Activation of Heparin Cofactor II by Fibroblasts and Vascular Smooth Muscle Cells*

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Inhibition of thrombin by heparin cofactor II (HCII) is accelerated by dermataan sulfate, heparan sulfate, and heparin. Purified HCII or defibrinated plasma was incubated with washed confluent cell monolayers, 125I-thrombin was added, and the rate of formation of covalent 125I-thrombin-inhibitor complexes was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Fibroblasts and porcine aortic smooth muscle cells accelerated inhibition of thrombin by HCII 2.3-7.5-fold but had no effect on other thrombin inhibitors in plasma. Human umbilical vein endothelial cells and mouse macrophage-derived cells did not accelerate the thrombin-HCII reaction. IMR-90 normal human fetal lung fibroblasts treated with heparinase or heparitinase accelerated the thrombin-HCII reaction to the same degree as untreated cells. In contrast, treatment with chondroitinase ABC almost totally abolished the ability of these cells to activate HCII while chondroitinase AC had little or no effect, suggesting that dermataan sulfate was responsible for the activity observed. [35S]Sulfate-labeled proteoglycans were isolated from IMR-90 fibroblast monolayers and conditioned medium and fractionated into two peaks on Sepharose CL-2B. The lower M, proteoglycans contained 74-76% dermataan sulfate and were 11-25 times more active with HCII than the higher M, proteoglycans which contained 68-97% heparan sulfate. The activity of the lower M, proteoglycans decreased 70-80% by degradation of the dermataan sulfate component with chondroitinase ABC. These results confirm that dermataan sulfate proteoglycans are primarily responsible for activation of HCII by IMR-90 fibroblasts. We suggest that HCII may inhibit thrombin when plasma is exposed to vascular smooth muscle cells or fibroblasts.

The serine protease derived from prothrombin during blood coagulation, has a number of biologic activities. The procoagulant activities of thrombin include activation of factors V, VII, VIII, and XIII, conversion of fibrinogen to fibrin, and stimulation of platelet aggregation and degranulation (1). Thrombin can also serve as an anticoagulant by activating protein C in the presence of thrombomodulin (2). In addition, thrombin is mitogenic for fibroblasts (3) and has chemotactic activity for monocytes and macrophage-derived cells (4). These properties suggest that thrombin may be involved in tissue repair in addition to its functions in homeostasis. Inhibitors of thrombin may modulate these various functions.

Heparin cofactor II (HCII) and antithrombin III (ATIII) are protease inhibitors in plasma that form 1:1 covalent complexes with their target proteases. ATIII inhibits all of the proteases of the intrinsic coagulation pathway including thrombin, factors Xa, IXa, Xla, XIIa, and kallikrein (5); of these proteases, HCII inhibits only thrombin (6). Both HCII and ATIII inhibit thrombin 1000 times more rapidly in the presence of heparin (5, 7). Dermataan sulfate also accelerates the inhibition of thrombin by HCII to a similar degree while having no appreciable effect on inhibition of thrombin by ATIII (8). Chymotrypsin (9) and leukocyte cathepsin G (6) are also inhibited by HCII, but these reactions are not accelerated by heparin or dermataan sulfate. Thus, in the presence of either glycosaminoglycan, thrombin is the preferred target protease for HCII.

ATIII appears to be an important inhibitor of the procoagulant activity of thrombin, since deficiency of ATIII is associated with thrombosis (10). Currently, it is thought that ATIII is activated in vivo by binding to heparin-like molecules on the surface of vascular endothelial cells (11, 12). Intravascular activation of HCII has not been demonstrated under physiologic conditions (13); however, intravenous administration of dermataan sulfate produces an antithrombotic effect in rabbits by activation of HCII (14). Although the physiologic function of HCII is unknown, we have postulated that HCII may inhibit thrombin in vivo in the vicinity of cells that synthesize significant quantities of dermataan sulfate. In this study, we have examined cultured cell monolayers for the ability to activate HCII. We find that HCII is activated by fibroblasts and vascular smooth muscle cells but not by endothelial cells and macrophage-derived cells. Furthermore, the ability of fibroblast monolayers and conditioned media to activate HCII is mediated primarily by dermataan sulfate proteoglycans synthesized by these cells.

EXPERIMENTAL PROCEDURES

Materials—Human HCII, ATIII, and thrombin were purified and assayed as previously described (7). Citrated normal human plasma was defibrinated by treatment with 1.0 unit/ml thrombin for 15 min at 22 °C followed by centrifugation prior to use. Thrombin was iodinated with carrier-free sodium [35S]iodide by the chloramine-T method.

1 The abbreviations used are: HCII, heparin cofactor II; ATIII, antithrombin III; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
method as previously described (15). The final specific activity was 2-4 x 10^6 cpm/unit of thrombin. Chondroitinase AC (Arthrobacter aurescens, EC 4.2.2.5), chondroitinase ABC (Proteus vulgaris, EC 4.2.2.4), heparanase (Flavobacterium heparinum, EC 4.2.2.7), and heparitinase (Flavobacterium heparinum, EC 4.2.2.8) were purchased from Miles, Elkhart, Ind. and diluted Diatomaceous earth was prewashed at 37°C prior to use to degrade the cells. PEG 8000 was purchased from Union Carbide. Tosyl-Gly-Pro-Arg-p-nitroanilide (Chromozym TH) was obtained from Boehringer Mannheim. Hirudin (grade IV), BSA, chromatography resins, and other chemicals were purchased from Sigma. Molecular weight standards were obtained from Bio-Rad. Human dermagen sulfate, a gift from Dr. Lennert Roden, University of Alabama, was treated with nitrous acid to remove contaminating heparin/heparan sulfate (16) prior to use.

**Cell Culture—** IMR-90 normal human fetal lung fibroblasts (17) were obtained from the National Mutant Cell Repository, Camden, NJ. Human adult skin fibroblasts, Chinese hamster ovary cells, and the mouse macrophage-derived cell line P388D1, were obtained from Dr. Stuart Kornfeld, Washington University. Another mouse macrophage-derived cell line, J774, was provided by Dr. George Wilner, Jewish Hospital, St. Louis. NIH 3T3 mouse fibroblasts were provided by Dr. Lee Ratner, Washington University. Porcine aortic smooth muscle cells were obtained from Dr. Richard Kenagy, University of Washington, Seattle. Cells were grown in Dulbecco's modified Eagle's medium containing 4 g/liter glucose at 37°C in a humidified 5% CO2 incubator. Cells were removed from Novexy roller bottles by incubating with 0.05% trypsin (Gibco) for 1-2 min at 37°C prior to use to degrade the cells. PEG 8000 was purchased from Union Carbide. Tosyl-Gly-Pro-Arg-p-nitroanilide (Chromozym TH) was obtained from Boehringer Mannheim. Hirudin (grade IV), BSA, chromatography resins, and other chemicals were purchased from Sigma. Molecular weight standards were obtained from Bio-Rad. Human dermagen sulfate, a gift from Dr. Lennert Roden, University of Alabama, was treated with nitrous acid to remove contaminating heparin/heparan sulfate (16) prior to use.

**Assay for the Effects of Cultured Cells on the Formation of Thrombin-Inhibitor Complexes—** Human fibroblasts and porcine smooth muscle cells were assayed between 10 and 17 passages. Cells were grown to confluence in 2-cm² wells of polystyrene 24-well tissue culture plates (Costar). Empty wells on the same plate, which served as controls, were conditioned with medium identical to that used to grow the cells. Medium was aspirated from empty and cell-containing wells. The wells were then washed three times with 1 ml of 150 mM NaCl, 1 mM CaCl2, 0.1% polyethylene glycol, 10 mM penicillin, 100 μg/ml streptomycin, and 10 mM HEPES, pH 7.4. NIH 3T3 fibroblasts were also supplemented with 1.1 mg/ml sodium pyruvate. Primary cultures of human umbilical vein endothelial cells were prepared by the method of Jaffe et al. (18) and were provided by Drs. Ikuro Maruyama and Philip Majerus, Washington University.

**Labeling of IMR-90 Fibroblasts with Sodium 35Sulfate—** Cells were passaged into 850-cm² polystyrene roller bottles, maleimide were added to make final concentrations of 150, 50, and 10 mM, respectively. Triton X-100 was added to a final concentration of 0.5%. The mixture was then applied to a 5-ml DEAE-Sephacel column equilibrated with 8 mM urea, 5 μg/ml BSA, 0.5% Triton X-100, 50 mM sodium acetate, pH 6.0 (8 mM urea buffer) containing 150 mM NaCl. The column was washed with 25 ml of equilibration buffer, followed by step elution with 20 ml of 8 mM urea buffer containing 0.4 M NaCl, and 20 ml of 0.25 M NaCl. The fraction containing 0.06 M NaCl was collected. Unincorporated 35Sulfate did not bind to this column, while proteoglycans eluted with 0.65 M NaCl.

**Isolation of Proteoglycans from Cell Monolayers—** Washed cell monolayers were extracted overnight at 4°C with constant rotation using 100 ml/bottle of 4 mM guanidine HCl, 100 mM 6-aminohexanoic acid, 100 mM 2-mercaptoethanol, 5 mM benzamidine, 5 mM phenylmethylsulfonyl fluoride, 50 mM NaNO2, EDTA, 2% Triton X-100, and 50 mM sodium acetate buffer, pH 5.8. The cell extract was concentrated with an Amicon PM10 membrane to a volume of 20 ml, dialyzed (Spectropor dialysis tubing M, cut-off, 12,000-14,000) against 8 mM urea buffer containing 150 mM NaCl, applied to a 5-ml DEAE-Sephacel column, and eluted as described above.

**Size Fractionation of Proteoglycans—** Proteoglycans eluted from the DEAE-Sephacel column were diluted with 5 volumes of 8 mM urea buffer without sodium chloride and then applied to a 1-ml DEAE-Sephacel column equilibrated with 8 mM urea buffer containing 150 mM NaCl. Proteoglycans were eluted with 2 ml of 4 mM guanidine HCl, 50 mM sodium acetate, 50 mM Tris-HCl, pH 7.0, containing 5 μg/ml BSA and 0.5% Triton X-100 directly onto a 1 x 107-cm Sepharose CL-2B column equilibrated with the same buffer. One-ml fractions were collected at a flow rate of 1 ml/min.

**Pronase Treatment—** Pooled fractions from the Sepharose CL-2B column were concentrated with an Amicon PM10 membrane to 2 ml and dialyzed against 1 mM NaCl, 20 mM CaCl₂, 100 mM Tris-HCl, pH 8.0. Samples were incubated at 57°C for 24 h with 270 μg of Pronase added at 0, 6, and 16 h. Reaction mixtures were heated to 100°C to inactivate the enzyme and dialyzed extensively against distilled water.

**Glycosaminoglycan Analysis—** The glycosaminoglycan composition of each Sepharose CL-2B peak was determined by measuring the percentage of [35S]sulfate susceptible to degradation by nitric acid, chondroitinase AC, or chondroitinase ABC. Samples containing 0.25 ml of Pronase-treated and dried evaporated material were applied to a 0.5% porcine skin dermatan sulfate, a gift from Dr. Lennert Roden, University of Washington, Seattle.

**Proteoglycan Analysis—** After assessing the percentage of material degraded by chondroitinase AC from that degraded by chondroitinase ABC, the percentage of material degraded by chondroitinase AC and ABC degradations were carried out in 0.5 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 unit/ml of enzyme. Samples were incubated at 37°C for 2 h. To separate degraded from undegraded material, each reaction mixture was chromatographed on a 0.5 x 45-cm Sephadex G-50 (fine) column equilibrated with 200 mM ammonium acetate-acetic acid, pH 5.0. Fractions (0.5 ml) were collected at a flow rate of 2 ml/h. Untreated samples of Pronase-treated and Pronase-treated and dried evaporated material were used as controls.

**Chromatographic Assay for Inhibition of Thrombin by HCII—** Samples of proteoglycans measured from IMR-90 model 6800 liquid scintillation counter using Scintiverse I (Fisher).

**Activation of HCII by Fibroblasts**
and the absorbance at 405 nm was determined. The absorbance decreased with increasing concentration of dermatan sulfate from 0 to 4 μg/ml. The specific activity of a [125I]sulfate-labeled sample was calculated as units/cpm, where 1 unit is equal to the inhibitory effect produced by 1 μg of standard dermatan sulfate. This assay cannot distinguish between the effects of heparin, dermatan sulfate, and heparan sulfate, all of which can accelerate the inactivation of thrombin by HCII (8).

RESULTS

Activation of HCII by Fibroblasts and Vascular Smooth Muscle Cells—We examined a variety of cells in monolayer culture for the ability to accelerate formation of the complex between [125I]thrombin and HCII. Monolayers were washed with buffer and incubated with purified HCII and [125I]thrombin as described under “Experimental Procedures.” Covalent complexes between [125I]thrombin and HCII in the supernatant buffer were detected by SDS-PAGE and autoradiography. Fig. 1A illustrates the effect of IMR-90 normal human fetal lung fibroblasts. More complex was formed when [125I]thrombin and HCII were incubated for 10 min in the presence of cells (lane 1) than in the absence of cells (lane 2). When [125I]thrombin alone was added to cells, no complexes were detected (lane 3). When an equimolar amount of ATIII was substituted for HCII, there was no increase in formation of the [125I]thrombin-ATIII complex in the presence of cells (Fig. 1B). The time course of [125I]thrombin incorporation into the covalent complex with purified HCII is shown in Fig. 2A. In this experiment, the cell monolayer increased the rate of complex formation 3.4-fold. IMR-90 fibroblasts had no effect on the rate of complex formation between [125I]thrombin and ATIII (Fig. 2B).

When [125I]thrombin was incubated with defibrinated plasma in the presence or absence of IMR-90 fibroblasts, 4 major complexes were detected by autoradiography as shown in Fig. 3. Complexes “a” and “b,” previously noted in our laboratory (8), have not been identified. Complexes “c” and “d” co-migrated with the complexes formed in the presence of purified HCII and ATIII, respectively. The amount of each complex formed at 5, 10, and 15 min is shown in Table I. Complexes a, b, c, and d were not affected by the presence of cells, while 2.6 times more complex c was formed in the presence of cells. These results agree with the experiments shown in Figs. 1 and 2 in which purified thrombin inhibitors were used. The results suggest that HCII is the major thrombin inhibitor in plasma that can be stimulated by fibroblasts.

Human skin fibroblasts, Chinese hamster ovary cells, and NIH 3T3 mouse fibroblasts accelerated formation of the [125I]thrombin-HCII complex from 2.3-7.5-fold under the conditions described in Table II. Porcine aortic smooth muscle cells accelerated complex formation 3.2-fold. In experiments with defibrinated plasma, smooth muscle cells only stimulated formation of the [125I]thrombin-HCII complex (data not shown). Smooth muscle cells did not accelerate complex formation between purified ATIII and [125I]thrombin (data not shown). Primary cultures of human umbilical vein endothelial cells and two mouse macrophage-derived lines (P388D1, and J774) did not activate HCII (Table II). In the case of endothelial cells, there was a slight but significant inhibition of HCII activity.
TABLE I

**Effect of IMR-90 fibroblasts on \(^{125}\text{I}-\text{thrombin-inhibitor reactions in plasma**}

Defibrinated plasma and \(^{125}\text{I}-\text{thrombin were incubated with IMR-90 fibroblasts or empty wells as described in the legend to Fig. 3. At the indicated times, samples of the supernatant solution were subjected to SDS-PAGE and autoradiography. The percentage of radioactivity in each band was determined by \(\gamma\) counting (100% equals the total radioactivity in the gel lane). The mean ± S.D. of triplicate wells is indicated.

| Complex* | 5 min | 10 min | 15 min |
|----------|-------|--------|--------|
|          | +Cells | −Cells | +Cells | −Cells | +Cells | −Cells |
| a        | 1.5 ± 0.1 | 1.5 ± 0.1 | 3.2 ± 0.2 | 3.4 ± 0.1 | 5.1 ± 0.2 | 5.2 ± 0.3 |
| b        | 3.8 ± 0.4 | 4.0 ± 0.1 | 6.2 ± 0.2 | 6.5 ± 0.2 | 8.1 ± 0.2 | 8.3 ± 0.3 |
| c (thrombin-HCII) | 1.2 ± 0.2* | 0.6 ± 0.2 | 2.9 ± 0.2* | 1.1 ± 0.2 | 4.4 ± 1.0* | 1.7 ± 0.2 |
| d (thrombin-ATIII) | 1.8 ± 0.3 | 2.5 ± 0.7 | 5.0 ± 0.4 | 5.8 ± 1.5 | 7.8 ± 0.8 | 9.1 ± 1.7 |

* Letters refer to bands labeled in Fig. 3.

TABLE II

**Effect of various cell types on the thrombin-HCII reaction**

Confluent monolayers of cells were incubated with 76 nM HCII and 7.3 nM \(^{125}\text{I}-\text{thrombin for 15 min as described in the legend to Fig. 1. For each cell type, the relative amount of radioactivity in the \(^{125}\text{I}-\text{thrombin-HCII complex was calculated by dividing by the mean value from "-cells" control. The mean ± S.D. of triplicate wells is shown. Each type was tested on at least 3 separate occasions with similar results.**

| Cell type | Relative amount of \(^{125}\text{I}-\text{thrombin-HCII complex} | p value\(^x\) |
|-----------|------------------------------------------------|-------------|
| IMR-90 normal human fetal lung fibroblasts | 3.9 ± 0.2 | 1.0 ± 0.2 | <0.001 |
| Human adult skin fibroblasts | 3.1 ± 0.2 | 1.0 ± 0.2 | <0.001 |
| NIH 3T3 mouse fibroblasts | 7.5 ± 0.8 | 1.0 ± 0.1 | <0.001 |
| Chinese hamster ovary cells | 2.3 ± 0.2 | 1.0 ± 0.3 | <0.01 |
| Porcine aortic smooth muscle cells | 3.2 ± 0.1 | 1.0 ± 0.3 | <0.01 |
| Human umbilical vein endothelial cells | 0.8 ± 0.02 | 1.0 ± 0.1 | <0.01 |
| P88D1 (mouse macrophage-derived cells) | 1.0 ± 0.2 | 1.0 ± 0.2 | NS\(^b\) |
| J774 (mouse macrophage-derived cells) | 1.0 ± 0.2 | 1.0 ± 0.1 | NS |

\(^x\) Calculated by Student’s two-tailed \(t\) test.

\(^b\) NS, not significant \((p > 0.1)\).

**Chondroitinase ABC Abolishes the Activation of HCII by IMR-90 Fibroblasts**—We have shown that HCII is activated by porcine skin dermatan sulfate and bovine liver heparan sulfate, although a 5-fold higher concentration of the latter is required (8). Since IMR-90 fibroblasts synthesize both dermatan sulfate and heparan sulfate as components of secreted and membrane-bound proteoglycans (22), either or both of these glycosaminoglycans could potentially account for the effect observed in Figs. 1 and 2.

Washed monolayers of IMR-90 fibroblasts were preincubated with enzymes to selectively degrade heparan sulfate or dermatan sulfate. No difference in adherence between enzyme-treated and control cells was apparent by light microscopy after the 2-h incubation. Cells were then washed three times and immediately assayed with \(^{125}\text{I}-\text{thrombin and HCII for complex formation (Fig. 4A). Preincubation with heparinase or heparitinase had no effect on the amount of complex formed. In contrast, preincubation of cell monolayers with chondroitinase ABC almost completely abolished the ability of the fibroblasts to stimulate complex formation. Chondroitinase AC had a small but reproducible effect. Since the hexosamine-uronic acid linkages in dermatan sulfate are susceptible to chondroitinase ABC and resistant to heparinase or heparitinase, the results shown in Fig. 4A suggest that dermatan sulfate is responsible for the activation of HCII by IMR-90 fibroblasts. We obtained similar results using normal adult skin fibroblasts (not shown).**

**Activation of HCII by Supernatant Medium from IMR-90 Fibroblasts**—Monolayers of IMR-90 fibroblasts were washed and incubated with serum-free medium for 2 h at 37°C. The supernatant medium was then removed and assayed with \(^{125}\text{I}-\text{thrombin and HCII. As shown in Fig. 4B, the supernatant**
medium accelerated complex formation 3.6-fold compared to supernatant medium from empty tissue culture wells. When chondroitinase ABC was present during the 2-h incubation, the supernatant medium did not accelerate complex formation (p > 0.1 in comparison to the "empty well" control). When chondroitinase AC was present during the 2-h incubation, the supernatant medium accelerated complex formation 2.5-fold. These results suggest that dermatan sulfate secreted by IMR-90 fibroblasts into the medium activates HCII.

Activation of HCII by Proteoglycans Purified from IMR-90 Fibroblasts—To confirm that dermatan sulfate from IMR-90 fibroblasts activates HCII, we fractionated the proteoglycans from these cells and determined their ability to accelerate inhibition of thrombin by HCII. The cells were metabolically labeled with [35S]sulfate and extracted with guanidine HCl and Triton X-100. The proteoglycans were then isolated by absorption to DEAE-Sephacel (see "Experimental Procedures" for details). Proteoglycans from the supernatant medium were isolated in a similar manner. Chromatography of the labeled proteoglycans on a Sepharose CL-2B column in the presence of guanidine HCl yielded two peaks for both the cell-associated (C-I and C-II) and supernatant material (M-I and M-II) (Fig. 5). The peaks were pooled as shown and a portion dialyzed against 150 mM NaCl, 0.1% polyethylene glycol, 20 mM Tris-HCl, pH 7.4. Dialyzed material was assayed with HCII and thrombin in the chromogenic substrate assay described under "Experimental Procedures." Pronase-treated samples were further treated with nitrous acid, chondroitinase AC, or chondroitinase ABC followed by gel filtration on a column of Sephadex G-50 to remove degraded material before being assayed. One unit of activity is defined as that equal to 1 μg of standard dermatan sulfate. Each value is the average of at least three determinations.

![Fig. 5. Size fractionation of IMR-90 fibroblast proteoglycans on Sepharose CL-2B. [35S]Sulfate-labeled proteoglycans were isolated from supernatant medium (panel A) and cell extract (panel B) as described under "Experimental Procedures." The proteoglycans were chromatographed on a 1 x 107-cm Sepharose CL-2B column equilibrated with 4 M guanidine HCl, 0.5% Triton X-100, 5 μg/ml BSA, 50 mM sodium acetate, and 50 mM Tris-HCl, pH 7.0. One-ml fractions were collected at a flow rate of 4 ml/h.](image)

| TABLE III |
| Specific activities of [35S]sulfate-labeled proteoglycans |

Proteoglycans were pooled as shown in Fig. 5 and tested (before and after Pronase treatment) for acceleration of the thrombin-HCII reaction with the chromogenic substrate assay described under "Experimental Procedures." Pronase-treated samples were further treated with nitrous acid, chondroitinase AC, or chondroitinase ABC followed by gel filtration on a column of Sephadex G-50 to remove degraded material before being assayed. One unit of activity is defined as that equal to 1 μg of standard dermatan sulfate. Each value is the average of at least three determinations.

| Cell extract | Before Pronase | After Pronase | Nitrous acid treated | Chondroitinase AC treated | Chondroitinase ABC treated |
|--------------|---------------|--------------|---------------------|--------------------------|---------------------------|
| C-I          | 1.8           | 3.9          | 30                  | 1.6                      | 2                         |
| C-II         | 46            | 43           | 46                  | 31                       | 5.8                       |
| Supernatant medium | | | | | |
| M-I          | 2.4           | 2.9          | 10                  | 4.6                      | 2.6                       |
| M-II         | 27            | 29           | 46                  | 58                       | 7.8                       |

| TABLE IV |
| Glycosaminoglycan compositions of [35S]sulfate-labeled proteoglycans |

Samples of each of the peaks shown in Fig. 5 (after treatment with Pronase) were treated with nitrous acid, chondroitinase ABC, or chondroitinase AC. The percentage of radioactivity degraded by each treatment was determined by chromatography on Sephadex G-50 (see "Experimental Procedures" for details).

| Cell extract | Heparan sulfate$^a$ | Dermatan sulfate$^b$ | Chondroitin 4-sulfate or chondroitin 6-sulfate$^b$ | % radioactivity |
|--------------|---------------------|----------------------|-----------------------------------------------|---------------|
| C-I          | 97                  | 2                    | 1                                             | 9              |
| C-II         | 14                  | 74                   | 12                                            | 12            |
| Supernatant medium | | | | |
| M-I          | 68                  | 6                    | 26                                            | 26            |
| M-II         | 8                   | 76                   | 16                                            | 16            |

* Nitrous acid sensitive and chondroitinase ABC resistant.
* Chondroitinase ABC sensitive minus chondroitinase AC sensitive.
* Chondroitinase AC sensitive.

$^a$ Nitrous acid sensitive and chondroitinase ABC resistant.
$^b$ Chondroitinase ABC sensitive minus chondroitinase AC sensitive.
$^c$ Chondroitinase AC sensitive.

After Pronase treatment, the glycosaminoglycan composition was determined for each of the Sepharose CL-2B peaks (Table IV). The higher molecular weight peaks from both the cell monolayer (C-I) and medium (M-I) contained predominantly heparan sulfate, while the lower molecular weight peaks (C-II and M-II) contained predominantly dermatan sulfate. Treatment of C-II and M-II with chondroitinase ABC degraded 86 and 92% of the labeled material, respectively (Table IV), and decreased the specific activity of the remaining intact glycosaminoglycan chains (i.e. heparan sulfate) by 70–90% (Table III). Nitrous acid treatment of peaks C-I and M-I degraded 97 and 68% of the labeled material, respectively (Table IV). The remaining intact glycosaminoglycans (i.e.
mixtures of dermatan sulfate and chondroitin 4- and/or 6-sulfate) had specific activities that were 4-17 times greater than the starting material. Treatment with chondroitinase AC, which will completely degrade chondroitin 4- and 6-sulfate, had relatively minor effects on the specific activities of the four proteoglycan peaks. These experiments indicate that the bulk of the activity with HCII is due to the dermatan sulfate component of the fibroblast proteoglycans.

**DISCUSSION**

In this paper we have shown that cultured monolayers of fibroblasts and aortic smooth muscle cells, as well as supernatant medium from fibroblasts, accelerate formation of the 125I-thrombin-HCII complex. In contrast, umbilical vein endothelial cells and macrophage-derived cells have no effect. These findings suggest that the function of HCII may be to inhibit thrombin under conditions of endothelial cell disrepair which lead to contact between plasma and subendothelial tissues.

Treatment of IMR-90 fetal lung fibroblasts with chondroitinase ABC, but not heparinase or heparitinase, greatly diminishes the ability of these cells to accelerate the thrombin-HCII reaction. Similarly, chondroitinase ABC treatment abolishes the activity of the supernatant medium from these cells. These experiments suggest that the acceleration of the thrombin-HCII reaction by fibroblasts depends primarily on the presence of dermatan sulfate on the cell surface and in the supernatant medium.

Chondroitinase AC consistently decreases 125I-thrombin-HCII complex formation by a small degree (Fig. 4). This enzyme cleaves the N-acetylatedomaminoglycan 1-4-glucuronic acid linkage, the only linkage found in chondroitin 4-sulfate and chondroitin 6-sulfate (23). However, we have previously shown that purified chondroitin 4-sulfate and chondroitin 6-sulfate do not activate HCII (8). The N-acetylatedomaminoglycan 1-4-glucuronic acid linkage also occurs in a minority of the repeating disaccharide units of dermatan sulfate (24) due to incomplete epimerization of glucuronic to iduronic acid during biosynthesis (25). Treatment of porcine skin dermatan sulfate with chondroitinase AC results in limited cleavage of the polymer (~5%) even after prolonged incubation (data not shown). This rules out the possibility that the chondroitinase AC used in our laboratory was contaminated with chondroitinase ABC. Moreover, the ability of porcine skin dermatan sulfate to activate HCII decreased ~5% after treatment with chondroitinase AC (data not shown). Thus, we believe that the effect of chondroitinase AC on the fibroblasts and the supernatant medium is most likely due to partial degradation of dermatan sulfate.

We confirmed the importance of dermatan sulfate by assaying the proteoglycans purified from IMR-90 fibroblasts. Vogel and Peterson (22) have previously characterized the [35S]sulfate-labeled proteoglycans synthesized by these cells. The gel filtration patterns they observed for the medium and cell-associated proteoglycans in the presence of guanidine HC1 were very similar to those obtained in our experiments (Fig. 5, A and B). In addition, the glycosaminoglycan compositions of the individual peaks were similar, except that we observed a higher proportion of dermatan sulfate in peak C-II. Vogel and Peterson (22) reported that the dermatan sulfate proteoglycan from conditioned medium (corresponding to M-II, Fig. 5A) contains 2-4 dermatan sulfate chains (M, = 25,000–40,000) linked to a core protein. In contrast, their data suggested that dermatan sulfate in the cell extract of IMR-90 fibroblasts (corresponding to C-II, Fig. 5B) may represent either a single chain linked to a small core protein or free glycosaminoglycan chains (M, = 28,000). Despite these differences in structure, M-II and C-II have comparable specific activities with HCII (Table III).

The increase in the rate of formation of the 125I-thrombin-HCII complex observed in our assay system with cell monolayers is relatively modest (2.2-7.5-fold) compared to that observed with optimal concentrations of purified dermatan sulfate (1500-fold at 250 µg/ml) or heparin (800-fold at 67 µg/ml) (8). The degree of acceleration of the thrombin-HCII reaction produced by the cell monolayers is approximately equal to that produced by 0.1-1.0 µg/ml purified porcine skin dermatan sulfate (not shown). However, local concentrations of dermatan sulfate in vivo may be much higher due to a greater ratio of cell surface (or extracellular matrix) to extracellular fluid volume. Vogel and Sapien (26) found that maintaining IMR-90 fibroblasts in a 0.5% newborn calf serum resulted in viable but nonproliferating cells which secrete 40% more dermatan sulfate and proportionally less heparan sulfate compared to cells grown in 10% serum. They speculated that low serum concentrations may better approximate in vivo conditions.

No acceleration of the thrombin-HCII reaction occurs in the presence of human umbilical vein endothelial cells or mouse macrophage-derived cells. Although Oohira et al. (27) found that dermatan sulfate accounts for 25% of the [35S]sulfate-labeled proteoglycans secreted by human umbilical vein endothelial cells, their methods were not sensitive enough to determine whether or not dermatan sulfate was present in an SDS extract of the cells. Therefore, it is possible that there is no dermatan sulfate on the cell surface. In the same paper, analysis of cultured bovine aortic endothelial cells, which also secrete dermatan sulfate, showed no dermatan sulfate in an SDS extract of the cells.

We have not observed acceleration of 125I-thrombin-ATIII complex formation in the presence of endothelial cells (data not shown). This may be due to the low surface (2 cm2) to volume (0.5 ml) ratio used in our assay system. Other investigators have shown acceleration of thrombin inhibition by ATIII using systems with large surface areas such as perfusion through a column of endothelial cells grown on microcarrier beads (11) or perfusion through a microvascular preparation (11, 28). In the study by Marcum et al. (28) approximately a 19-fold rate enhancement of thrombin inhibition by ATIII was seen by perfusion through the blood vessels of a rat hindquarter preparation. This rate enhancement could be abolished by first perfusing the system with heparinase to degrade cell surface heparan sulfate. Heparan sulfate molecules purified from cloned bovine aortic endothelial cells have been shown to activate ATIII (29).

In this study, we have identified a potential physiologic site for the inhibition of thrombin by HCII. A break in the integrity of the endothelial surface would expose these proteins to fibroblasts and smooth muscle cells and their extracellular matrices which could increase the rate of thrombin inhibition by HCII. Platelet factor 4, a glycosaminoglycan-binding protein released by platelets upon degranulation (30, 31), is a potential negative regulator of this process, since platelet factor 4 prevents dermatan sulfate from accelerating the inhibition of thrombin by HCII in vitro (32).

The mitogenic effect of thrombin on human fibroblasts is dependent on its esterolytic activity (33). Since HCII inhibits both the proteolytic and esterolytic activities of thrombin (7), formation of the thrombin-HCII complex could block thrombin-induced mitogenesis. Thrombin is also mitogenic for macrophage-derived cells, but this effect is independent of esterolytic activity. However, thrombin-induced mitogenesis in
these cells can be blocked by the thrombin inhibitor hirudin (34). The chemotactic effect of thrombin on monocytes and macrophages can be inhibited by formation of a thrombin-inhibitor complex with either hirudin or ATIII (35). Hence, it is possible that HCII may inhibit thrombin-induced macrophage chemotaxis or mitogenesis. We suggest that HCII may inhibit thrombin in the subendothelial or extravascular tissues, thereby modulating thrombin’s hemostatic, mitogenic, or chemotactic effects.

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REFERENCES
1. Davie, E. W., Fujikawa, K., Kurachi, K., and Kisiel, W. (1979) Adv. Enzymol. Relat. Areas Mol. Biol. 48, 277–318
2. Esmen, C. T. (1984) Prog. Hemostasis Thromb. 7, 25–54
3. Chen, L. B., and Buchanan, J. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 131–135
4. Bar-Shavit, R., Kahn, A., Fenton, J. W., II, and Wilner, G. D. (1983) J. Cell Biol. 96, 282–285
5. Rosenberg, R. D. (1977) Semin. Hematol. 14, 427–439
6. Parker, K. A., and Tollefsen, D. M. (1985) J. Biol. Chem. 260, 3501–3505
7. Tollefsen, D. M., Majerus, D. W., and Blank, M. K. (1982) J. Biol. Chem. 257, 2162–2169
8. Tollefsen, D. M., Pestka, C. A., and Monacho, W. J. (1983) J. Biol. Chem. 258, 6713–6716
9. Church, F. C., Noyes, C. M., and Griffith, M. J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6431–6434
10. Egeberg, O. (1965) Thromb. Diath. Haemorrh. 13, 516–530
11. Busch, C., and Owen, W. G. (1982) J. Clin. Invest. 69, 726–729
12. Marcus, J. A., Fritze, L., Galli, S. J., Karp, G., and Rosenberg, R. D. (1983) Am. J. Physiol. 245, H725–H733
13. MacIntosh, S., Jakubowski, S. H., and Owen, W. G. (1984) Fed. Proc. 43, 1946 (abstr.)
14. Buchanan, M. R., Boneu, B., O’osu, F. A., and Hirsh, J. (1985) Blood 65, 198–201
15. Tollefsen, D. M., Feagler, J. R., and Majerus, P. W. (1974) J. Biol. Chem. 249, 2646–2651
16. Teien, A. N., Abildgaard, U., and Höök, M. (1976) Thromb. Res. 8, 859–863
17. Nichols, W. W., Murphy, D. G., Cristofalo, V. J., Toji, L. H., Greene, A. E., and Dwight, S. A. (1977) Science 196, 60–63
18. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) J. Clin. Invest. 52, 2745–2766
19. Laemmli, U. K. (1970) Nature 227, 680–685
20. Tollefsen, D. M., and Blank, M. K. (1981) J. Clin. Invest. 68, 580–596
21. Yanagishita, M., and Hascall, V. C. (1984) J. Biol. Chem. 259, 10289–10299
22. Vogel, K. G., and Peterson, D. W. (1981) J. Biol. Chem. 256, 13235–13242
23. Suzuki, S. (1972) Methods Enzymol. 28, 911–917
24. Fransson, L. Å., Coster, L., Malmström, A., and Sjöberg, I. (1974) Biochem. J. 143, 369–378
25. Malmström, A., Fransson, L. Å., Höök, M., and Lindahl, U. (1975) J. Biol. Chem. 250, 3419–3425
26. Vogel, K. G., and Sapién, R. B. (1982) Biochem. J. 207, 369–379
27. Oohira, A., Wight, T. N., and Bornstein, P. (1983) J. Biol. Chem. 258, 2014–2021
28. Marcum, J. A., McKenney, J. B., and Rosenberg, R. D. (1984) J. Clin. Invest. 74, 341–350
29. Marcum, J. A., Atha, D. H., Fritze, L. M. S., Nawroth, P. C., Stern, D., and Rosenberg, R. D. (1986) J. Biol. Chem. 261, 7507–7517
30. Levine, S. P., and Wohl, H. (1976) J. Biol. Chem. 251, 324–328
31. Handin, R. I., and Cohen, H. J. (1976) J. Biol. Chem. 251, 4273–4282
32. Tollefsen, D. M., and Pestka, C. A. (1985) J. Clin. Invest. 75, 496–501
33. Glenn, K. C., Carney, D. H., Fenton, J. W., II, and Cunningham, D. D. (1980) J. Biol. Chem. 255, 6609–6616
34. Bar-Shavit, R., Kahn, A. J., Mann, K. G., and Wilner, G. D. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 976–980
35. Bar-Shavit, R., Kahn, A., Wilner, G. D., and Fenton, J. W., II (1983) Science 220, 728–731
36. Tollefsen, D. M., and Pestka, C. A. (1985) Blood 66, 769–774