Brief Definitive Report

HISTIDINE-RICH GLYCOPROTEIN INHIBITS THE ANTIPROLIFERATIVE EFFECT OF HEPARIN ON SMOOTH MUSCLE CELLS

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Vascular smooth muscle cell proliferation may occur in response to endothelial cell injury and is thought to play a major role in the genesis of the atheromatous lesion (1). Aside from the stimulatory effect of several smooth muscle cell mitogens such as those derived from platelets, endothelial cells, and macrophages (2–4), smooth muscle cell proliferation can also be markedly inhibited by several effectors released from arterial cells, among which are the heparinoid glycosaminoglycans (5). Heparin has an antiproliferative effect that appears to be independent of its anticoagulant activity (5, 6); and, from in vitro structure-function studies (6), it is now apparent that heparin requires O-sulfation and N-substitution with dodecasaccharides to show maximum inhibitory activity. Also, confluent bovine endothelial cells generate a heparinase-sensitive material that can inhibit smooth muscle cell proliferation (7) that has been shown to be released by platelet-derived endoglycosidases that can cleave heparin and heparan sulfate (8).

It is now recognized that heparin interacts with multiple proteins, including antithrombin III (9), platelet factor 4 (10), fibrinogen (11), fibronectin (11), albumin (9), thrombospondin (12), and heparin cofactor II (13). A specific interaction takes place with histidine-rich glycoprotein (HRGP), both in purified systems and in plasma, with consequent neutralization of heparin anticoagulant activity (14). However, the role of HRGP in the modulation of arterial cell proliferation remains undefined. We report herein studies that have examined the effect of HRGP and other known heparin-binding proteins on the antiproliferative activity of heparin on cultured bovine arterial smooth muscle cells.

Materials and Methods

Materials. Porcine intestinal heparin was obtained from Elkins-Sinn, Inc. (Cherry Hill, NJ). Fatty acid–free albumin and fibrinogen were purchased from Sigma Chemical Co. (St. Louis, MO). DME and heat-inactivated FCS were obtained from Gibco Laboratories

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(Grand Island, NY). Purified fibronectin was kindly supplied by Dr. Domenick Falcone (Cornell University Medical College).

Cell Culture. Cells were cultured at 37°C in a humidified, flowing CO_2 incubator. All media contained 4 mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml amphotericin. Bovine smooth muscle cells were grown in DME containing 10% FCS.

Isolation and Culture of Smooth Muscle Cells. Smooth muscle cells were obtained from adult bovine aortas by a modification of the explant technique of Ross (15). Cells were harvested as previously described (15). Cells were subpassaged up to three times after primary culture before the initiation of the experiments.

Assay of Cell Proliferation. Smooth muscle cells harvested by trypsin treatment from semiconfluent cultures (15) were plated into 24-well tissue culture plates (Linbro Chemical Co., Hamden, CT; Flow Laboratories, Inc., McLean, VA) at an initial density of 5–7 × 10^3 cells/well in growth medium. 24 h later, the medium was removed and replaced with the control or test medium at 1 ml/well. Cells were harvested at 1 and 6–7 d after replacement with control or test medium. Cells were not fed during the experiment after the initial medium change. Cells were harvested (15) and counted in a Coulter counter in duplicate. Cultures were routinely examined to assure complete removal of cells from the plates after trypsinization and the absence of cellular debris, which is indicative, in part, of cell lysis. We also routinely performed trypan-blue exclusion tests to assess cell viability after treatment of cell cultures. Cell viability was consistently >95%.

Control cultures, after attachment for 24 h, were placed in DME plus 10% FCS. Experimental cell cultures were exposed to heparin alone and to varying concentrations of HRGP, fibrinogen, fibronectin, or albumin in the presence and absence of heparin in 1 ml DME plus 10% FCS. Preliminary experiments were performed using varying doses of heparin (0.1–100 μg/ml). We demonstrated a dose-related inhibition of smooth muscle cell proliferation at day 7 by the addition of heparin (data not shown). A single heparin dose (50 μg/ml) was chosen for all subsequent studies because of consistent inhibition at this dose. Elkins-Sinn porcine intestinal heparin was chosen after comparison of several other sources. These other sources of heparin demonstrated inconsistent antiproliferative activity. At each dose level, eight separate analyses were done at each time point.

Histidine-rich Glycoprotein Isolation and Characterization. HRGP from fresh human plasma was purified by the procedure of Morgan (16). Pancreatic trypsin inhibitor (2 μg/ml; Worthington Biochemical Corp., Freehold, NJ), as well as the serine protease inhibitors, *p*-nitrophenyl-*p'*-guanidino benzoate HCl and PMSF (final concentration 10^{-5} M; Sigma Chemical Co., St. Louis, MO), were added to the plasma and to all buffers throughout the purification procedure to prevent proteolysis of HRGP.

Results

Heparin Effect on Bovine Smooth Muscle Cell Proliferation. Bovine arterial smooth muscle cells incubated with control media, showed typical spindle appearance. In contrast, cells grown in the presence of heparin (50 μg/ml) appeared less dense, with areas which had more sparse, non-confluent cell growth. Cell proliferation was inhibited 51% to 68% in the presence of heparin compared to control cell growth at seven days. Cell viability did not appear to be influenced by the presence of heparin.

Effect of HRGP on Heparin Anti-proliferative Activity. Smooth muscle cells were plated at sparse density as above. At 24 h, cells were washed and medium containing 10 μg/ml or 50 μg/ml of HRGP in the presence and absence of heparin (50 μg/ml) and heparin alone (50 μg/ml), were added to the wells. The cells were incubated and cell counts were obtained on days 1 and 7. The inhibition of smooth muscle cell growth noted at day 7 in the presence of heparin was reversed when 50 μg/ml HRGP was added (Fig. 1). Cell counts at day 7 demonstrated a progressive reversal of heparin inhibition with HRGP. Cells

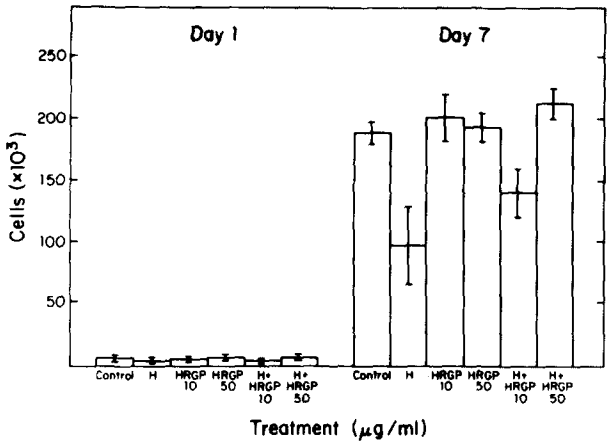

FIGURE 1. Effect of HRGP on bovine smooth muscle cell proliferation. Cells in culture were exposed to: DME (control), 50 μg Elkins-Sinn porcine intestinal heparin (*H*), 10 and 50 μg/ml concentration of human HRGP, or a mixture of heparin and HRGP (*H* + *HRGP*). At days 1 and 7, cells were harvested by trypsinization and counted in a Coulter counter in duplicate. Eight separate experiments were done at each time point. Bars indicate SEM.

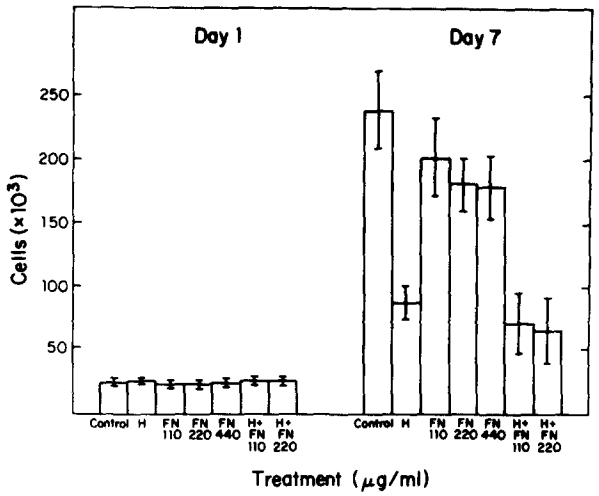

FIGURE 2. Effect of fibrinogen (*FN*) on smooth muscle cell proliferation. Cells in culture were grown with medium alone, with heparin (*H*), or with human fibrinogen (110, 220 μg/ml) in the presence or absence of heparin. Cells were counted at 1 and 7 d. Eight separate experiments were done at each time point. Bars indicate SEM.

incubated in the presence of HRGP alone showed no difference in cell proliferation compared with control cells at day 7 (Fig. 1). Additional controls consisted of cells exposed to fibrinogen, fibronectin, or albumin. There was no reversal of the heparin antiproliferative effect when fibrinogen (Fig. 2), albumin (Fig. 3), or fibronectin (data not shown) were used.

Discussion

We investigated the effects of various serum proteins on heparin inhibition of vascular smooth muscle cell proliferation. The rationale was based on previous

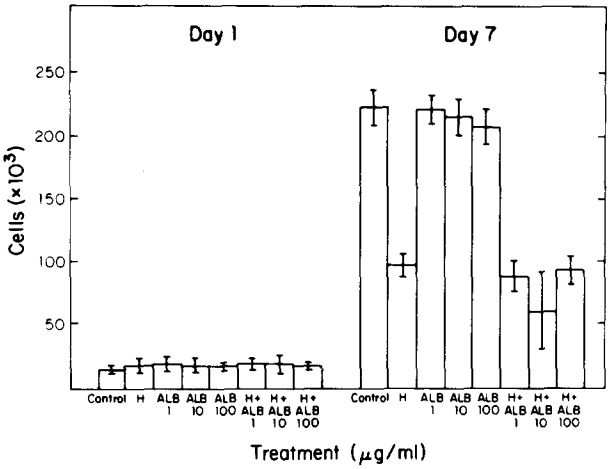

FIGURE 3. Effect of human albumin (*ALB*) on smooth muscle cell proliferation. Cells were grown with medium alone, heparin (*H*), or with human albumin (1, 10, 100 μg/ml), with or without heparin. Cells were counted at 1 and 7 d. Eight separate experiments were done at each time point. Bars indicate SEM.

studies demonstrating that heparin is a potent inhibitor of smooth muscle cell proliferation (5–8). This antiproliferative effect is independent of heparin anticoagulant activity (5), and is associated with a reduction in DNA, RNA, and protein synthesis (17). This effect may be mediated in vivo by platelet endoglycosidase-releasable, heparinase-sensitive heparinoids that may be generated by the endothelium (8).

In this study, we were able to confirm the findings of others (7, 8) that there is a dose-related inhibition of bovine arterial smooth muscle cells by heparin. This effect appeared reproducible and consistent with only Elkins-Sinn heparin, reflecting the probable heterogeneity of biological activity in heparins from various commercial sources recently noted by Castellot et al. (18). Inhibition of cell growth was best attained at 50 μg/ml heparin and ranged from 51 to 68%. This higher dose requirement, in contrast with the amount used in other studies (6), may reflect the use of non–growth arrested cells. Castellot et al. (7) have shown that quiescent, growth-arrested cells are more sensitive to growth medium containing heparin than exponentially growing cells.

HRGP was evaluated for possible effects on heparin's antiproliferative activity. HRGP has diverse biological effects, but the exact function of this glycoprotein remains unclear. We were able to demonstrate a dose-related reversal of the heparin antiproliferative effect with HRGP. Using 50 μg HRGP, the heparin effect was abolished (Fig. 1). This effect was noted despite substoichiometric concentrations of HRGP. This may reflect the known heterogeneity of heparin fragments in unfractionated commercial heparin, with only a portion of the heparin present possessing antiproliferative activity or the need for a threshold level of free heparin to induce inhibition. Castellot et al. (6) have noted a dose-related effect with heparin that is maximal with 12-residue fragments or larger but present at the hexasaccharide level. HRGP could alter heparin activity by an independent, direct proliferative effect on the smooth muscle cell itself or by

interfering directly or indirectly with heparin by high-affinity binding with the polysaccharide (14). A direct mitogenic effect appears unlikely since HRGP alone did not increase smooth muscle cell proliferation (Fig. 1). HRGP may also interfere with the heparin inhibitory effect by binding and blocking the heparin binding site on the smooth muscle cell, which has now been putatively identified (19). In contrast to HRGP, fibrinogen (Fig. 2), albumin (Fig. 3), or fibronectin (data not shown) did not appear to have an affect on cell proliferation or on heparin inhibition of cell growth. Similarly, antithrombin III and a crude preparation of platelet factor 4 do not reverse the inhibitory effect of heparin on smooth muscle cell mitogenesis, as shown by Hoover et al. (20), suggesting that a HRGP-heparin-smooth muscle cell interaction is specific.

Arterial injury induced by de-endothelialization results in a multicellular response with the influx of platelets, macrophages, and lymphocytes, and the probable release of numerous soluble mediators. HRGP, in addition to its presence in plasma, is present in both macrophages and platelets. The ability of HRGP to specifically influence the inhibitory effect of heparin on smooth muscle cell growth may reflect the complex regulation of this cell population within the injured, atheromatous vessel wall, permitting transient unrestrained cell growth for vessel repair.

Summary

Histidine-rich glycoprotein (HRGP), an α-glycoprotein in human plasma that is also present in platelets and macrophages, binds heparin with high affinity and neutralizes its anticoagulant activity. We now report that HRGP specifically inhibits the antiproliferative effect of heparin on arterial smooth muscle cells while other heparinoid-binding proteins do not influence mitogenesis. The multicellular inflammatory response to endothelial injury characterized, in part, by the influx of platelets and macrophages, may be associated with HRGP release into the arterial microenvironment. This release of HRGP may allow smooth muscle cell proliferation and atherogenesis by inhibiting the action of endothelial cell–derived heparinoid substances.

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