Journal of Supramolecular Structure 14:33–46 (1980)
Control of Cellular Division and Development: Part A: 153–166

Inhibition of Cell Proliferation and Protease Activity by Cartilage Factors and Heparin

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Proliferating rat smooth muscle cells and fibroblasts have membrane-associated protease activity. High concentrations of heparin inhibited membrane-associated protease activity and cell proliferation, while low concentration of heparin promoted smooth muscle cell proliferation. The inhibition of protease activity and proliferation was abolished when heparin was treated with protamine sulfate or when acid treated fetal calf serum was used. Heparin required the presence of an acid labile factor(s) in serum for the inhibition of protease activity and proliferation. Heparin and antithrombin III in the presence of acid-treated fetal calf serum did not inhibit cell proliferation or protease activity. Cartilage factors isolated from bovine nasal cartilage containing trypsin inhibitory activity, but not papain inhibitory activity, inhibited rat smooth muscle and fibroblast proliferation and surface associated protease activity. The cartilage factors did not require acid-labile components in the fetal calf serum for the inhibitory activity. The inhibitory activity due to heparin and cartilage factors was not permanent under our experimental condition. Protein synthesis was not inhibited by heparin or the cartilage factors. In rat smooth muscle cells and fibroblasts, the expression of surface-associated protease activity was related to the proliferative state of the cells. Surface protease activity was only present on proliferating cells. When surface protease activity was inhibited by high concentrations of heparin in the presence of an acid-labile serum component(s) or cartilage factors, cell proliferation was also inhibited.

Key words: **smooth muscle cell proliferation, fibroblast proliferation, membrane proteases, protease inhibitors, heparin, cartilage factors**

The role of proteolytic enzymes in the cellular events accompanying cell proliferation is not clearly understood. Proteases have been implicated in the triggering of lymphocytes [1–3]. A relationship between cell proliferation and surface-associated protease activity has been reported [4, 5]. Proliferating cells have surface protease activity, while a loss of surface protease activity was observed before the termination of cell proliferation [5]. Conversely, protease inhibitors have been shown to inhibit cell proliferation [6–8]. Schnebli and Burger [6] have reported that a number of protease inhibitors including N-α-tosyl-L-lysyl-chloromethyl ketone (TLCK) inhibit the growth of transformed cells. The inhibition occurs either in G_2 phase of the cycle or during mitosis [8]. Several laboratories have demonstrated the inhibitory action of protease inhibitors on lymphocyte activation [9–15].

Received April 9, 1980; accepted July 21, 1980.

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The effect of heparin and various acid mucopolysaccharides on cell growth have been contradictory. Acid mucopolysaccharides have been reported to promote tumor growth [16]. Yang and Jenkin [17] have reported that heparin, at a concentration of 5 μg/ml promotes the growth of prepuce cells, but did not stimulate the growth of BHK-21, MK-2, or Novikoff rat hepatoma cells. Fisher [18] demonstrated that heparin, at concentrations from 20–500 μg/ml inhibited the mitosis of heart fibroblasts, while Lippman [19] reported inhibition of mouse L cells with heparin at 50 μg/ml. Lippman and Mathews [20] have shown that some heparin preparations inhibit mouse L-M cells in suspension culture. The inhibition was due to the attachment of heparin to the cell surface and occurred in late mitosis of the cell cycle. No correlation was observed between the anticoagulant activity of the heparin preparations and the antiproliferative activity. Commerical heparin preparations have also been reported to suppress intimal smooth muscle cell proliferation in a rat model of arterial endothelial injury. Clowes and Karnovsky [21] observed that following arterial de-endothelization in the Sprague-Dawley rat, the intimal smooth muscle cell proliferation could be inhibited by pig mucosa heparin.

Cartilage-derived factors [22–29] have been shown to inhibit tumor-induced vascular proliferation, tumor growth, in vitro bone resorption, and several proteases. The cartilage factors inhibit the growth of endothelial cells [25, 28] and the collagenolytic activity derived from osteosarcoma cells [25] but do not affect the growth of adult fibroblasts [25]. High levels of inhibitor have been found in poorly vascularized tissues such as blood vessel walls, cornea, and dentin [30, 31].

In this investigation we have studied the interaction of commercial heparin on proliferating cultured rat smooth muscle cells. The demonstration that pig mucosa heparin inhibited intimal smooth muscle proliferation in the Sprague-Dawley rat [21] lead to our investigation on the effect of heparin on the proliferation of cultured rat smooth muscle cells. Surface protease activity was measured in order to determine if the expression of protease activity was related to cell proliferation. The effect of high concentration of heparin and antithrombin III on proliferating rat smooth muscle cells was also evaluated. The interaction of cartilage-derived factors on rat smooth muscle cells and fibroblasts was studied in order to determine if cellular protease activity was altered by cartilage-derived factors. In this paper we present evidence that heparin and cartilage-derived factors – under experimental conditions in which cell proliferation is inhibited – also affect the expression of cellular protease activity.

MATERIALS AND METHODS

Cells and Materials

Rat aortic fibroblasts and smooth muscle cells were obtained from Sprague-Dawley rat abdominal aorta. Rat aortic smooth muscle cells (passages 5–12) were dispersed in trypsin (0.25% w/v)–EDTA (0.5 mM) and grown in Dulbecco's modified Eagles medium containing either 10% fetal calf serum (FCS), 5% FCS, 5% pooled human serum, or 5% pooled rat (Sprague-Dawley) serum according to the method described by Ross [32]. Rat smooth muscle cells were also grown in Dulbecco's modified Eagles medium containing 10% acid treated FCS. The FCS was adjusted to pH 3.2 by the addition of 1 N hydrochloric acid incubated at room temperature for 2 hr and then neutralized with 1 N sodium hydroxide. The acid treatment removes some of the endogenous protease inhibitors [33]. Rat aortic fibroblasts were dispersed in trypsin (0.25% w/v)–EDTA (0.5 mM) and

grown in Dulbecco's modified Eagles medium containing either 10% FCS or 10% acid-treated FCS.

^{3}H-acetic anhydride (400 mCi/mmole), L-^{3}H-amino acid mixture (1 mCi/ml), and Riafluor scintillation cocktail were purchased from New England Nuclear (NEN). Dulbecco's modifed Eagles medium and bovine fetal calf serum were obtained from Flow Laboratories, Inc. Bovine nasal cartilage was obtained from Max Insel Cohen, Inc., Livingston, NJ. The ovalbumin, myoglobulin, ribonuclease and antithrombin III were from the Sigma Chemical Co. Papain was purchased from Worthington, while trypsin 12X Nat. Form was from I.C.N. Pharmaceuticals. Bovine serum albumin was obtained from Miles (Pentex). Protamine sulfate (1 mg inactivates 90 U.S.P. units of heparin) was obtained from Lilly. Porcine intestinal mucosa heparin (Panheparin, 10,000 U.S.P. units/ml in isotonic sodium chloride, NDC 0074-6738, 02) was purchased from Abbot Laboratories, Chicago. The Sepharose 4B was obtained from Pharmacia Fine Chemicals. The Sprague-Dawley rats were purchased from the Charles River Breeding Laboratories.

Surface Protease Activity

The surface protease determination was based on the measurement of TCA-soluble radioactive peptides released from ^{3}H-labelled casein [4, 5]. The ^{3}H-acetyl-casein assay is capable of detecting proteolysis produced by 50–100 pg of trypsin [34, 35]. The ^{3}H-acetyl-casein, which was prepared by acetylating casein with ^{3}H-acetic anhydride, had a specific activity of 10,099 dpm per pmol, assuming a molecular mass of 121,800 daltons. In order to measure protease activity bound to the cell surface, the cell cultures grown in Falcon petri dishes were washed 6X with Dulbecco's modified Eagles medium without serum. The medium was removed and 300 μl ^{3}H-labelled casein (60 μg casein, 5.3×10^6 dpm), which was previously dialyzed against serum-free medium, was added to the plates together with fresh serum-free medium (600 μl). The plates were then incubated for 180 min at 37°C under 7% CO_2 in room air. The reaction was terminated by the addition of unlabelled 3% casein (100 μl) dissolved in 1 M KCl followed by chilled 6% TCA (200 μl). The mixture was allowed to remain overnight in an ice slurry after which the samples were centrifuged at 27,000g for 40 min and, 200 μl of supernatant was added to 10 ml of Riafluor (NEN) and then the radioactivity was determined. Control plates without cells were incubated for 24 hr in Dulbecco's modified Eagles medium containing 10% FCS. The plates were washed 6X with Dulbecco's modified Eagles medium without serum. The medium was removed and 300 μl ^{3}H-labeled casein (60 μg casein, 5.3×10^6 dpm) was added to the plates together with serum-free medium (600 μl). The plates were incubated and the reaction was terminated as described for the plates containing cells. The TCA soluble counts measured on control plates without cells were subtracted from the TCA soluble counts measured on plates containing cells. Approximately 2.0% of the total radioactivity in the ^{3}H-acetyl casein was TCA soluble following TCA precipitation on the control plates after incubation for 180 minutes. In the plates containing cells from 2.0%–6.3% of the total ^{3}H-acetyl casein, radioactivity was TCA soluble after incubation for 180 minutes. Following each experiment, the cells were trypsinized and counted utilizing a hemocytometer. The viability of cells was examined by Eosin-y exclusion at the end of the experiment. In some experiments the cells were trypsinized, washed 3X, centrifuged, lysed, and the cell button counted in Riafluor. Less than 0.02% of the total amount of ^{3}H-labelled casein was measured in the cell button. Surface protease is only reported under experimental conditions in which no secreted protease was detected.

Secreted Protease Activity

In order to determine if neutral proteolytic activity was secreted during the assay, replicate plates containing serum-free medium (900 μl) were incubated for 180 min at 37°C under 7% CO_2 in room air. The medium was removed, centrifuged at 21,000g for 5 min, and an aliquot (100 μl) incubated for 120 min at 37°C with ^{3}H-labelled casein (40 μl). The reaction was terminated with unlabelled 3% casein (100 μl) and 6% TCA (200 μl) under the conditions described above. An aliquot (100 μl) was counted for radioactivity. The results indicated that under our experimental conditions, no secreted protease activity was detected in the cultured cells. The results do not exclude the possibility that proteases are being secreted, but are being inactivated very rapidly.

Interaction of Heparin With Rat Smooth Muscle Cells

In order to determine the effect of heparin on smooth muscle cell proliferation, heparin (0–333.3 units/ml) was added to the medium containing 5% FCS 24 hr after the cells were subcultured. In experiments in which heparin was inactivated with protamine sulfate, protamine sulfate (3.3 mg/ml) was added to the heparin (33.3 units/ml). The precipitate was removed by centrifugation and the inactivated heparin was added to the medium containing 5% FCS. In experiments in which heparin was added to rat serum or human serum, the cells were subcultured in Dulbecco's modified Eagles medium containing either 5% pooled rat (Sprague-Dawley) serum or 5% pooled human serum. Heparin (333.3 units/ml) was added in either 5% rat serum or 5% human serum 24 hr after subculturing. In one experiment, the heparin was replaced by Dulbecco's modified Eagles medium containing 5% FCS at 48 hours. In the other experiments, no changes in medium or heparin were made during the experiment. At 72 hr and 96 hr, the cells were trypsinized and counted in duplicated plates employing a hemacytometer. Surface protease activity, secreted protease activity, and cell counts were performed 60 min after the heparin was added and at 24-hr intervals.

In one experiment, 72 hr after subculturing, the cells were treated for 30 min with either: 1) 5% FCS; 2) heparin (33.3 units/ml) in serum-free medium; or 3) heparin (33.3 units/ml) in 5% FCS. Surface protease assay was then performed in serum-free medium. Rat smooth muscle cells were also cultured in Dulbecco's modified Eagles medium containing 10% acid-treated FCS. Twenty-four hr after subculturing, either: 1) heparin (333.3 units/ml); 2) antithrombin III (3.0 units/3 ml); 3) antithrombin III (10.0 units/3ml); 4) heparin (333.3 units/ml) + antithrombin III (3.0 units/3 ml); or 5) heparin (333.3 units/ml) + antithrombin III (10.0 units/3 ml) was added to Dulbecco's modified Eagles medium containing 10% acid-treated fetal calf serum (ATFCS). No further changes in medium, ATFCS, antithrombin III, or medium were made during the experiment. Cell counts were performed on duplicate plates at 72 hr, while surface protease activity was performed at 1 hr and 24 hr following subculturing.

Preparation of Cartilage-Derived Factors

The carilage-derived factors were prepared according to the methods described by Keuttner et al [23] and Langer et al [22]. Finely minced bovine nasal cartilage (500 gm) was suspended and extracted in 2500 ml of 1 M guanidine-HCl, pH 6.0, for 24 hr at 4°C. Following removal of the minced tissue by centrifugation, the extracted material was dialyzed exhaustively against distilled water at 4°C. The extract was then centrifuged at 27,000g for 30 min and the supernatant was lyophilized. The guanidine extract (0.5 gm) was dissolved in 40 ml of 0.05 M phosphate buffer, pH 8.1. The cartilage extract was then

applied to a trypsin-Sepharose 4B affinity column (2.5 × 8 cm, 50.0 ml/hr) equilibrated with 0.05 M phosphate buffer, pH 8.1. The affinity column was prepared using 0.5 gm of trypsin per 100 ml of packed Sepharose 4B [22]. The column was washed with distilled water and the fraction used in our studies eluted with 0.01 N HCl. Trypsin inhibitory activity was measured on an aliquot of each fraction. The aliquot was preincubated for 30 min at 4°C with trypsin (5 ng) in 0.2 M phosphate buffer, pH 8.1, and ^{3}H-labelled casein was added and proteolysis was measured as previously described [34, 35]. Each fraction with trypsin inhibitory activity was also assayed for papain inhibitory activity utilizing papain (5 ng) and (^{3}H)-labelled casein at pH 6.0 in the presence of cysteine (2 mM). The fraction used in this investigation contained trypsin inhibitory activity, but not papain inhibitory activity.

SDS-polyacrylamide gel electrophoresis on the fraction used in the studies was performed [36] using bovine serum albumin (Mr, 67,000), ovalbumin (Mr, 43,000), myoglobulin (Mr, 16,900), and ribonuclease (Mr, 12,600) as markers. The cartilage fraction used in this investigation contained components of molecular mass of 14,000 daltons, 21,000 daltons, 24,000 daltons, and 32,500 daltons.

Interaction of Cartilage Factors With Rat Smooth Muscle Cells on Rat Fibroblasts

Cartilage-derived factors (5 μg, 30 μg) were added to Dulbecco's modified Eagles medium containing either 5% FCS or 10% ATFCS 24 hr after the rat smooth muscle cells or fibroblasts were subcultured. Surface protease activity, secreted protease activity, and cell number were determined 1 hr after the cartilage factors were added and at 24-hr intervals.

Protein Synthesis

One hr and 24 hr after the heparin (333.3 units/ml) was added to the rat smooth muscle cells, duplicate plates were pulsed for 4.5 hr with L-^{3}H amino acid mixture (1.0 mCi/ml, 10 μl per plate). Rat smooth muscle cells with and without cartilage factor (30 μg/plate) and rat fibroblasts with and without cartilage factors (30 μg/plate) were pulsed for 4.5 hr with L-^{3}H-amino acid mixture (1.0 mCi/ml, 50 μl per plate), 1 hr and 24 hr after the factors were added to the plates. Protein was precipitated with TCA, filtered, washed, and the radioactivity determined by scintillation counting with 10 ml Aquasol (NEN).

RESULTS

The effect of different concentrations of heparin on rat smooth muscle cell (RSMC) proliferation is shown in Table I. In 5% FCS, the cell number increases from an average of 8.2×10^4 cells at 0 hr to 2.2×10^5 cells at 72 hours. At low concentrations, heparin promotes the growth of rat smooth muscle cells. In the presence of low concentrations of heparin (0.3 units/ml, 3.3 units/ml) the cell number increases from an average of 8.2×10^4 cells to 3.7×10^5 cells and 2.9×10^5 cells at 72 hr, respectively. Higher concentrations of heparin (33.3 units/ml, 333.3 units/ml) inhibit the growth of RSMC. Greater inhibition was observed at 72 hr compared to 96 hours. The inhibition was almost completely abolished when the heparin was treated with protamine sulfate. Inhibition was decreased when the plates containing heparin (333.3 units/ml) were changed to 5% FCS at 48 hours. In one experiment (not shown in Table I), in which the RSMC with and

TABLE I. Effect of Heparin on Rat Smooth Muscle Cell Proliferation

	% of Control cell number		
Treatment	1 hr	72 hr	96 hr
Control (5% FCS)	100	100	100
Heparin (0.3 units/ml)	100	168	ND
Heparin (3.3 units/ml)	100	132	ND
Heparin (33.3 units/ml)	100	70	83
Heparin (33.3 units/ml) + protamine sulfate (3.3 mg/ml)	100	94	ND
Heparin (333.3 units/ml)	100	50	70
Heparin (333.3 units/ml) + 5% FCS at 48 hr	100	73	88
Heparin (333.3 units/ml) dissolved in 5% rat serum	100	48	ND
Heparin (333.3 units/ml) dissolved in 5% human serum	100	67	ND

Twenty-four hr after cells were subcultured, heparin was added to the cells. No further changes in medium, serum, or heparin were made, except in the experiment where medium plus 5% FCS was added at 48 hours. Approximately 170 units per mg heparin. Percent of control cell number calculated from the average cell number from two plates counted in duplicate. These data are typical results from a minimum of two independent experiments. ND, not determined.

without heparin (33.3 units/ml) were changed daily, the inhibition of cell growth in the heparin-treated plates was not enhanced at 72 hr or 96 hr compared to the plates in which heparin was added 24 hr after the cells were subcultured. Inhibition of cell growth was also observed when the cells were grown in heparin (333.3 units/ml) in rat serum or human serum.

Surface protease activity on RSMC treated with heparin in serum-free medium and 5% FCS is shown in Table II. Seventy-two hr after the cells were subcultured, either serum-free medium, or heparin in serum-free medium containing 5% FCS was incubated with the cells for 30 minutes. Surface protease activity was then measured for 3 hr in serum-free medium. Surface protease activity was partly inhibited with heparin plus serum-free medium. Complete inhibition of protease activity was observed in the presence of FCS. The effect of heparin on RSMC protease activity is presented in Table III. In the absence of heparin, protease activity was detected at 1 hr, 24 hr, and 48 hours. An increase in the cell number was observed at 24 hr and 48 hours. The presence of protease activity at 48 hr and the increase in cell number at 72 hr (not shown in Table III) was also observed. The presence of protease activity was observed before an increase in cell number was detected. In the presence of heparin, no protease activity was observed at 24 hours. When protease activity was completely inhibited, no increase in cell number was observed. The inhibition of surface protease activity was not observed at 24 hr, and the cells doubled as determined by the increase in the cell number at 48 hours. Under our experimental conditions, the inhibition of protease activity and cell proliferation lasted only for 24 hours. In RSMC, the amount of protease activity did not correlate with the rate of cell proliferation. At 24 hr surface protease activities of $11{,}309 \pm 2{,}191$ and $20{,}514 \pm 3{,}595$ per 10^5 cells were observed, and in both cases the cells doubled at 48 hours. No significant cell death was observed as examined by Eosin-y exclusion at 24 hr or 48 hours.

TABLE II. Surface-Associated Protease Activity in Heparin-Treated Proliferating Rat Smooth Muscle Cells

Treatment	Surface protease activity ($dpm/10^5$ cells)
Control (5% FCS)	22,100 ± 1762
Heparin (33.3 units /ml) in serum-free medium	5639 ± 2867
Heparin (33.3 units /ml) in 5% FCS	0

RSMC cells (72 hr after subculturing) were treated for 30 min with: 1) 5% FCS; 2) heparin (33.3 units/ml) in serum-free medium; 3) heparin (33.3 units/ml) in 5% FCS. Surface protease activity was then determined in serum-free medium for 3 hr as described in Materials and Methods. Each value represents mean ± SEM. Approximately 170 units per mg heparin. These data are typical results from a minimum of two independent experiments.

Surface protease activity and cell proliferation of RSMC were also measured in the presence of acid-treated fetal calf serum (ATFCS) (Tables IV, V). In the presence of Dulbecco's modified Eagles medium containing 10% ATFCS, the cells increased from an average of 2.6×10^5 cells at 0 hr to 5.5×10^5 cells at 72 hours. Heparin (333.3 units/ ml) in the presence of ATFCS did not inhibit RSMC cell proliferation or protease activity (Tables IV, V). Human antithrombin III (3.0 units/3 ml, 10.0 units/3 ml) did not significantly inhibit cell growth in RSMC (Table IV). Surface-protease activity with antithrombin III (10.0 units/3 ml) was decreased at 1 hr, but not at 24 hr (Table V). No significant inhibition of cell proliferation was observed in the presence of 10% ATFCS, heparin, (333.3 units/ml), and antithrombin III (10.0 units/3 ml) (Table IV). No inhibition of protease activity was observed with heparin (333.3 units/ml) containing antithrombin III (10.0 units/3 ml) (Table V). Thus, ATFCS in the presence of heparin or heparin and antithrombin III did not significantly inhibit cell proliferation or protease activity.

Cartilage factors at a concentration of 5 μg/plate did not inhibit cellular proteases or proliferation. The effect of cartilage factors (30.0 μg/plate) on protease activity on RSMC and rat fibroblasts (RF) is in Table VI. In the RMSC treated with cartilage factors, no surface protease activity was observed at 1 hr and no increase in cell number was observed at 24 hours. RSMC without the cartilage factors contained protease activity at 1 hr and 24 hr, and the cells increased at 24 hr and 48 hours. In the RF without cartilage factors, no surface protease activity was observed at 1 hr, and the cell number only increased from $50 \pm 6 \times 10^4$ cells to $68 \pm 6 \times 10^4$ cells at 24 hours. Protease activity was seen at 24 hr, and a significant increase in cell number was measured at 48 hours. In RF treated with 30 μg of cartilage factors, no protease activity was present at 1 hr or 24 hr, and the cell number did not double at 48 hours. Surface protease activity was present at 48 hours. Thus, cartilage-derived factors also inhibit cellular protease activity as well as inhibiting cell proliferation. No correlation was observed between the amount of cellular protease activity and the rate of cell proliferation. When surface protease was present, the cells proliferated, while very little cell proliferation was observed when the protease activity was inhibited. In order to determine if the inhibition by the cartilage factors occurred in the presence of ATFCS, the cartilage-derived factors were added to RSMC and RF treated with ATFCS. The results are shown in Table VII. Acid treatment

TABLE III. Effect of Heparin on Rat Smooth Muscle Cell Surface Protease Activity and Proliferation

	1 hr		24 hr		48 hr	
	Surface protease activity (dpm/10^5 cells)	Cell number (X 10^3)	Surface protease activity (dpm/10^5 cells)	Cell number (X 10^3)	Surface protease activity (dpm/10^5 cells)	Cell number (X 10^3)
RSMC	11,572 ± 439	150 ± 12	11,309 ± 2,191	290 ± 18	23,670 ± 1,754	620 ± 35
RSMC + heparin (333.3 units/ml)	0	150 ± 12	20,514 ± 3,595	200 ± 24	39,450 ± 2,630	410 ± 29

At 0 time 200,000 RSMC were dispersed. Twenty-four hr later, either 5% fetal calf serum or 5% fetal calf serum and heparin (333.3 units/ml) was added to the cells. No further changes in medium, fetal calf serum, or heparin were made during the experiment. Approximately 170 units per mg heparin. Surface protease activity and cell counts were performed on triplicate plates 1 hr, 24 hr, and 48 hr after the heparin was added. Each value represents mean ± SEM. These data are typical results from a minimum of three independent experiments. RSMC, rat smooth muscle cells.

TABLE IV. Effect of Heparin and Antithrombin III on Rat Smooth Muscle Cell Proliferation

	% of Control cell number	
Treatment	1 hr	72 hr
Control (10% ATFCS)	100	100
+ heparin (333.3 units/ml)	100	96
+ antithrombin III (3.0 units/ml)	100	90
+ antithrombin III (10.0 units/3 ml)	100	98
Antithrombin III (3.0 units/3 ml) + heparin (333.3 units/ml)	100	86
Antithrombin III (10.0 units/3 ml) + heparin (333.3 units/ml)	100	93

Twenty-four hr after RSMC were subcultured in medium containing 10% ATFCS, heparin and/or antithrombin III were added to the cells. No further changes in medium, ATFCS, heparin, or antithrombin III were made. Percent calculated from average cell number from two plates counted in duplicate. Approximately 170 units per mg heparin. These results are typical from a minimum of two independent experiments. ATFCS, acid-treated fetal calf serum.

TABLE V. Surface Protease Activity in Heparin- and Antithrombin III-Treated Rat Smooth Muscle Cells

	Surface protease activity (dpm/10^5 cells)	
Treatment	1 hr	24 hr
Control (10% ATFCS)	9016 ± 1949	4626 ± 842
+ heparin (333.3 units/ml)	9707 ± 3340	7369 ± 3472
+ antithrombin III (10.0 units/3ml)	3461 ± 1055	16,905 ± 6441
Antithrombin III (10.0 units/3 ml) + heparin (333.3 units/ml)	9121 ± 2270	13,576 ± 3842

Twenty-four hr after RSMC were subcultured in medium containing 10% ATFCS, heparin and/or antithrombin III were added to the cells. No further changes in medium, ATFCS, heparin, or antithrombin III were made. Surface protease activity was determined 1 hr and 24 hr after heparin and/or antithrombin III was added. Approximately 170 units per mg heparin. These results are typical from a minimum of two independent experiments.

of the FCS did not affect the ability of the cartilage factors to inhibit cell proliferation. The cartilage factors did not require an acid-labile component of FCS to inhibit cellular protease and cell proliferation in RSMC and RF. At the end of the experiments, the cells were viable as examined by Eosin-y exclusion. Protein synthesis in RSMC and RF treated with heparin and cartilage factors are shown in Table VIII. The RSMC and the RSMC + heparin were pulsed for 4.5 hr with 10.0 μl of L-^{3}H amino acid mixture (1.0 mCi/ml), while the RSMC, RSMC and cartilage factors, RF, and RF and cartilage-derived factors were pulsed for 4.5 hr with 50 μl of L-^{3}H amino acid mixture (1.0 mCi/ml). No differences were observed in protein synthesis 1 hr or 24 hr after the heparin was added to the RSMC. Protein synthesis was also similar 1 hr and 24 hr after the cartilage factors were added to

TABLE VI. Effect of Cartilage Factors on Surface Protease Activity and Cell Proliferation

	1 hr		24 hr		48 hr	
	Surface protease activity (dpm/10^5 cells)	Cell number ($\times 10^3$)	Surface protease activity (dpm/10^5 cells)	Cell number ($\times 10^3$)	Surface protease activity (dpm/10^5 cells)	Cell number ($\times 10^3$)
RSMC	12,098 ± 2630	340 ± 17	5260 ± 1578	420 ± 17	ND	770 ± 29
RSMC + cartilage factors (30.0 μg)	0	340 ± 12	2472 ± 350	350 ± 13	ND	540 ± 17
RF	0	50 ± 6	36,820 ± 5699	68 ± 6	25,511 ± 1052	106 ± 6
RF + cartilage factors (30.0 μg)	0	50 ± 6	0	51 ± 6	44,973 ± 3069	70 ± 6

Twenty-four hr after cells were subcultured, either 5% FCS or 5% FCS and cartilage factors was added. No further changes were made. Surface protease activity and cell counts were performed on triplicate plates 1 hr, 24 hr, and 48 hr after the cartilage factors were added. Each value represents mean ± SEM of triplicate determinations on three separate plates. ND, not determined; RSMC, rat smooth muscle cells; RF, rat fibroblasts.

TABLE VII. Inhibition of Cell Proliferation by Cartilage Factors in Acid-Treated Fetal Calf Serum

	% of Control cell number	
Treatment	1 hr	72 hr
RF + 10% ATFCS	100	100
RF + 10% ATFCS + cartilage factors (30.0 μg)	100	50
RSMC + 10% ATFCS	100	100
RSMC + 10% ATFCS + cartilage factors (30.0 μg)	100	53

Twenty-four hr after the cells were subcultured, either 10% ATFCS or 10% ATFCS and cartilage factors were added to the cells. No further changes were made. Percent of control cell number was calculated from the average cell number from two plates counted in duplicate. The results are typical of a minimum of two independent experiments.

TABLE VIII. Protein Synthesis in Cells Treated With Heparin and Cartilage-Derived Factors

	dpm per 10^5 cells	
Treatment	1 hr	24 hr
RSMC + 5% FCS	5394	10,975
RSMC + 5% FCS + heparin (333.3 units/ml)	5497	9994
RSMC + 5% FCS	89,157	51,811
RSMC + 5 FCS + cartilage factors (30.0 μg)	84,949	66,300
RF + 5% FCS	81,530	49,970
RF + 5% FCS + cartilage factors (30.0 μg)	71,799	55,756

The RSMC and RSMC + heparin were pulsed with 10 μl of L-^{3}H-amino acid mixture for 4.5 hr, 1 hr, and 24 hr after the heparin was added. The RSMC, RSMC + cartilage factors, RF, and RF + cartilage factors were pulsed with 50 μl of L-^{3}H-amino acid mixture for 4.5 hr, 1 hr, and 24 hr after the cartilage factors were added. The results are the average of counts on duplicate plates. These data are typical results from a minimum of two independent experiments.

RSMC or RF. Thus, the presence of heparin and cartilage-derived factors for 1 hr and 24 hr did not affect protein synthesis under our experimental conditions.

DISCUSSION

The presence of membrane-associated protease(s) has been confirmed by several investigators [37–41]. Quigley [42] has shown that plasminogen activator was associated with a specific membrane fraction in Rous virus transformed chick embryo fibroblasts. Fulton and Hart [43] have characterized a plasma membrane-associated plasminogen activator on thymocytes. Our studies have shown that proteases other than plasminogen

activator are associated with the cell surface of many cell types [3–5]. Under our experimental condition, ^{3}H-acetyl casein is a poor substrate for plasminogen activator from human and rat fibroblasts and RSMC (unpublished results).

The function of membrane-associated proteases in the cell is unknown at the present time. In this study, we have observed that the surface protease is present in proliferating RSMC and RF. Protease activity was followed by an increase in the cell number. The amount of protease activity was not directly related to the rate of cell proliferation. In order to determine if the inhibition of RSMC proliferation due to pig mucosa heparin was similar to the inhibition of intimal smooth muscle cells in the Sprague-Dawley rat, the effect of heparin on cultured Sprague-Dawley smooth muscle cells was evaluated. The addition of heparin at high concentrations to RSMC resulted in the complete inhibition of cell proliferation and surface associated protease activity. The inhibition of cellular protease activity and proliferation was not permanent. Twenty-four hr after the RSMC were incubated with heparin, cellular protease activity reappeared and cell growth resumed. Replacing the heparin each day did not prolong the inhibition of cell proliferation. The inhibition of cell proliferation required an acid-labile component in the fetal calf serum since inhibition was not observed with ATFCS. Acid treatment of FCS is known to inactivate protease inhibitors [33]. Heparin as an anticoagulant interacts with antithrombin III [44, 45]. In order to determine if the interaction of heparin with antithrombin III produced the inhibition of cell proliferation and protease activity, antithrombin III was added to heparin in the presence of RSMC and ATFCS. Our results indicated that heparin and antithrombin III in the presence of ATFCS did not inhibit cell proliferation or cellular protease activity. Either heparin and antithrombin III plus another heat-labile component of FCS was responsible for the inhibition, or heparin and a completely different acid-labile component of FCS were responsible for the inhibition. Heparin and antithrombin III alone did not inhibit cell proliferation or protease activity. The experiments with the ATFCS demonstrated that high concentrations of a glycoconjugate such as heparin were not sufficient to inhibit cell proliferation. Although the commercial heparin which contained multiple components was not further purified, the inhibitory effect of heparin could be removed with protamine sulfate. The heparin was not cytotoxic to the RSMC using methods previously described [35]. Protein synthesis of RSMC was not affected by the presence of heparin in the cultures. The effect of low and high concentrations of heparin also demonstrated that heparin at low concentrations promote cell proliferation, while high concentrations inhibit cell proliferation.

The molecular events involved in the heparin inhibition of RSMC proliferation are not understood. Cell-surface heparan sulfate has been reported to be released from cultured Chinese hamster cells when the cells were incubated with heparin [47]. Heparin has also been shown to interfere with the binding of hormones to specific receptors [48]. Recently, Baker et al [46] have reported that low concentrations of heparin (0.2 μg/ml) prevent the binding of protease nexin, a protease inhibitor complex, to diploid human foreskin fibroblasts. In our experiments, 0.2 μg/ml of heparin promotes RSMC proliferation. At the present time, it is not clear if there is a relationship between the protease-nexin complex to cells and the ability of cells to proliferate. At low concentrations of heparin, a decrease in protease-nexin complexes on the cell may be related to an increase in cell proliferation.

In this investigation, the effect of cartilage factors isolated from bovine nasal septum on RSMC and RF were also evaluated in order to determine if cellular proteases were also inhibited in the presence of cartilage-derived factors. The cartilage factors used in the

investigation contained a minimum of 5 components on SDS polyacrylamide gels. Langer et al [28] have shown that the cartilage fraction which contains 4 proteins (Mr, 14,000–28,000) inhibits the vascularization and growth of V_2 carcinoma in the rabbit cornea. Roughley et al [26] have demonstrated that bovine nasal cartilage contains fractions with inhibitory activity against trypsin (Mr, 7,000), papain (Mr, 13,000), and collagenase (Mr, 22,000). The authors suggest that the trypsin inhibitor is Trasylol. Rifkin and Crowe [27] have purified to homogeneity a major trypsin inhibitor from bovine cartilage. The inhibitor was identical to Trasylol, a commercial preparation of Kunitz inhibitor found in bovine tissues and plasma. At the present time, it is not clear if the inhibitory effect of the cartilage factors is due to a single component or many components. It is not known if the effect on cellular protease activity is due to the same component(s) as the effect on cell proliferation. We observed that cartilage factors inhibit cell proliferation and cellular protease activity on RSMC and RF. The effect on RF contradicts the report of Eisenstein et al [25] that the cartilage factors do not affect adult fibroblast proliferation. In comparing our study with the Eisenstein et al [25] report, we utilized higher concentrations of affinity chromatography-purified cartilage components. We did not observe any inhibition of RSMC or RF proliferation or protease activity when 5 μg/plate of cartilage factors were used. The inhibition was not affected by the use of ATFCS, which was not the case when high concentrations of heparin were interacted with ATFCS and RSMC. The cartilage factors do not require an acid labile component in fetal calf serum to inhibit cell proliferation and protease activity. The inhibition was only observed for 24 hr, but the cartilage factors were not added each day. Higher concentrations of factors over several days may inhibit proliferation and protease activity for longer periods. Protein synthesis was not inhibited by cartilage factors.

The functional role of proteases associated with the membrane has not been determined in the present study. Linsley et al [49] have reported that epidermal growth factor (EGF) receptor-complex binding undergoes proteolytic modifications in murine 3T3 cells and human foreskin fibroblasts. Two distinctic proteases, one of unknown specificity and one similar to trypsin, seem to be involved in the cleavages of EGF-receptor complexes [50]. It is not clear if the proteases involved in EGF-receptor complex modification are associated with the plasma membrane. Recently, Bach et al [5] suggested that plasma membrane proteases control the binding of IgE to specific receptors in rat peritoneal mast cells. The present study suggests that protease(s) associated with the plasma membrane are expressed when cells are proliferating and that heparin and cartilage factors, under conditions in which cell proliferation is inhibited, inhibit the cellular protease activity.

ACKNOWLEDGMENTS

The assistance of L. Leibowitz in the preparation of this manuscript is acknowledged. Mel Tiell kindly provided the pooled rat serum. We thank Drs. D. Hamerman, P. Barland, T. Spaet, and A. Grayzel for help and discussion. This research was supported by grants from the U.S. Public Health Service (AG 01732 and HL 16387), New York Heart Association, The Cystic Fibrosis Foundation, and The Arthritis Foundation. Victor B. Hatcher is a Senior Investigator of The New York Heart Association.

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