molecular Medicine at Children’s Hospital Boston, Harvard Medical School, Boston, Massachusetts, USA

Summary

C1 inhibitor (C1INH) is a serpin that regulates both complement and contact (kallikrein-kinin) system activation. It consists of a serpin domain that is highly homologous to other serpins and an amino terminal non-serpin mucin-like domain. Deficiency of C1INH results in hereditary angioedema, a disease characterised by episodes of angioedema of the skin or the mucosa of the gastrointestinal tract or the oropharynx. Although early data suggested that angioedema was mediated via complement system activation, the preponderance of the data indicate that bradykinin is the mediator. In the past few years, it has become apparent that C1INH has additional anti-inflammatory functions independent of protease inhibition. These include interactions with leukocytes that may result in enhanced phagocytosis, with endothelial cells via E- and P-selectins that interfere with leukocyte rolling and in turn results in suppression of transmigration of leukocytes across the endothelium, and interactions with extracellular matrix components that may serve to concentrate C1INH at sites of inflammation. In addition, C1INH suppresses gram negative sepsis and endotoxin shock, partly via direct interaction with endotoxin that interferes with its interaction with macrophages, thereby suppressing tumour necrosis factor-α and other inflammatory mediators. C1INH treatment improves outcome in a number of disease models, including sepsis and other bacterial infections, possibly malaria, ischaemia-reperfusion injury (intestinal, hepatic, muscle, cardiac, brain), hyper-acute transplant rejection, and other inflammatory disease models. Recent data suggest that this effectiveness is the result of mechanisms that do not require protease inhibition, in addition to both complement and contact system activation.

Keywords

C1 inhibitor; complement, kallikrein-kinin system, sepsis, ischaemia-reperfusion injury

Introduction

Oscar Ratnoff, in addition to his landmark contributions related to the biochemistry and biology of coagulation, was a pioneer in the analysis of the mechanism of complement activation. He, with Irwin Lepow and others, was the first to isolate and characterise the first component of complement (C1) from human plasma and to demonstrate its esterolytic activity (1–3). In 1957, they described a heat-labile factor in human plasma that inhibited the esterase activity of C1 and which could not be identified with any known component of complement; it was subsequently named C1-inhibitor (C1INH) (3). C1INH is the only known plasma protease inhibitor that regulates complement activation, is the major regulator of contact system activation, and is able to inactivate several fibrinolytic and coagulation system proteases. A major biological role of C1INH is the regulation of vascular permeability, as is best illustrated by patients with hereditary angioedema (HAE), which results from deficiency of C1INH protease inhibitor activity. C1INH also suppresses inflammation via inhibition of both the complement and kallikrein-kinin systems. However, many of the beneficial effects of C1INH are not the result of protease inhibition. This review will briefly discuss C1INH, its protease inhibition activity and its deficiency, and will then focus on evidence suggesting that the beneficial effects of C1INH are at least partially the result of interaction with both endogenous components (endothelial cells, leukocytes, extracellular matrix) and with exogenous infectious agents.

C1 inhibitor, a serpin with multiple targets

C1INH is an acute-phase protein that, based on its structure and inhibitory mechanism, belongs to the superfamily of serine-protease inhibitors (serpins). It is a single 105 kDa polypeptide chain consisting of a serpin domain that contains near its carboxy terminus the protease recognition region (the “reactive center loop”) and a heavily glycosylated amino terminal domain (approximately 100 amino acid residues) that shares no homology with other serpin family members. This domain plays no role in protease inhibition; its elimination has no effect on protease inhibitory activity (4). Protease inactivation by serpins, including C1INH, involves...
two steps. First, the target protease recognises the reactive centre loop exposed at the surface of C1INH which mimics the substrate specificity of the protease and cleaves the Arg444-Thr445 peptide bond. Cleavage triggers a molecular rearrangement in which the serpin and the protease become irreversibly distorted with covalent bond formation between the inhibitor and the active site serine of the protease (5). Proteases inactivated by C1INH include C1r, C1s, MASP1 and MASP2 of the complement system; factor XII and plasma kallikrein of the contact system; factor XI and thrombin of the coagulation system; and plasmin and tissue plasminogen activator of the fibrinolytic system (for details and references, see [6–8]) (Table 1).

Vascular permeability

People with hereditary angioedema (HAE) are heterozygous for deficiency or dysfunction of C1INH. HAE is characterised by recurrent episodes of acute, localised increases in vascular permeability, which may affect the skin or the mucosa of the oropharynx and gastrointestinal tract. Virginia Donaldson (who had trained with Oscar Ratnoff) first demonstrated that HAE patients had low plasma levels of C1INH (9), although the previous year Landerman had described a deficiency of kallikrein inhibitory capacity in patients with HAE (10). Therefore, studies over the next several years focused on the potential role of the complement system in mediation of angioedema. In the late 1970s and 1980s the role of C1INH in regulation of contact system activation began to be appreciated. This led to analysis of the contact system in HAE.

During attacks of angioedema the complement system is activated: C4 and C2 levels, which may be low even in the absence of symptoms, fall (11, 12), activated C1 circulates in plasma (13, 14), as do C1INH-C1r and -C1s complexes (15). Activation of the contact system during attacks of angioedema was suggested by the observation that fluid from suction-induced skin blisters in patients with HAE contained activated kallikrein (16) (Table 2). Furthermore, plasma from patients during angioedema contained decreased levels of high-molecular-weight kininogen (HK) and prekallikrein (17). Other studies showed that, during attacks, plasma HK was cleaved in a manner consistent with bradykinin release, and that cleavage decreased during recovery (18). Finally, in 1998, Nussberger et al. demonstrated the presence of bradykinin in plasma during angioedema attacks (19).

Donaldson et al. described a vascular permeability-enhancing and smooth muscle-contracting activity that appeared in HAE

<table>
<thead>
<tr>
<th>Table 1: C1 inhibitor biological functions.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protease inhibition</strong></td>
</tr>
<tr>
<td>Complement system</td>
</tr>
<tr>
<td>Contact system</td>
</tr>
<tr>
<td>Fibrinolytic system</td>
</tr>
<tr>
<td>Coagulation system</td>
</tr>
<tr>
<td>Complement system</td>
</tr>
<tr>
<td>Extracellular matrix</td>
</tr>
<tr>
<td><strong>Interactions with endogenous components</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Interactions with cells</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Interactions with infectious agents</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

© Schattauer 2010

Thrombosis and Haemostasis 104.5/2010
Evidence | Ref.
--- | ---
Active kallikrein present in induced blister fluid in skin of HAE patients | (16)
Decreased HK & prekallikrein in HAE plasma during attacks | (17)
HK cleavage during HAE attacks | (18, 87)
Members of a family that express a dysfunctional C1INH that inhibited contact system proteases but did not inhibit C1r & C1s did not have HAE | (29–31)
Kinin generated in vitro in C1INH-depleted C2 deficient plasma but not in C1INH-depleted HK, prekallikrein or factor XII deficient plasma | (28)
Kinin generated in HAE plasma in vitro identified as bradykinin | (28)
Bradykinin present in HAE plasma during attacks | (19)
Vascular leak in C1INH deficient mouse mediated by bradykinin | (32)

Table 2: Bradykinin mediates angioedema in C1INH deficiency.

C1 inhibitor, more than a protease inhibitor

Interaction with cells, extracellular matrix, and endogenous proteins

Although the primary function of serpins is protease inhibition, many also have additional biological functions. For C1INH, a number of such interactions have been reported (Table 1). Chang et al. reported that C1INH binds to human peripheral blood lymphocytes, monocytes and neutrophils but not to human erythrocytes (34). Binding is independent of inhibitory activity and is suppressed by desialylation indicating that carbohydrate is required for binding. Potential cell surface receptors and the consequences of this interaction have not been elucidated. Interestingly, C1INH and reactive center-cleaved, inactive C1INH (iC1INH) reduced the number of viable bacteria in the cecal ligation and puncture model of sepsis in mice and enhanced the bactericidal activity of blood neutrophils and peritoneal exudate leukocytes in vitro (35). These observations combined with reports showing C1INH-mediated enhancement of neutrophil chemotaxis in plasma activated with lipopolysaccharide (LPS), zymosan or antigen-antibody complexes (36) suggest a role for C1INH in innate defenses against bacterial infections.

Another example of activities that are independent of protease inhibition is the ability of C1INH to inhibit, in a dose-dependent manner, the adhesion of U937 cells to human umbilical vein endothelial cells (37). Inhibition of leukocyte-endothelial cell adhesion was mediated by the binding of C1INH to P- and E-selectins at the surface of endothelial cells. Sialyl LewisX moieties on C1INH N-linked carbohydrate mediates the binding to selectins (37). In addition, C1INH and iC1INH inhibit leukocyte-endothelial cell adhesion under flow conditions in vitro and after tumour necrosis factor (TNF)-α-induced leukocyte rolling in vivo (38). Bergamaschi et al. had previously described C1INH binding to endothelial cells in culture and to liver sinusoidal endothelial cells, which protected the liver from ischaemia-reperfusion (IR)-induced damage (39, 40). Binding was enhanced by prolonged incubation at 4°C and without loss of C1s inhibitory activity. Modulation of selectin-mediated cell adhesion by C1INH may be important in the physiologic suppression of inflammation and may explain, in part, its protective role during inflammatory diseases.

Thrombosis and Haemostasis 104.5/2010 © Schattauer 2010
Interactions of C1INH with extracellular matrix components and with endogenous proteins that do not require protease inhibition have been reported. Although the biologic relevance of these reactions has not been established, it has been hypothesized that they may concentrate C1INH at extra-vascular sites of inflammation resulting in local regulation of complement and contact system activation. C1INH binds to the extracellular matrix proteins type IV collagen, laminin and entactin (41). In addition, tissue transglutaminase cross-links C1INH to immobilized fibrin (42). C1INH, in vitro, regulates alternative complement pathway activation via reversible binding to C3b, by interfering with the C3b-factor B interaction (43). Recently, we demonstrated binding of C1INH at the surface of Chinese hamster ovary cells expressing CD36 or chondroitin sulfate A (CSA). This binding was completely abrogated by pre-incubation of C1INH with soluble CD36 or CSA (Mejia, P., unpublished data).

Sepsis and endotoxin shock

The symptoms of gram negative bacterial endotoxin shock are due primarily to a complex glycolipid, endotoxin lipopolysaccharide (LPS), found in the outer membrane of these organisms. This syndrome is characterised by extensive triggering of multiple endogenous mediators. Administration of LPS to humans or experimental animals as well as gram-negative bacterial infections result in physiological changes including fever, hypotension, hypoglycemia, disseminated intravascular coagulation and shock (44). The complement and contact systems are implicated in the pathophysiology of sepsis; both can be activated directly by LPS and/or cell wall components of gram-negative bacteria (45–47). As described, C1INH is the major inhibitor of both systems. Moreover, increased levels of proteolytically inactivated C1INH in patients with fatal septic shock, while antigenic levels of C1INH remain normal, point to a deficiency of functional C1INH during sepsis (48). This may result in insufficient control of complement and contact systems activation. Therefore, it has been reasoned that therapy with C1INH may be beneficial for patients with septic shock. Abundant data demonstrate a positive effect on survival and improvement of biochemical and physiological parameters following C1INH administration using animal models of sepsis and endotoxin shock (reviewed in [49–51]). Studies analyzing the efficacy of C1INH in human sepsis have been limited and have generated less convincing results. High doses of C1INH are well tolerated but modest beneficial effects were observed (reviewed in [49–51]). Further studies are necessary to determine whether there is a clinical benefit from C1INH therapy in human sepsis and endotoxin shock.

Our studies demonstrate a C1INH protective effect independent of protease inhibition in mouse models of sepsis and endotoxin shock. Active C1INH and iC1INH protected mice from lethal gram-negative endotoxemia and in the cecal ligation and puncture (CLP) model of peritonitis and sepsis (35, 52). Protection from endotoxin shock occurred through direct binding to the LPS molecule, preventing its interaction with, and activation of, macrophages and endothelial cells (52). Binding of C1INH to LPS depends on N-linked glycosylation and on positively charged residues within the amino-terminal non-serpin domain (53, 54). Improved survival in the CLP model was explained in part by an enhancement in phagocytic activity and decreased levels of TNF-α following C1INH treatment. These results suggest that effects other than complement and contact system inhibition, including C1INH inhibition of leukocyte rolling and transmigration, also play an important role in protection. A recent report emphasizes the role of C1INH as a multi-faceted inhibitor of inflammatory responses (55). Using pig and human serum and whole blood, both C1INH and iC1INH reduced Escherichia coli-induced pro-inflammatory cytokines and chemokines. Specific complement inhibitors had a minor effect on these mediators demonstrating that the C1INH anti-inflammatory effect was independent of complement inhibition.

The binding of C1INH to Salmonella typhimurium LPS has been well characterised (53, 54). ELISA and native PAGE shift experiments have shown the interaction of C1INH with a wide range of gram-negative enteric bacterial LPS. The binding of both native and iC1INH results in inhibition of LPS-induced pro-inflammatory cytokine production. Notably, C1INH binds only to a restricted number of whole live enteric bacteria. The likelihood of an interaction between C1INH and the surface of the microorganism seems to be determined by the length of the polysaccharide chain of the endotoxin molecule because mutant bacteria expressing a truncated LPS lacking the O-antigen allow C1INH binding (Mejia P, unpublished data).

C1INH interactions with other bacteria also have been described. Bordetella pertussis, the causative agent of whooping cough, binds high levels of human C1INH which results in resistance to serum killing of the bacteria (56). This interaction requires protease inhibitory activity by C1INH and is not abrogated by N-deglycosylation. Another example of C1INH use by pathogenic microorganisms to evade complement-mediated killing is the finding that StC, a metalloprotease secreted by E. coli O157:H7, cleaves C1INH within the amino-terminal domain and localizes it to cell membranes resulting in enhanced inhibition of complement activation at sites of lytic complex formation (57, 58).

Malaria

Severe Plasmodium falciparum malaria is a complex multisystem disorder (59). Mechanisms involved in the pathology during the erythrocytic stage include parasite replication, parasite toxins release, pro-inflammatory cytokine production, cytoadherence of parasitized RBCs (pRBCs) to vascular endothelium and to other infected and uninfected RBCs, and sequestration of pRBCs within the small vessels of many tissues (60). The effect of C1INH on the pathogenic mechanisms induced by P. falciparum was assessed recently, inspired by the effectiveness of C1INH in gram-negative bacterial endotoxin shock and structural similarities be-
IR injury

IR injury is a relatively common clinical event implicated in a variety of pathological conditions. C1INH has been used in animal models of IR injury and in some human clinical studies for more than a decade. C1INH exerts significant protective effects against reperfusion injury, especially in the myocardial IR model. Intravenous C1INH at, or just before, reperfusion attenuates troponin and creatine kinase release, reduces infarct size and prevents neutrophil infiltration (reviewed in [49–51]). This led to the first use of C1INH in patients receiving emergency coronary surgery for failed percutaneous transluminal coronary angioplasty in 1998 (62). Other studies showed that C1INH was safe and reduced complement activation (reviewed in [49–51]). Cardiac troponin I levels were reduced in the C1INH-treated patients suggesting suppression of myocardial damage. A controlled randomised double-blind study confirmed these findings and showed that C1INH treatment resulted in reduction of cardiac enzyme levels and improvement of clinical parameters (63). We recently analysed the cardioprotective effect of intravenous C1INH in a mouse myocardial IR model (Lu F, unpublished data). In both wild type and C3 deficient mice, both C1INH and iC1INH reduced infarct size, decreased neutrophil infiltration and attenuated cardiac enzyme elevation. Therefore, the anti-inflammatory effect of C1INH very likely is mediated by both inhibition of complement activation and by other mechanisms. Further analysis of the precise mechanism of this protection is needed. We have hypothesised that the protective effect may be due to the suppression of leukocyte infiltration secondary to the interaction of C1INH with endothelial selectins, as described above (37, 38).

Beneficial effects of C1INH have been reported in a variety of other IR models (reviewed in [6, 49–51]). C1INH, in a murine model of transient brain ischemia, reduced both general and local deficits and ischemic volume, with decreased accumulation of leukocytes, and decreased expression of P-selectin, ICAM-1, pro-inflammatory cytokines and procaspase-3, together with improvement in appearance and motor performance (64). C1q-deficient mice are susceptible to brain IR injury and C1INH is neuroprotective in C1q-deficient mice, which indicates that this action is independent from classical complement pathway activation (65). This is consistent with mediation of damage via lectin pathway activation and/or with C1INH-mediated effects that do not depend on protease inhibition. In animal models of hepatic IR injury, C1INH treatment resulted in a reduction of leukocyte adhesion and an increase in the number of perfused sinusoids (reviewed in [49–51]). C1INH treatment of skeletal muscle IR injury prevented plasma creatine kinase elevation, reduced injury and improved contractile function in reperfused muscles (reviewed in [49–51]). In intestinal IR injury, C1INH suppresses tissue injury and attenuates the accumulation of neutrophils (66–69). Both active C1INH and iC1INH decreased leukocyte rolling in the mesenteric circulation after the onset of reperfusion, which indicates, in agreement with other data, that C1INH inhibitor may directly suppress leukocyte migration (37, 38, 68).

Transplantation

Complement activation and endothelial cell dysfunction are involved in the inflammatory events in the early phase of transplant rejection. C1INH has been used in in vitro, ex vivo and in vivo xenotransplantation models to prevent such activation and related pathological processes. Soluble human C1INH prevented the activation of porcine endothelial cells incubated with human serum (reviewed in [49–51]). Surface-bound C1INH was shown to be quite effective in down-regulating complement-mediated xenogeneic cell lysis and xenograft rejection (reviewed in [49–51]). In an ex vivo pig-to-human perfusion model, the survival of C1INH-perfused kidneys was significantly longer than controls and the activation of complement, platelets and neutrophils was markedly reduced (70). Similar findings were obtained from other ex vivo transplantation models (71, 72). In lung transplantation models, pre-treatment with C1INH prevented early pulmonary dysfunction and organ damage (73, 74). Two clinical cases suggested that C1 inhibitor may play a role in the management of capillary leak syndrome after lung and liver transplantation (75, 76). C1INH reduced cell adherence and infiltration in transplantation-related hepatic IR injury (77). In an experimental heart transplantation model, intracoronary administration of C1INH significantly improved right ventricular function (78). As discussed previously, C1INH added to the organ preservation solution during storage bound to endothelial cells and retained the ability to inhibit C1s, which suggested that endothelial targeting with C1INH may be useful to reduce complement activation and tissue injury (39, 40).

Other inflammatory diseases

The effect of C1INH also has been investigated in animal models for a variety of other inflammatory diseases. In experimental pancreatitis, C1INH substitution reduced mortality and ameliorated the course of disease (79). These results were confirmed by Yamauchi et al., who demonstrated that the combined application of C1INH and antithrombin III improved survival in severe haemorrhagic pancreatitis, but the beneficial effects on edema and the histopathological lesion were not significant (80). In contrast, high
doses of C1INH failed to have beneficial effect in other models of experimental pancreatitis (81). Experience using C1INH in human pancreatitis is very limited (82, 83), which makes it difficult to judge whether this therapy might be of clinical benefit. In the dextran sulfate sodium-induced colitis model in mice, C1INH ameliorated colon inflammation and suppressed leukocyte infiltration (Lu F, unpublished data).

In a porcine model of thermal injury, C1INH reduced organ alteration, improved microcirculation and suppressed bacterial translocation into the gastrointestinal tract in the acute stage (84, 85). In burned patients, activation of the complement and coagulation systems may play an important role in the development of the capillary leak syndrome and inflammatory tissue destruction. Application of C1INH could prove to be an effective means to suppress such damage after thermal trauma (86).

Conclusions

C1INH has been used for treatment of attacks of angioedema for many years. For this disease, it is quite effective and well tolerated with few, if any, side effects. Over the past few years, as outlined here, it has been considered for use in a variety of inflammatory diseases including IR injury (cardiac, cerebral, muscle, liver, gastrointestinal), hyper-acute transplant rejection, gram negative sepsis, particularly endotoxin shock, in addition to a number of other conditions. In animal models of these diseases, C1INH has been surprisingly effective and, to some extent, is beginning to be used in human diseases. It has been assumed that the effectiveness of C1INH in these diseases was entirely a function of its suppression of activation of the complement and contact systems, each of which are involved to some extent in the mediation of all these conditions. However, data has begun to emerge, from our laboratory and from others, that activities that do not depend on protease inactivation are also involved. The potential activities that may be involved include a direct effect on inhibition of the activity of gram negative bacterial endotoxin, enhancement of phagocytosis, and suppression of leukocyte rolling and transmigration of leukocytes across the endothelium to sites of inflammation, in addition to other less well-defined activities. Future studies should continue to clarify the precise mechanism of the C1INH effect in these diseases.

Acknowledgements

The studies described from the author’s laboratory were supported by National Institutes of Health Grants RO1-AI057366 and R37-HD22082.

References


© Schattauer 2010

Downloaded from www.thrombosis-online.com on 2018-04-15 | ID: 1001066444 | IP: 54.70.40.11
For personal or educational use only. No other uses without permission. All rights reserved.
boration of hereditary angioedema. Mol Immunol 1982; 19: 1365 (Ab- 
stract).
edema: Characterization of plasma kinin and vascular permeability-enhancing 
29. Wisnieski J, Krauss TC, Yike I, et al. Unique C1 inhibitor dysfunction in a 
kidney without angioedema. I: A mutant C1 inhibitor that inhibits C1s but not C1r. J 
kidney without angioedema. II: Identification of an Ala443-Val substitution and 
fuctional analysis of the recombiant mutant protein. J Clin Invest 1995; 95: 1299- 
3105.
31. Zahedi R, Wisnieski J, Davis III AE. Role of the P2 residue of complement 1 in-
hibitor (Ala443) in determination of target protease specificity. J Immunol 1997; 159: 
983–988.
32. Han ED, MacFarlane RC, Mulligan AN, et al. Increased vascular permeability in 
C1 inhibitor-deficient mice is mediated by the bradykinin type 2 receptor. J Clin 
34. Chang N-S, Boackle RJ, Leu RW. Characteristics of C1 inhibitor binding to 
no 2007; 179: 3966–3972.
36. Smith CW, Hollers JC, Bing DH, et al. Effects of human C1 inhibitor on comple-
37. Cai S, Davis III AE. Complement regulatory protein C1 inhibitor binds to selec-
tins and interferes with endothelial-leukocyte adhesion. J Immunol 2003; 171: 
4786–4791.
38. Cai S, Dole V, Bergmeier W, et al. A direct role for C1 inhibitor in leukocyte ad-
effect of organ preservation solution on endothelial cells during cold storage. 
degrees complement activation in vitro and during ex vivo reperfusion of pig 
41. Patston PA, Schapira M. C1 inhibitor cross-linking by tissue transglu-
42. Jiang H, Wang X, Zhang H, et al. Component 1 inhibitor is a regulator of the al-
44. Kalter ES, van Dijk WC, Timmerman A, et al. Activation of purified human plas-
ma prekallikrein triggered by cell wall fractions of Escherichia coli and Staphy-
45. Morrison DC, Kline LF. Activation of the classical and properdin pathways of 
47. Lathem WW, Bergsbaken T, Welch RA. Potentiation of C1 esterase inhibitor by 
1077–1087.
49. Schofield L, Grau GE. Immunological processes in malaria pathogenesis. Nat Rev 
50. Caro HN, Sheikh NA, Taverne J, et al. Structural similarities among malarial to-
xisins insulin second messengers, and bacterial endotoxin. Infect Immun 1996; 64: 
3438–3441.
51. Bauernschmitt R, Bohrer H, Hagl S. Rescue therapy with C1-esterase inhibitor 
concentrate after emergency coronary surgery for failed PTCA. Intensive Care 
52. Liu D, Cai S, Gu X, et al. C1 inhibitor prevents endotoxin shock via a direct inter-
53. Liu D, Gu X, Scalfid I, et al. N-linked glycosylation is required for C1 inhibitor-
mediated protection from endotoxin shock in mice. Infect Immun 2004; 72: 
54. Davis III et al. C1 inhibitor functions
Ratnoff Symposium 2009

© Schattauer 2010

Thrombosis and Haemostasis 104.5/2010

Downloaded from www.thrombosis-online.com on 2018-04-15 | ID: 1001066444 | IP: 54.70.40.11
For personal or educational use only. No other uses without permission. All rights reserved.


