ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 337, No. 2, January 15, pp. 291–299, 1997
Article No. BB969787

Crovidisin, a Collagen-Binding Protein Isolated from Snake Venom of *Crotalus viridis*, Prevents Platelet–Collagen Interaction[1]

Chao-Zong Liu and Tur-Fu Huang[2]
Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan

Received March 29, 1996, and in revised form October 11, 1996

By means of liquid chromatography consisting of gel filtration, anionic exchange, and C8 reverse-phase HPLC, a selective inhibitor of collagen-induced platelet aggregation, named crovidisin, has been purified to homogeneity from the venom of *Crotalus viridis* snake. Crovidisin is a single-chain, 53-kDa protein with a selective inhibitory activity on collagen-induced aggregation of human washed platelets without affecting those elicited by thrombin, sodium arachidonate, and ADP. Partial sequencing of tryptic digests of crovidisin reveals that partial sequence of crovidisin appears to be identical to that of catrocollastatin, a collagen antagonist occurring in the venom of *Crotalus atrox* snake. Crovidisin dose-dependently blocked aggregation of human washed platelets triggered by 5 and 10 μg/ml of collagen with IC_{50} of 0.17 and 0.47 μM, respectively. Not only platelet aggregation but also release reaction, thromboxane formation, and increase of intracellular Ca^{2+} level of platelets in response to collagen were all completely abolished by crovidisin. In the presence of crovidisin, the Mg^{2+}-dependent adhesion of platelets to collagen was diminished in a dose-dependent manner, while the glycoprotein IIb/IIIa-mediated platelet–fibrinogen interaction was unaffected. When collagen was pretreated with crovidisin and followed by three washes with phosphate-buffered saline, the antiadhesion activity of crovidisin was unaffected. In addition, collagen fibers emitted fluorescence after incubation with fluorescein isothiocyanate-conjugated crovidisin, indicating that crovidisin binds directly to collagen fibers. In conclusion, crovidisin blocks the interaction between platelets and collagen fibers through its binding to collagen fibers, resulting in the blockade of collagen-mediated platelet functions such as adhesion, release reaction, thromboxane formation, and aggregation. © 1997 Academic Press

***Key Words: Crotalus viridis;* snake venom; crovidisin; collagen antagonist; platelet aggregation.**

Subendothelial collagen serves as an anchorage for circulating platelets in the processes of hemostasis and thrombosis (1). Through membrane GPIa/IIa, platelets adhere to exposed subendothelial collagen and become activated, releasing ADP as well as thromboxane A_2 to further activate platelets. Subsequent binding of fibrinogen to GPIIb/IIIa of the activated platelets results in platelet aggregation, eventually forming hemostatic plug to arrest blood loss under normal conditions, but forming thrombus under a pathological environment.

There are profound disturbances in hemostatic processes of the victims who have been bitten by the snakes of Viperidae and Crotalidae families (2). Studies of these snake venoms have revealed that snake venom proteins affect platelet functions markedly by acting as either inducers or inhibitors (3). Of these proteins, the most prominent inducers are trimucytin and aggretin which were isolated from *Trimeresurus mucrosquamatus* (Taiwan habu snake) and *Calloselasma rhodostoma* (Malayan pit viper) venoms, respectively. Both inducers cause platelet activation and aggregation through the activation of endogenous phospholipase C by acting as GPIa/IIa agonists (4, 5). On the other hand, disintegrins are a group of low-molecular-weight proteins occurring in various snake venoms (6) and have been named according to their inhibitory activity on GPIIb/IIIa, a member of integrins (7). The first documented disintegrin, trigramin, was reported by Huang *et al.* (8). Near its carboxyl terminal it was found to contain an Arg-Gly-Asp (RGD) sequence (9),

[1] This work was financially supported by grants from the Department of Health, Taiwan (DOH 85-HR-526) and National Science Council of Taiwan (NSC85-2331-B002-059BC).

[2] To whom correspondence should be addressed. Fax: 886-2-341-7930.

0003-9861/97 $25.00

which has been demonstrated to play an important role in matrix–cell interaction (10). The study of these venom proteins not only enriches our understanding of the mechanism underlying the detrimental symptoms following snakebite (11), but also provides useful tools for exploring the mystery of life science (12) as well as opening a new avenue for developing antithrombotic agents (13).

Recently, some inhibitors which specifically interfere with collagen-mediated platelet functions have been found in snake venoms, including a 50-kDa protein from *Bothrops atrox* (14), jararhagin from *Bothrops jararaca* (15), and catrocollastatin from *Crotalus atrox* (16). The action mechanism of jararhagin has been referred to be a collagen receptor (GPIa/IIa) antagonist, whereas the other two have not been documented. Our laboratory found that snake venom of *Crotalus viridis* possessed an inhibitory activity on platelet aggregation elicited by various inducers and this inhibitory activity was mainly attributed to the action of crotavirin, a member of disintegrins, by acting as a GPIIb/IIIa antagonist (17). Concurrently, we also found that there was another platelet aggregation inhibitor in this venom. This principle inhibited the platelet aggregation caused by collagen but not that by thrombin. The purpose of this study was to characterize this selective inhibitor of collagen-induced platelet aggregation and to study its mechanism of action.

MATERIALS AND METHODS

Materials. Lyophilized crude venom powder of *C. viridis* was purchased from Latoxan (Rosans, France). Molecular weight standards for SDS/PAGE and gels used for chromatography, including Sephadex G-100, G-10, and DEAE Sephadex A-50, were obtained from Pharmacia (Uppsala, Sweden). Bovine thrombin was obtained from Parke Davis (Detroit, MI). Sodium arachidonate, Fura-2/AM, type I collagen (bovine achillis tendon), human fibrinogen, prostaglandin E_1, ADP, apyrase, firefly lantern extract (mixture of luciferin and luciferase), and BSA[3] were obtained commercially from Sigma Chemical Co. (U.S.A.). Heparin was from Novo Nordisk (Bagsvaerd, Denmark) and fluorescence dyes, BCECF/AM, and FITC were purchased from Molecular Probes, Inc. (U.S.A.). Trifluoroacetic acid (TFA) and acetonitrile were from Merck (U.S.A.). Iodoacetic acid and urea were commercially obtained from Fluka Chemical Co. (Japan) and Kanto Chemical Co. (Japan), respectively. Monoclonal antibodies, 6F1 (against glycoprotein Ia/IIa) (18) and 7E3 (against glycoprotein IIb/IIIa) (19), were kindly provided by Dr. B. S. Coller (Mount Sinai Medical Center, New York, NY). Crotavirin, a member of disintegrins, was purified from *C. viridis* snake venom following the protocol described by Liu *et al.* (17).

Purification of crovidisin from Crotalus viridis venom. Crude venom powder (0.1 g) was first fractionated by a Sephadex G-100 column (1.6 × 70 cm) eluted with 0.01 M ammonium bicarbonate. Antiplatelet activity of each fraction was assessed on thrombin- and collagen-induced aggregation of human washed platelets. Fractions that selectively inhibited collagen-induced platelet aggregation were pooled and lyophilized and a DEAE Sephadex A-50 column (1.6 × 40 cm) was employed for the further purification with a buffer gradient from 0.2 M ammonium acetate (pH 8.2) to 0.8 M ammonium acetate (pH 6.4) at a flow rate of 15 ml/h. Crovidisin was finally purified to homogeneity by HPLC using a reverse-phase Ultrapore C8 column (0.46 × 25 cm, Beckman) equilibrated with solution A (0.1% TFA in water, v/v) at a flow rate of 1 ml/min, and a gradient of solution B (a mixture of water (20%), acetonitrile (80%), and TFA (0.1%), v/v) from 25 to 80% in 40 min was used to elute the applied proteins. Protein eluates were monitored by UV detector at 280 nm and dried with a SpeedVac concentrator. After reconstituting these proteins with distilled water, their antiplatelet activities were subsequently tested.

SDS/PAGE. The molecular weight of crovidisin was estimated by SDS/PAGE on a 12% polyacrylamide gel following the method described by Laemmli (20) in the presence or absence of reducing agent, β-mercaptoethanol (4%, v/v). Following electrophoresis, protein bands on the slab gel were stained with Coomassie blue R-250 (0.1%).

S-carboxymethylation of crovidisin. This reaction was carried out by adding 20 μl of iodoacetic acid (1 M in H_2O) to the reduced protein (800 μg in 1 ml of 8 M urea, 15 mM Tris–HCl, pH 8.5, and 20 mM β-mercaptoethanol). The reaction mixture was incubated 2 min at 25°C in the dark and the modified protein was isolated free of reagents by a Sephadex G-10 column (1 × 30 cm).

Measurement of amino acid composition of crovidisin. The amino acid composition of crovidisin was measured according to the method of Heinrikson and Meredith (21). After hydrolysis in 6 N HCl for 1 h at 150°C, free amino acids derived from S-carboxymethylated crovidisin were coupled with phenyl isothiocyanate (PITC) for 30 min at room temperature and subsequently quantified by reverse-phase HPLC by comparing with amino acid standards coupled with PITC under similar conditions.

Preparation of human platelet suspension. Human platelet suspension was prepared following the protocol of Mustard *et al.* (22). Blood samples obtained from healthy adults who did not receive medication in the past 2 weeks were anticoagulated with acid citrate/dextrose and centrifuged at 150*g* at room temperature (25°C) for 9 min to obtain platelet-rich plasma (PRP). After being incubated with heparin (5 units/ml) and prostaglandin E_1 (1 μM) for 5 min at room temperature, platelets of PRP were pelleted by centrifugation at 500*g* at room temperature for 7 min and subsequently washed two times with Tyrode solution (NaH_2PO_4, 0.4 mM; NaCl, 136.9 mM; KCl, 2.7 mM; $NaHCO_3$, 11.9 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; BSA, 3.5 mg/ml; pH 7.35). Heparin (5 units/ml), prostaglandin E_1 (1 μM) and apyrase (0.5 units/ml) were included in the first suspension medium, while the last wash contained only apyrase (0.5 units/ml). Platelet count was adjusted to be 3.75×10^8 platelets/ml for aggregation assay. BCECF-loaded platelet suspension was prepared basically according to the procedure described above. To uptake BCECF/AM, platelet pellets obtained from PRP were suspended in the modified Tyrode solution (Ca^{2+}-free, 2 mM of Mg^{2+}) containing 5 mg/ml of BCECF/AM and incubated at 37°C for 30 min. Dye-loaded platelets were pelleted and washed two times with the modified Tyrode solution as described above and prostaglandin E_1 (2 μM) was included in all processes. Finally, BCECF-loaded platelets were suspended in modified Tyrode solution containing prostaglandin E_1 (2 μM) and adjusted to be 3×10^8 platelets/ml for adhesion assay.

Measurement of platelet aggregation, ATP release. Platelet aggregation was measured with a lumi-aggregometer (Payton Scientific) turbidimetrically (23). Mixtures (total 500 μl) of 400 μl of human platelet suspension (3.75×10^8 platelets/ml), test sample, and an appropriate volume of Tyrode solution have been incubated at 37°C for 3 min under a stirring condition (900 rpm) prior to the addition

[3] Abbreviations used: GP, glycoprotein; BSA, bovine serum albumin; TFA, trifluoroacetic acid; PITC, phenyl isothiocyanate; BCECF/AM, 2′,7′-bis(2-carboxyethyl)-5-(and -6-)carboxyfluorescein, acetoxymethyl ester; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PRP, platelet-rich plasma.

of inducers. The extent of aggregation was expressed as the increase of light transmission at 6 min after the addition of aggregation inducer. Percentage aggregation of platelets was calculated as the final extent of aggregation relative to that of control (as 100% aggregation). ATP release reaction was monitored by bioluminence change (24) with 10 μl mixture of luciferin and luciferase.

Measurement of the intracellular Ca^{2+} level. The concentration of intracellular free Ca^{2+} was determined using a fluorescent probe, Fura-2, according to the method of Pollock and Rink (25). Fura-2-loaded platelets were prepared essentially as the washed platelets except washing with Ca^{2+}-free Tyrode solution and allowed platelets to uptake Fura-2/AM (1 μM) for 30 min in the first wash. The intracellular Ca^{2+} level of Fura-2-loaded platelets (3×10^8/ml) in the presence of 1 mM of Ca^{2+} was measured by the fluorescence change (excitation at 339 nm, emission at 500 nm) with a Hitachi fluorescence spectrophotometer. The intracellular Ca^{2+} concentration was calculated by the equation described by Grynkiewicz *et al.* (26).

Measurement of thromboxane formation. Thromboxane formation of platelets caused by collagen in the presence of vehicle, crovidisin, or crotavirin was stopped by adding indomethacin (50 μM) and EDTA (2 mM) at 6 min following the addition of collagen. After centrifugation, the content of thromboxane B_2, a stable metabolite of thromboxane A_2, in the supernatant was determined by a EIA kit (Cayman Chemical Co.).

Platelet adhesion assay. This assay was carried out according to the protocol described by Santoro (27) with a minor modification. Matrix, type I collagen (100 μg/ml) in acetic acid (25 mM) or fibrinogen (100 μg/ml) in phosphate-buffered saline (PBS), was coated onto plate wells (Falcon, 96-well) for 4 h at room temperature (25°C) and subsequently blocked with BSA (5 μg/ml) solution for 1 h. The coated wells were rinsed with PBS (100 μl) for three times and then an aliquot of 50 μl of BCECF-loaded platelet suspension (3×10^8/ml) pretreated with vehicle, crovidisin, or monoclonal antibodies for 10 min was added into matrix-coated wells. Platelets were allowed to settle onto collagen or fibrinogen for 10 and 20 min at room temperature (25°C), respectively. Nonadherent platelets were removed by aspiration and a gentle wash with 200 μl of the modified Tyrode solution. Adherent platelets were lysed with 100 μl of Triton X-100 (1%) and the plate was subsequently subjected to a fluorescent reader (Cytofluor 2300, Millipore) equipped with excitation and emission filters centered at 485 and 530 nm, respectively, for the measurement of fluorescence intensity. Percentage adhesion was calculated according to a regression plot of fluorescence intensity versus the fixed number of BCECF-loaded platelets (ranged from 1.5 to 100% of total added cells) in 100 μl of Triton X-100 (1%, v/v).

FITC conjugation of proteins and their interaction with collagen. Conjugation of crovidisin and BSA with FITC was carried out according to the procedure described by Liu *et al.* (28). FITC conjugation did not affect crovidisin's inhibitory activity. Similar conjugating efficacy was obtained with crovidisin and BSA. Prior to the binding assay, a 96-well plate was precoated with collagen as described above. Various concentrations of FITC–crovidisin or FITC–BSA (in PBS) were allowed to interact with coated collagen at room temperature for 10 min, followed by three washes with PBS and subsequently the plates were subjected to fluorescence measurement by a fluorescent reader (Cytofluor 2300). The fluorescence intensity of collagen fibers was measured using excitation and emission wavelengths at 485 and 530 nm, respectively. The fluorescence image of the collagen fibers was also taken by a Nikon fluorescent microscope equipped with a camera.

Cleavage of S-carboxymethylated crovidisin with trypsin. Trypsin (TPCK treated, Worthington Biochemical Corp.) digestion of the modified crovidisin was carried out at pH 8.8 for 2 h at 37°C with an enzyme to protein ratio of 1:50 by weight and tryptic peptides were isolated by a reverse-phase HPLC using a C18 column (LiChrospher WP 300, Merck) with a linear gradient from 0 to 60% of solution B (a mixture of water (20%) and acetonitrile (80%)) for 40 min at a flow rate of 1 ml/min.

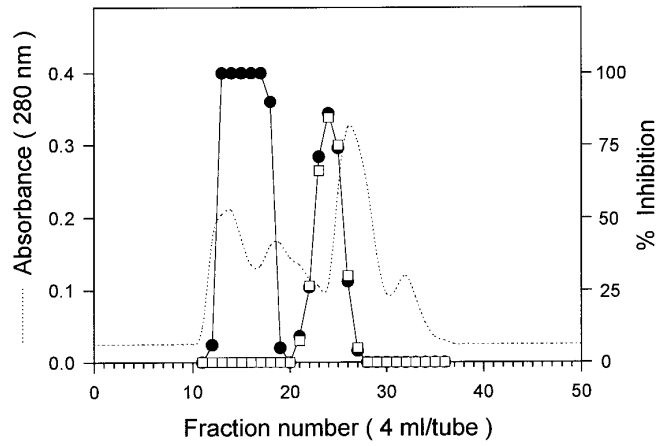


FIG. 1. Chromatography of crude venom of *Crotalus viridis* snake on a Sephadex G-100 column. Crude venom powder (0.1 g) was fractionated on a Sephadex G-100 column (1.6 × 180 cm) eluted with 0.01 M ammonium bicarbonate at a flow rate of 6 ml/h and absorbance (at 280 nm) of the eluent was continuously recorded (· · ·). An aliquot (10 μl) of each fraction was assayed for antiplatelet activity on collagen (10 μg/ml, ●) and thrombin (0.2 U/ml, □)-induced platelet aggregation.

Peptide sequencing. Amino acid sequences of some tryptic peptides were determined by using an Applied Biosystems 477A gas-pulsed liquid-phase sequencer equipped with an on-line 120A PTH–amino acid analyzer.

RESULTS

Isolation and Characterization of Crovidisin

Crude venom of *C. viridis* was first fractionated by gel filtration on a Sephadex G-100 column and absorbance of the eluent at 280 nm was continuously monitored. Platelet aggregation in response to collagen and thrombin was found to be inhibited by aliquot (in 10 μl) from fractions 22 to 27, in which a member of disintegrin family, termed crotavirin, has been demonstrated to account for this inhibitory action (17). In addition, fractions that exhibited a selective inhibitory activity on collagen-induced platelet aggregation (Fig. 1) were pooled, lyophilized, and further purified by anionic exchanger (DEAE-Sephadex A-50). Fractions (ranging from 83 to 100) that inhibited collagen (10 μg/ml)-induced platelet aggregation (Fig. 2) were desalted and finally purified by reverse-phase HPLC. Using a linear gradient of acetonitrile, the active principle was eluted at the retention time of 36 min (Fig. 3) and SDS/PAGE of this protein showed a single band with a molecular weight of 53 and 62 kDa, respectively, under nonreduced and reduced conditions (Fig. 4). This specific inhibitor of collagen-induced platelet aggregation was named as crovidisin. Amino acid analysis revealed that crovidisin is a glycine- and lysine-rich protein (Ta-

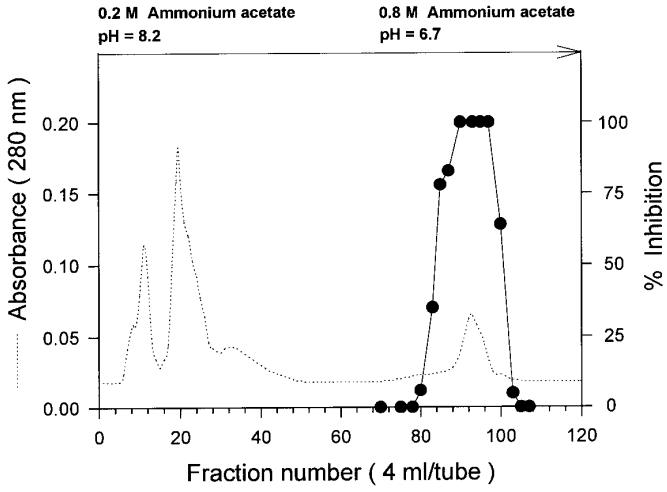


FIG. 2. Chromatography of active principle from a Sephadex G-100 column on a DEAE-Sephadex A-50 column. The active material from the Sephadex G-100 column was further purified by a DEAE-Sephadex A-50 column (1.6 × 40 cm) as described under Materials and Methods. Only collagen-induced platelet aggregation (●) was tested to reveal active fractions.

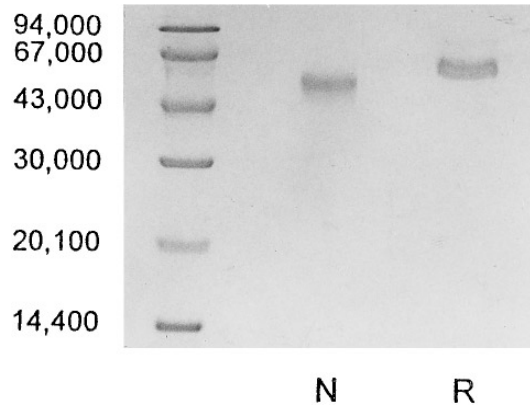


FIG. 4. SDS/PAGE of crovidisin. Crovidisin (5 μg) was analyzed on 12% SDS/polyacrylamide gel in the presence (R) or absence (N) of reducing agent β-mercaptoethanol (4%, v/v). The standard proteins used for calibration of the apparent molecule weight were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

ble I) and its composition was found to be similar to that of another snake (*Crotalus atrox*)-derived inhibitor, catrocollastatin, whose amino acid sequence has been deduced from its cDNA (16).

Effect of Crovidisin on the Aggregation and ATP Release of Human Washed Platelets Elicited by Collagen

Crovidisin inhibited collagen-induced aggregation of human washed platelets in a dose-dependent manner and its potency depended on the concentration of collagen used for triggering aggregation (Fig. 5). IC_{50} of crovidisin on platelet aggregation elicited by 5 and 10 μg/ml of collagen were estimated to be 0.17 and 0.47 μM,

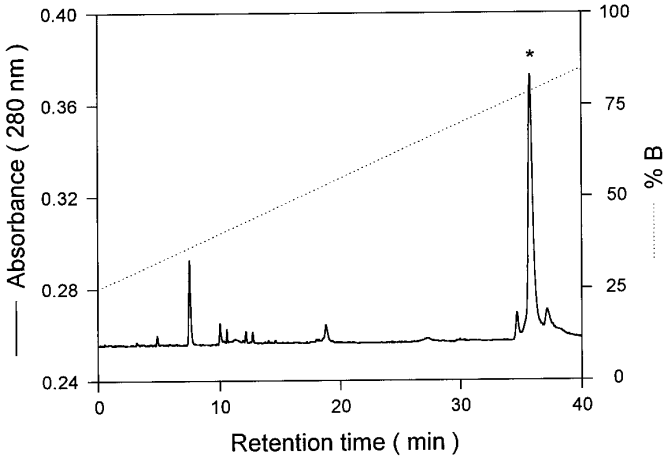


FIG. 3. Reverse-phase HPLC of crovidisin. Partially purified crovidisin (100 μg) was applied to an Ultrapore C8 column equilibrated with solution A (0.1% TFA in H_2O, v/v) and proteins were eluted by an increasing gradient of acetonitrile as indicated (· · ·) of solution B (mixture of 20% H_2O and 80% acetonitrile containing 0.1% TFA). Crovidisin was eluted at 36 min (*).

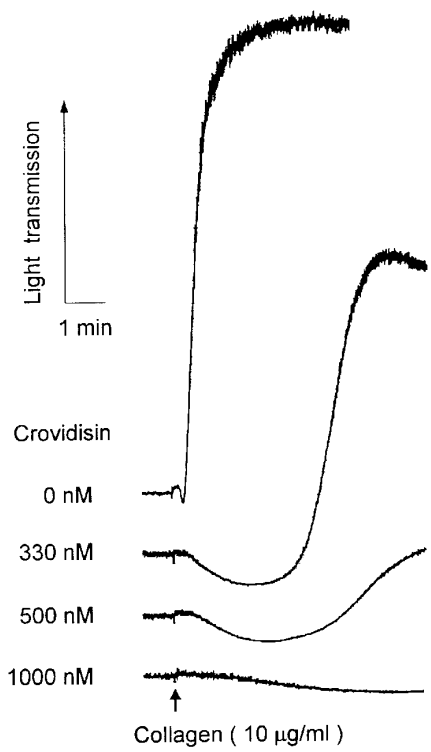
TABLE I

Amino Acid Composition of Crovidisin

Amino acid	Residues/mole	
	Crovidisin	Catrocollastatin
Asp/Asn	71	75
Glu/Gln	48	69
Ser	18	35
Gly	48	45
His	13	20
Thr	16	25
Ala	26	34
Arg	18	15
Pro	26	30
Tyr	29	37
Val	29	38
Met	12	16
Ile	31	31
Leu	35	40
Cys	36	39
Phe	18	16
Lys	42	40
Trp	ND	4
Total	516	609

Note. Amino acids are expressed as three-letter abbreviations and the nearest integer of each amino acid residue is shown. Asparagine and glutamine were determined as aspartic acid and glutamic acid, respectively. ND, not determined. The amino acid composition of catrocollastin was taken from the sequence deduced from cDNA (16).

respectively. At the concentration (1 μM) that completely inhibited collagen (10 μg/ml)-induced platelet aggregation, crovidisin did not exert any significant effect on platelet aggregation caused by ADP, thrombin, and sodium arachidonate (Fig. 6). Collagen-induced ATP release was also dose-dependently attenuated by crovidisin and completely abolished when platelet aggregation was blocked (data not shown). Pretreatment of platelets with crovidisin for a longer period (10 min) did not cause a higher degree of inhibitory activity to be attained (data not shown). Furthermore, when platelets failed to aggregate in response to collagen (10 μg/ml) in the presence of crovidisin (1 μM), a further addition of collagen overcame the inhibitory activity of crovidisin and elicited a full aggregation (data not shown).



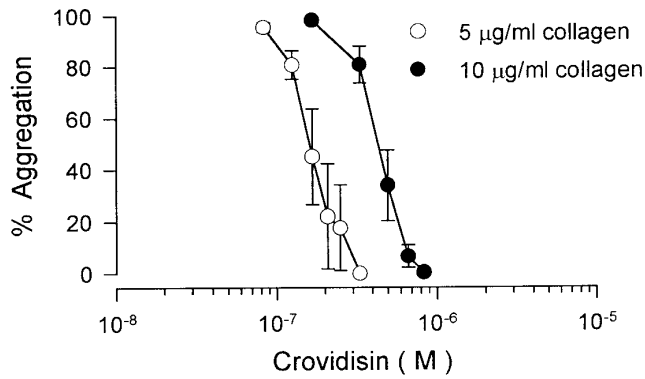


FIG. 5. Effect of crovidisin on collagen-induced platelet aggregation. Human washed platelets (3×10^8/ml) was preincubated with vehicle or various concentrations of crovidisin for 3 min at 37°C. Then, collagen (10 μg/ml) was added to trigger platelet aggregation (upward tracing). (Top) Typical tracings of platelet aggregation in four different experiments. Dose–inhibition curves of crovidisin on the platelet aggregation induced by 5 μg/ml (○) and 10 μg/ml (●) of collagen are shown (bottom). The percentage aggregation of platelets is calculated based on the extent of aggregation at 6 min after addition of collagen compared to that of control (without crovidisin). Data are expressed as means ± SEM (n = 4).

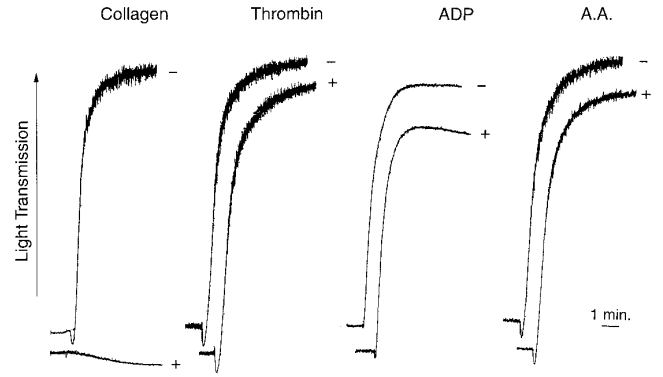


FIG. 6. Effects of crovidisin on platelet aggregation caused by collagen, thrombin, sodium arachidonate, and ADP. After pretreatment of platelet suspension with 1 μM crovidisin (+) for 3 min, various inducers, including collagen (10 μg/ml), thrombin (0.2 U/ml), sodium arachidonate (AA, 100 μM), or ADP (20 μM), were added into the platelet suspension to trigger platelet aggregation. Human fibrinogen (200 μg/ml) was added in the case of ADP-induced platelet aggregation. One of three similar experiments is represented.

Effect of Crovidisin on Thromboxane Formation of Platelets Caused by Collagen

Thromboxane A_2 formation is a landmark of collagen-induced activation of platelets. Thromboxane B_2, a stable metabolite of thromboxane A_2, in the suspension of platelets in response to collagen (10 μg/ml) was measured in the presence or absence of inhibitors crovidisin (1 μM) and crotavirin (0.5 μM), a glycoprotein IIb/IIIa antagonist (17). Our results showed that only negligible amount of thromboxane B_2 (0.35 ± 0.047 ng/ml, n = 4) was detected in the unstimulated platelet suspension, but collagen (10 μg/ml) markedly elevated the thromboxane B_2 content (68.4 ± 6.5 ng/ml, n = 4). Crovidisin completely blocked collagen-induced thromboxane B_2 formation (0.52 ± 0.14 ng/ml, n = 4; 99% inhibition). In contrast, there was no significant attenuation on collagen-induced thromboxane B_2 formation in the presence of crotavirin (56.7 ± 10.7 ng/ml, n = 4) compared with that of control ($P > 0.05$).

Effect of Crovidisin on the Intracellular Ca^{2+} Level Increase by Collagen

Collagen (10 μg/ml) caused an increase in intracellular Ca^{2+} level of platelets as reflected by the increase

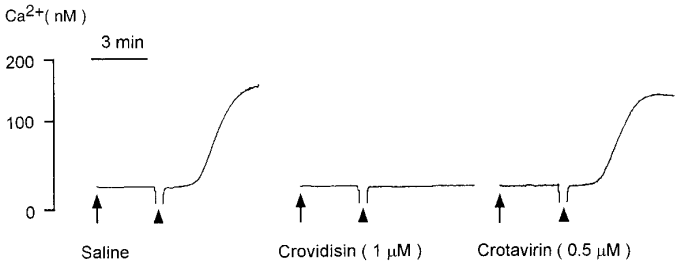


FIG. 7. Effects of crovidisin and crotavirin on the increase of intracellular Ca^{2+} concentration of human washed platelets triggered by collagen. Fura-2/AM-loaded platelets were preincubated with saline, crovidisin (1 μM), or crotavirin (0.5 μM) for 3 min and then collagen (10 μg/ml, ▲) was added to induce an increase in the level of cytosolic Ca^{2+}. One of four separate experiments is represented.

of fluorescence intensity with a latent period and this activity was completely abolished by crovidisin (1 μM) but not affected by crotavirin (0.5 μM) (Fig. 7).

Effect of Crovidisin on Platelet Adherence to Immobilized Collagen and Fibrinogen

In order to see whether crovidisin prevents platelet–collagen interaction, platelets were preloaded with a fluorescent dye, BCECF, and adhesion of platelets to collagen was assessed. The adhesion of BCECF-loaded platelets to coated collagen or fibrinogen was determined in the modified Ca^{2+}-free Tyrode solution containing 2 mM $MgCl_2$ and 2 μM PGE_1. As shown in Fig. 8, adhesion of platelets to collagen was progressively

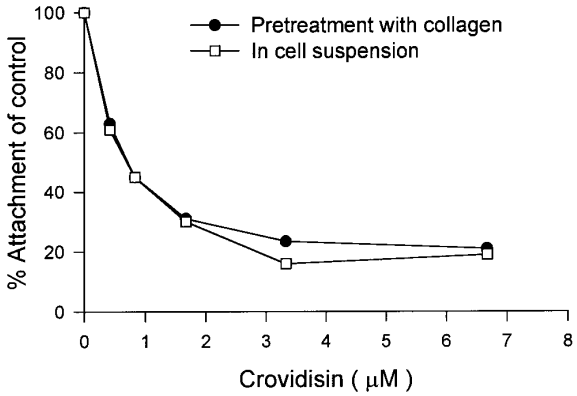


FIG. 8. Effects of crovidisin and monoclonal antibodies (6F1 and 7E3) on the adhesion of platelets to immobilized collagen and fibrinogen. Adhesion assays were performed as described under Materials and Methods. Mg^{2+}-dependent adhesion of platelets to collagen-coated surface (100 μg/ml, □) was determined in the presence of vehicle or various concentrations (ranging from 0.83 to 6.7 μM) of crovidisin. Concurrently, platelet adherence to crovidisin-pretreated collagen (10 min, then washed out) was also measured (●). All experiments were carried out in triplicate and data were expressed as means of triplicate results. Control efficiency of the adhesion of platelets to collagen and fibrinogen in the absence of inhibitors was 5.9 and 7.2% of total platelets added into the well, respectively.

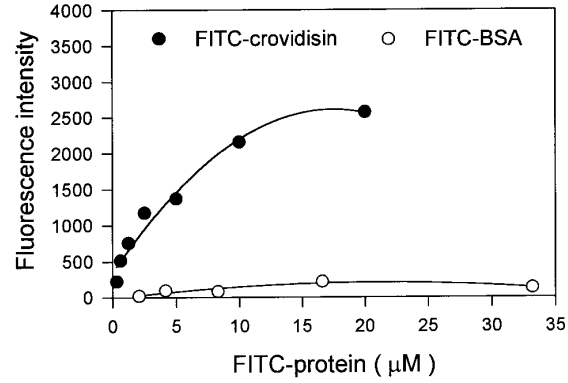


FIG. 9. Binding of FITC conjugated-crovidisin and BSA to collagen fibers. Various concentrations of FITC–crovidisin and FITC–BSA (50 μl) were incubated with coated collagen for 10 min at 25°C followed by three washes with PBS (200 μl). The fluorescence intensity of collagen fibers was determined with a Cytofluor 2300 fluorescent reader. Data were expressed as means of triplicate results.

diminished in the presence of increasing dose of crovidisin, and the maximal effect (about 80% inhibition) of crovidisin was achieved at 3.3 μM. After pretreatment of collagen fibers with crovidisin for 10 min followed by three washes with PBS, the interaction between platelets and collagen remained defective. On the other hand, GPIIb/IIIa complex-mediated platelet–fibrinogen interaction was not significantly affected by crovidisin, even at concentration up to 6.7 μM (data not shown). Unlike crovidisin, crotavirin, a member of disintegrins, inhibited the adhesion of platelets to fibrinogen (96% inhibition) and only slightly affected the adhesion of platelets to collagen (11% inhibition) at the concentration of 4.4 μM. 6F1 and 7E3 are two murine monoclonal antibodies raised against platelet membrane GPIa/IIa and GPIIb/IIIa, respectively. At 20 μg/ml, 6F1 blocked the adhesion of BCECF-loaded platelets to immobilized collagen to an extent of 60% with only a slight effect on the interaction between platelets and fibrinogen (10% inhibition). Conversely, 7E3 (20 μg/ml) completely blocked platelet–fibrinogen interaction (97% inhibition), exhibiting only a slightly inhibitory activity (13% inhibition) on platelet–collagen interaction.

Interaction of FITC-Crovidisin with Collagen

After incubation with various concentrations of FITC-conjugated crovidisin (in PBS) for 10 min followed by three washes with PBS, collagen fibers emitted fluorescence (Fig. 9) and their fluorescence intensity was dependent on the concentration of FITC–crovidisin added (Fig. 9). On the other hand, there was no significant fluorescence increase if FITC–BSA was used instead of FITC–crovidisin (Fig. 9). Even after the extensive wash (10 times) with PBS, FITC–crovidisin

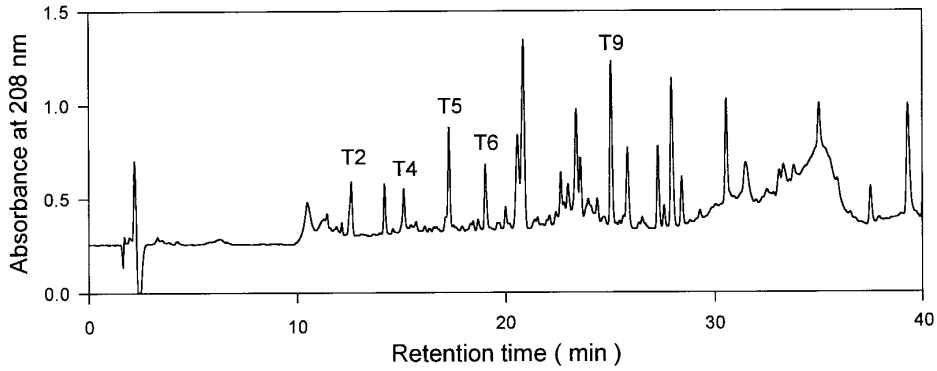


FIG. 10. HPLC elution profile of trypsin digest of S-carboxymethylated crovidisin. For details of separation, see Materials and Methods.

firmly bound to collagen fibers as evidenced by the strong fluorescence intensity of collagen (data not shown).

Partial Sequence Analysis of Crovidisin

The HPLC peptide map of the trypsin-digested crovidisin fragments was shown in Fig. 10 and the amino acid sequences of some tryptic peptides of crovidisin were shown in Fig. 11. These amino acid sequences of crovidisin show almost complete identity (50 of 51 residues) to that of catrocollastatin comparing their partial sequences of the digested fragments. Catrocollastatin, a collagen antagonist derived from *C. atrox* venom, is a multidomain molecule composed of a N-terminal domain, a metalloproteinase domain, a disintegrin-like domain, and a cysteine-rich C-terminal domain (16). Crovidisin differs from catrocollastatin only in a proline residue in place of serine at position 576 in the C-terminal domain of catrocollastatin (Fig. 11).

DISCUSSION

In the present study, a platelet aggregation inhibitor existing in snake (*C. viridis*) venom has been isolated and characterized to be a selective inhibitor of collagen-induced platelet aggregation. This novel inhibitor, named crovidisin, is a 53-kDa protein which selectively blocked platelet aggregation elicited by collagen in a dose-dependent manner without affecting those triggered by other inducers such as thrombin, ADP, and sodium arachidonate (Figs. 5 and 6). Crovidisin inhibited not only aggregation but also the activating processes, including granule release, thromboxane formation, and increase of cytosolic free calcium of platelets in response to collagen. This inhibitory profile of crovidisin is different from that of crotavirin, another platelet aggregation inhibitor occurring in the same venom which blocked the association of fibrinogen with GPIIb/IIIa complex without showing a significant effect on the activating process, suggesting that crovidisin acts at the upstream step, whereas crotavirin blocks the downstream of platelet aggregation caused by collagen. Platelets did not adhere to collagen in the presence of crovidisin (Fig. 9), confirming our inference that crovidisin interferes with the initial interaction between platelets and collagen. However, two possibilities exist that crovidisin may affect collagen-mediated platelet functions by acting on either collagen or collagen receptor expressed on the platelet membrane. We did not detect any binding of crovidisin to platelets (data not shown). The inhibitory effect of crovidisin on platelet aggregation was surmountable by a second challenge of collagen, indicating that collagen receptors on the platelet membrane remain accessible to collagen after crovidisin treatment. Furthermore, after pretreatment of collagen with crovidisin followed by successive PBS washes, the subsequent platelet–collagen interaction was still blocked, indicating that crovidisin may directly bind to collagen fibers in a rather irreversible manner (Fig. 9). As shown in Fig. 9, FITC–crovidisin directly bound to collagen fibers as the fluorescence intensity increased progressively as the concentration

Catrocollastatin (in metalloproteinase domain)		^{205} VVDKAMVTKNNGDLDKIKTR ^{224}
Crovidisin fragment	T4	AMVTK
	T6	NNGDLDK
Catrocollastatin (in disintegrin-like domain)		^{445} DCCEQCKFSKSGTECRASMS ^{464}
Crovidisin fragment	T2	SGTECR
Catrocollastatin (in C-terminal domain)		^{556} GRLYCKDNSPGQNNPCKMFYSNEDEHKGMVLPGTK ^{590}
Crovidisin fragment	T5	LYCKDNSPGQNNSCK
	T9	MFYSNEDEHKGMVLPGTK

FIG. 11. Partial amino acid sequences of crovidisin. Trypsin-digested fragments (T2, T4, T5, T6, and T9) were sequenced, mapped, and compared to those sequences deduced from c-DNA of catrocollastatin (16).

of this probe increased. On the other hand, FITC–BSA did not significantly bind to collagen. In addition, the interaction between crovidisin and collagen was in a divalent cation-independent manner because EDTA (2 mM) did not attenuate crovidisin binding to collagen, and the inhibitory activity of crovidisin on the adhesion of platelets to collagen was not affected by EDTA when collagen was pretreated with this inhibitor (data not shown). Although its mechanism of action is different from that of 6F1, a monoclonal antibody raised against glycoprotein Ia/IIa (also called $\alpha 2\beta 1$ or VLA2), crovidisin like 6F1 predominantly blocked platelet–collagen interaction without affecting GPIIb/IIIa-mediated platelet–fibrinogen interaction that was markedly inhibited by 7E3, a monoclonal antibody to GPIIb/IIIa, and by a snake venom-derived GPIIb/IIIa antagonist, crotavirin. This indicates that crovidisin selectively interferes with the interaction between collagen and platelets through binding to collagen fibers. However, whether crovidisin binds to denatured collagen type I or other types of collagen still remains to be elucidated.

Both molecular weight and amino acid composition of crovidisin were shown to be similar to those of catrocollastatin, a snake venom protein from *C. atrox*, which also inhibits platelet aggregation and adhesion to collagen. The cDNA-derived amino acid sequence of catrocollastatin reveals that this inhibitor contains an N-terminal domain, a metalloproteinase domain, a disintegrin-like domain, and a cysteine-rich C-terminal domain (16). From the partial amino acid sequence analysis, crovidisin was found to contain some identical sequences that appeared in the metalloproteinase domain, disintegrin-like domain, and C-terminal domain of catrocollastatin (Fig. 11). These results indicate that crovidisin may be a multidomain molecule composed of four different domains like catrocollastatin. Jararhagin is another inhibitor of collagen-induced platelet aggregation purified from *Bothrops jararaca* snake venom whose amino acid sequence shows high homology to that of catrocollastatin (16, 29). Despite their sequence similarity with that of catrocollastatin, crovidisin and jararhagin act as collagen antagonists through extremely different ways. Jararhagin was found to be a GPIa/IIa antagonist (15), whereas crovidisin's action mechanism was attributed to its binding to collagen fibers (this study). Why these two snake venom-derived collagen antagonists have different working mechanisms remains to be elucidated and these studies may promote our knowledge regarding the platelet–collagen interaction at the molecular level.

Many metalloproteinases, including hemorrhagic and nonhemorrhagic metalloproteinases, have been described in various snake venoms (30). Hemorrhagic metalloproteinases can degrade collagenous basement membrane and other connective tissue collagen with consequent weakening of the integrity of the vessel wall causing hemorrhagic effects (31). The metal ion, especially zinc ion, in the active site of these proteinases is required for enzymatic activity and in some cases it is important for maintaining the native structure of the protein (31, 32). Since crovidisin was proposed to contain the so-called metalloproteinase domain like catrocollastatin, it is reasonable to infer that this enzymatic activity may contribute to its inhibitory action. But our data revealed that it is unlikely in this case. First, a prolonged incubation of collagen with crovidisin did not enhance its inhibitory effect on platelet adhesion. Second, EDTA did not block crovidisin's inhibitory activity on the interaction between platelets and collagen. Third, the binding of crovidisin to collagen was not affected by EDTA (data not shown).

In addition to the normal functions, collagen may serve as a pathological mediator through its interaction with cells such as in arterial thrombosis (33) and in proliferative vitreoretinopathy which is the leading cause of failure of retinal reattachment surgery (34). Much effort has been devoted to understanding the mechanism regarding the collagen–cell interaction and designing inhibitors to block the interaction between collagen and cells. As a protein that can associate with collagen to prevent collagen-mediated platelet adhesion and aggregation, crovidisin may become an useful tool in elucidating the mechanism of collagen–cell interaction at a molecular level. Furthermore, in our unpublished data, crovidisin dose-dependently blocked the aggregation of human washed platelets caused by trimucytin, a snake venom protein which has been demonstrated to cause activation as well as aggregation of platelets by acting as a GPIa/IIa agonist (4). Further study on the interaction of crovidisin–collagen and crovidisin–trimucytin at a molecular level may provide a new avenue for developing inhibitors to prevent collagen-mediated detrimental effects.

ACKNOWLEDGMENTS

We express our gratitude to Dr. Barry S. Coller for his providing monoclonal antibodies of 6F1 and 7E3. We also thank C. C. Wang and H. L. Lee for assistance in determination of the amino acid composition and the sequence of crovidisin, respectively.

REFERENCES

1. Sixma, J. J., van Zanten, G. H., Banga, J. D., Nieuwenhuls, H. K., and de Groot, P. G. (1995) *Semin. Hematol.* **32,** 89–98.
2. Teng, C. M., Chen, Y. H., and Ouyang, C. (1984) *Biochim. Biophys. Acta* **786,** 204–212.
3. Teng, C. M., and Huang, T. F. (1991) *Platelets* **2,** 77–78.
4. Teng, C. M., Ko, F. N., Tsai, I. H., Hung, M. L., and Huang, T. F. (1993) *Thromb. Haemostasis* **69,** 286–292.
5. Huang, T. F., Liu, C. Z., and Yang, S. H. (1995) *Biochem. J.* **309,** 1021–1027.
6. Gould, R. J., Polokoff, M. A., Friedman, P. A., Huang, T. F., Holt,

J. C., Cook, J. J., and Niewiarowski, S. (1990) *Proc. Soc. Exp. Biol. Med.* **195,** 168–171.
7. Hynes, R. O. (1987) *Cell* **48,** 549–554.
8. Huang, T. F., Holt, J. C., Lukasiewicz, H., and Niewiarowski, S. (1987) *J. Biol. Chem.* **262,** 16157–16163.
9. Huang, T. F., Holt, J. C., Kirby, E. P., and Niewiarowski, S. (1989) *Biochemistry* **28,** 661–666.
10. Ruoslahti, E., and Pierschbacher, M. D. (1987) *Science* **238,** 491–497.
11. Ouyang, C., Yeh, H. I., and Huang, T. F. (1986) *Toxicon* **24,** 633–643.
12. Weskamp, G., and Blobel, C. P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2748–2751.
13. Weller, T., Alig, L., Müller, M. H., Kouns, W. C., and Steiner, B. (1994) *Drugs Future* **19,** 461–476.
14. Smith, J. B., Dangelmaier, C., and Selak, M. (1991) *FEBS Lett.* **283,** 307–310.
15. De Luca, M., Ward, C. M., Ohmori, K., Andrews, R. K., and Berndt, M. C. (1995) *Biochem. Biophys. Res. Commun.* **206,** 570–576.
16. Zhou, Q., Smith, J. B., and Grossman, M. H. (1995) *Biochem. J.* **307,** 411–417.
17. Liu, C. Z., Peng, H. C., and Huang, T. F. (1995) *Toxicon* **33,** 1289–1298.
18. Coller, B. S., Beer, J. H., Scudder, L. E., and Steinberg, M. H. (1989) *Blood* **74,** 182–192.
19. Coller, B. S. (1985) *J. Clin. Invest.* **76,** 101–108.
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
21. Heinrikson, R. L., and Meredith, S. C. (1984) *Anal. Biochem.* **136,** 65–74.
22. Mustard, J. P., Perry, D. W., Ardlie, N. G., and Packham, M. A. (1972) *Br. J. Haematol.* **22,** 193–204.
23. Born, G. V. R., and Cross, M. J. (1963) *J. Physiol.* **168,** 178–195.
24. De Luca, M., and McElory, W. D. (1978) *Methods Enzymol.* **57,** 3–15.
25. Pollock, W. K., and Rink, T. J. (1986) *Biochem. Biophys. Res. Commun.* **139,** 308–314.
26. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.
27. Santoro, S. A. (1986) *Cell* **46,** 913–920.
28. Liu, C. Z., Wang, Y. W., Shen, M. C., and Huang, T. F. (1994) *Thromb. Haemostasis* **72,** 919–925.
29. Paine, M. J. I., Desmond, H. P., Theakston, R. D. G., and Crampton, J. M. (1992) *J. Biol. Chem.* **267,** 22869–22876.
30. Iwanaga, S., and Suzuki, T. (1979) *in* Handbook of Experimental Pharmacology (Lee, C. Y., Ed.), Vol. 52, pp. 61–158, Springer-Verlag, New York.
31. Iwanaga, S., and Takeya, H. (1993) *in* Methods in Protein Sequence Analysis (Imahori, K., and Sakiyama, F., Eds.), pp. 107–115, Plenum, New York.
32. Fox, J. W., Campbell, R., Berrerly, L., and Bjarnason, J. B. (1986) *Eur. J. Biochem.* **156,** 65–72.
33. Schafer, A. I., and Handin, R. I. (1979) *Prog. Cardiovasc. Dis.* **22,** 31–52.
34. Glaser, E. M., Cardin, A., and Biscoe, B. (1987) *Ophthalmology* **94,** 327–332.