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Heparin inhibits *c-fos* and *c-myc* mRNA expression in vascular smooth muscle cells

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Heparin is a potent inhibitor of vascular smooth muscle cell (VSMC) growth. In this paper we show that heparin suppressed the induction of *c-fos* and *c-myc* mRNA in rat and calf VSMC. This effect of heparin is closely associated with its growth-inhibitory activity, as shown by isolating and characterizing a strain of rat VSMC that was resistant to heparin's antiproliferative effect; heparin did not suppress *c-fos* mRNA induction in these cells. Moreover, neither a nonantiproliferative heparin fragment or other glycosaminoglycans that lack growth-inhibitory activity repressed *c-fos* or *c-myc* mRNA levels. The effect of heparin on *c-fos* mRNA induction was selective for specific mitogens, as heparin inhibited *c-fos* mRNA induction in phorbol 12-myristate 13-acetate (TPA) stimulated but not epidermal growth factor (EGF) stimulated VSMC. The effect of heparin on gene expression is independent of ongoing protein synthesis, and inhibition of *c-fos* mRNA is at the transcriptional level. These results suggest that heparin may selectively inhibit a protein kinase C–dependent pathway for protooncogene induction and that this may be one mechanism used by heparin to inhibit cell proliferation.

Introduction

Vascular smooth muscle cell (VSMC)[1] proliferation is controlled by a number of regulators, including peptide growth factors and cell–cell and cell–matrix interactions. Smooth muscle cells are the predominant cell type in atheromatous plaques, and their proliferation is thought to be a key step in the development of atherosclerotic lesions (Ross, 1986). Studies investigating the molecular and biochemical mechanisms regulating VSMC mitogenic responses have shown that serum or growth factor stimulation of quiescent VSMC results in the rapid and transient induction of *c-fos* and *c-myc* mRNA (Kindy and Sonnenshein, 1986). These protooncogenes encode nuclear proteins that are thought to function as transcriptional regulators (Franza *et al.*, 1988; Mitchell and Tjian, 1989); antisense mRNA expression and antibody injection studies suggest the expression of both genes may be necessary for mitogen-stimulated cells to initiate DNA synthesis (Holt *et al.*, 1986; Heikkila *et al.*, 1987; Nishikura and Murray, 1987; Riabowol *et al.*, 1988).

Our laboratory is interested in the regulatory role of heparin in the growth of VSMC. The growth of VSMC is negatively controlled by factors including interferons (Heyns *et al.*, 1985), transforming growth factor β, (Majack, 1987), and heparin/heparan sulfates. Heparin, a glycosaminoglycan composed of repeating glucosamine and uronic acid sugar residues, is a potent inhibitor of VSMC proliferation. Both anticoagulant and nonanticoagulant fractions of heparin inhibit VSMC proliferation in arterial injury models of atherosclerosis (Clowes and Karnovsky, 1977; Guyton *et al.*, 1980), and in in vitro culture systems of VMSC (Hoover *et al.*, 1980; Benitz *et al.*, 1986; Fager *et al.*, 1988). Heparan sulfates may play a physiological role in VSMC growth regulation, as both vascular endothelial cells and VSMC can produce growth-inhibitory heparan sulfate species (Castellot *et al.*, 1981; Fritze *et al.*, 1985).

[1] Abbreviations: bFGF, basic fibroblast growth factor; CS, calf serum; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FCS, fetal calf serum; PKC, protein kinase C; SDS, sodiumdodecyl sulfate; TPA, phorbol 12-myristate 13-acetate; VSMC, vascular smooth muscle cells.

A number of studies have been performed to understand the mechanisms by which heparin inhibits growth. Heparin does not act merely by binding serum components, as VSMC are still stimulated to proliferate by serum depleted of heparin binding factors, and heparin is able to inhibit the growth of VSMC in response to this serum (Reilly *et al.,* 1986). Radiolabeled and fluoresceinated heparin probes have been used to show binding of heparin to specific high-affinity ($K_d = 10^{-9}$ M), protease-sensitive binding sites on VSMC (Castellot *et al.,* 1985; Resink *et al.,* 1989). It is not proven, however, that binding of heparin to high-affinity binding sites is necessary for antiproliferative activity. Metabolic studies show heparin does not affect the rate of protein synthesis; however, heparin modulates the patterns of gene products in VSMC. Examination of specific proteins synthesized after heparin treatment revealed that heparin induces the secretion of a novel 60 000-MW collagen protein (Majack and Bornstein, 1985), stimulates the secretion of apolipoprotein E (Majack *et al.,* 1988), and inhibits release of a protein immunologically related to major excreted protein (Cochran *et al.,* 1988). Heparin also inhibits the incorporation into the extracellular matrix of thrombospondin, a protein implicated in VSMC growth regulation (Majack *et al.,* 1985). The role, if any, of these metabolic changes in the antiproliferative mechanism of heparin's action is not known.

The ability of heparin to inhibit early mitogenic signals is an area of active research. In one study heparin was found to have little effect on serum-stimulated *c-myc* and *c-fos* mRNA while inhibiting *c-myb* mRNA levels in calf VSMC (Reilly *et al.,* 1989). Studies from this laboratory have used Balb/c 3T3 cells to examine the mitogen specificity of heparin's effects (Wright *et al.,* 1989b). These studies found heparin inhibits serum and phorbol 12-myristate 13-acetate (TPA) but not epidermal growth factor (EGF) induction of proliferation, changes in morphology, and *c-fos* and *c-myc* mRNA induction. In this report we use VSMC, which are potential biological target cells for heparin/heparan sulfates, and provide evidence that heparin does suppress serum-stimulated *c-fos* and *c-myc* gene expression in VSMC. We have expanded previous studies by demonstrating that heparin's inhibition of the protooncogene mRNA levels correlates with heparin's growth-inhibitory action. Heparin's inhibition of gene expression is independent of ongoing protein synthesis, operative at the transcriptional level, and specifically affects protein kinase C (PKC)-dependent gene induction. Suppression of protooncogene mRNA expression may thus represent one mechanism used by heparin to inhibit VSMC proliferation.

Results

To determine the effect of heparin on specific gene expression, growth-arrested rat and calf VSMC were exposed to serum plus or minus 200 μg/ml heparin, total cellular RNA was isolated, and the levels of specific transcripts were determined by Northern analysis. After serum stimulation of rat VSMC, *c-fos* mRNA accumulated, reaching peak levels by 30 min and becoming undetectable by 2 h. Levels of *c-myc* mRNA rose more slowly, peaking at 2 h and slowly declining through 12 h. Heparin markedly inhibited the accumulation of both *c-fos* and *c-myc* mRNA while inhibiting thrombospondin mRNA to a lesser extent (Figure 1A). Densitometric measurements of at least three experiments showed an average repression by heparin of 78.5% for *c-fos* mRNA levels, 66% for *c-myc* mRNA levels, and 42% for thrombospondin mRNA levels compared with serum-stimulated levels of these mRNAs. A50 is a constitutively expressed gene of unknown identity (Nguyen *et al.,* 1983); A50 mRNA levels were not affected by heparin treatment and were used to demonstrate uniform RNA loading. As evident from the time course, *c-fos* or *c-myc* mRNA levels did not peak later in heparin-treated cells, indicating heparin did not act merely to delay the induction of these genes. Heparin also suppressed *c-fos* and *c-myc* mRNA accumulation in calf VSMC (Figure 1B). Kinetic analysis again showed no delay of the induction of *c-fos* or *c-myc* in heparin-treated calf VSMC (data not shown). The inhibition of *c-fos* gene expression constitutes one of the earliest demonstrable actions of heparin, because *c-fos* mRNA was substantially repressed within 30 min of heparin treatment.

To determine whether heparin's suppression of *c-fos* and *c-myc* mRNA induction was due to a general inhibition of RNA synthesis, growth-arrested VSMC were stimulated with serum in the presence or absence of heparin, and RNA synthesis was measured by the use of incorporation of [^{3}H]uridine into trichloroacetic acid (TCA)-precipitable material. Triplicate experiments in rat and calf VSMC indicated that heparin only slightly inhibited [^{3}H]uridine incorporation at 30 min (11.2% $\pm$ 7.2% [SD]) or at 2 h (8.8% $\pm$ 8%). This relatively slight inhibition by heparin of total RNA synthesis, in combination with the observation that heparin had no effect

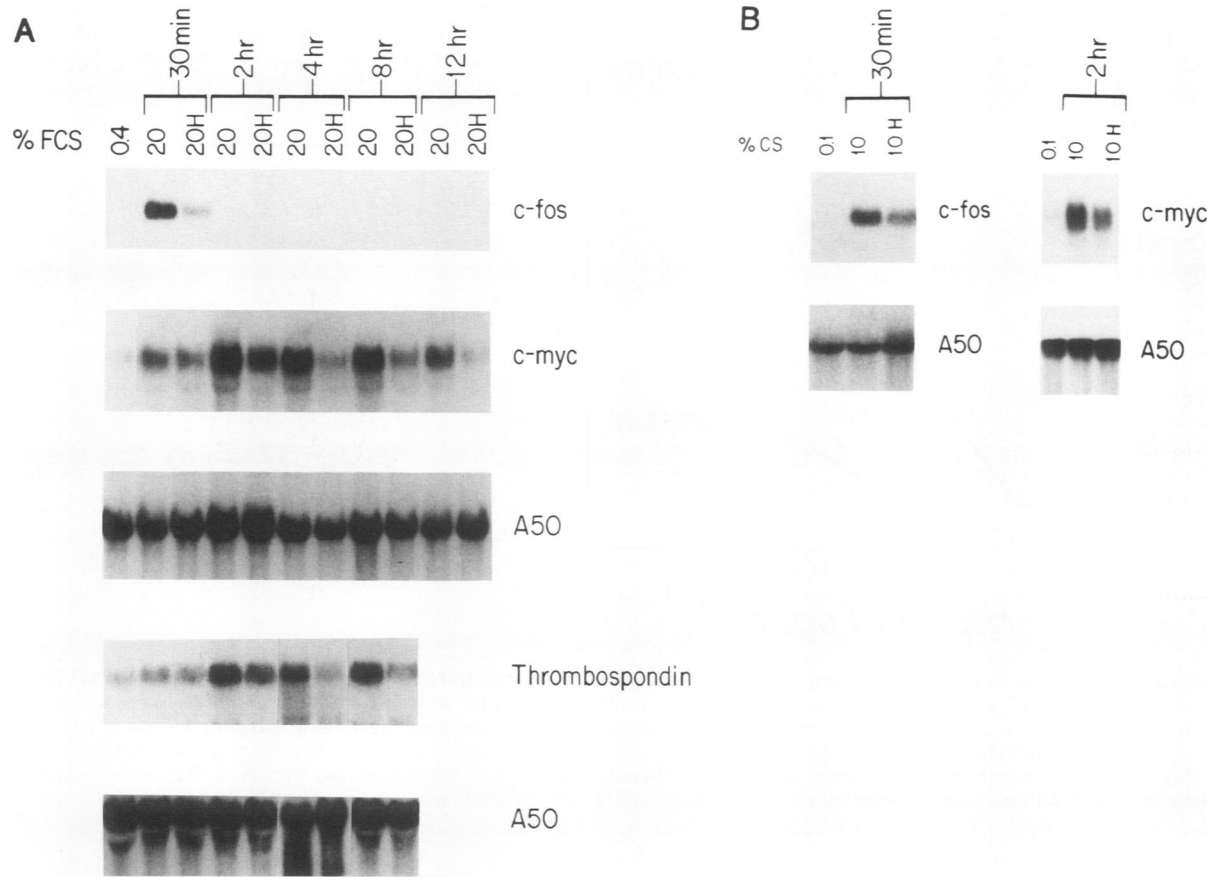

Figure 1. **Heparin inhibits serum-stimulated protooncogene expression in VSMC.** (A) Rat VSMC were growth-arrested in G_0 by treatment with 0.4% FCS for 72 h and were released from G_0 by feeding with fresh medium containing 20% FCS with or without heparin (200 μg/ml). Total RNA was isolated at the indicated time points and analyzed by Northern blot procedures. The same filter was analyzed for *c-fos* mRNA, washed and reprobed for *c-myc* mRNA, and washed and reprobed for A50 mRNA. A second filter was used to analyze thrombospondin and A50 mRNA. (B) Calf aortic SMC were growth-arrested for 72 h in 0.1% CS and stimulated with fresh medium containing 10% CS with or without heparin; total RNA was isolated at 30 min and 2 h; *c-fos*, *c-myc*, and A50 mRNA levels were analyzed by Northern blots.

on A50 mRNA levels, strongly indicates that heparin does not act to nonspecifically inhibit RNA synthesis.

To help identify specific signaling or metabolic processes important for heparin's antiproliferative ability, we isolated heparin-resistant VSMC cultures by the use of classic drug-selection methods. Rat VSMC were cultured continuously in medium containing heparin (200 μg/ml), and cells that grew even in the presence of heparin were selected. Both wild-type and heparin-resistant cells stained with an antibody specific for muscle cell actin (data not shown). Heparin, even at high doses, did not significantly repress the growth of the resistant cells, which were ~100-fold less sensitive than normal VSMC to its antiproliferative effect (Figure 2A). The induction of *c-fos* mRNA by serum, while significantly inhibited by heparin in normal rat VSMC, was not suppressed by heparin in the resistant cells (Figure 2B). Thus, heparin's ability to inhibit gene expression closely correlated with its antiproliferative effect and suggests heparin may operate through the same mechanism for both of these inhibitory actions.

To ascertain the specificity of heparin's inhibition of *c-fos* and *c-myc* mRNA levels, several other highly charged glycosaminoglycans were tested. Hyaluronic acid, dermatan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate did not inhibit the serum-stimulated induction of *c-fos* or *c-myc* mRNA (Figure 3A and B). Unlike heparin, these glycosaminoglycans do not inhibit the proliferation of VSMC (Castellot *et al.*, 1981). To more stringently correlate the antiproliferative activities of heparin with its effects on gene expression, we next tested whether *c-fos* mRNA expression could be suppressed by

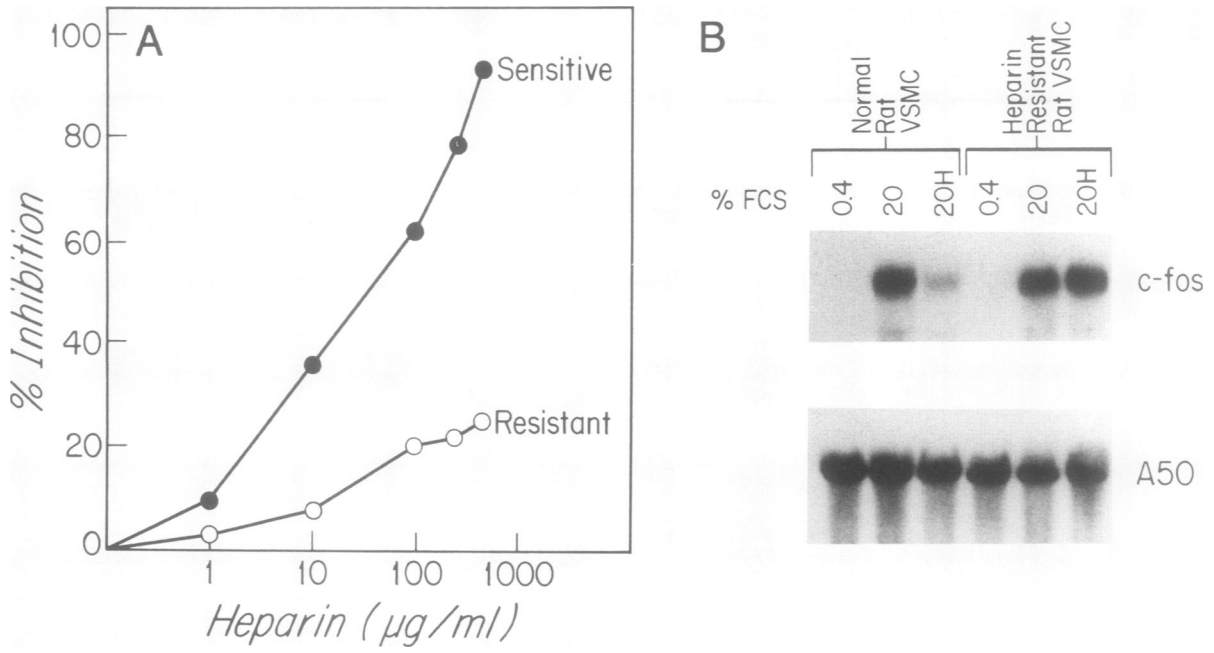

Figure 2. **Heparin does not inhibit serum-stimulated mitogenesis or *c-fos* mRNA induction in heparin-resistant rat VSMC.** (A) Normal rat VSMC and heparin-resistant VSMC were growth-arrested for 48 h in 0.4% FCS and then released from G_0 with 20% FCS in the presence of indicated doses of heparin; cell number was determined after 6 d with the use of a Coulter counter. Percent inhibition of proliferation was measured as [1 − (cell number in 20% FCS plus heparin/cell number in 20% FCS)] × 100. (B) Growth-arrested normal and heparin-resistant rat VSMC were stimulated with 20% FCS with or without heparin, total RNA was isolated after 30 min, and *c-fos* and A50 mRNA levels were assayed by Northern analysis.

a hexasaccharide fragment of heparin that has low antiproliferative activity (Wright *et al.*, 1989a). Northern analysis showed that, unlike native heparin, the heparin fragment did not inhibit serum-stimulated *c-fos* mRNA accumulation (Figure 3C). In concurrent DNA synthesis assays, quiescent rat VSMC were stimulated with 20% fetal calf serum (FCS) in the presence or absence of heparin (200 μg/ml) or the heparin fragment (200 μg/ml). Measurement of [^{3}H]thy-

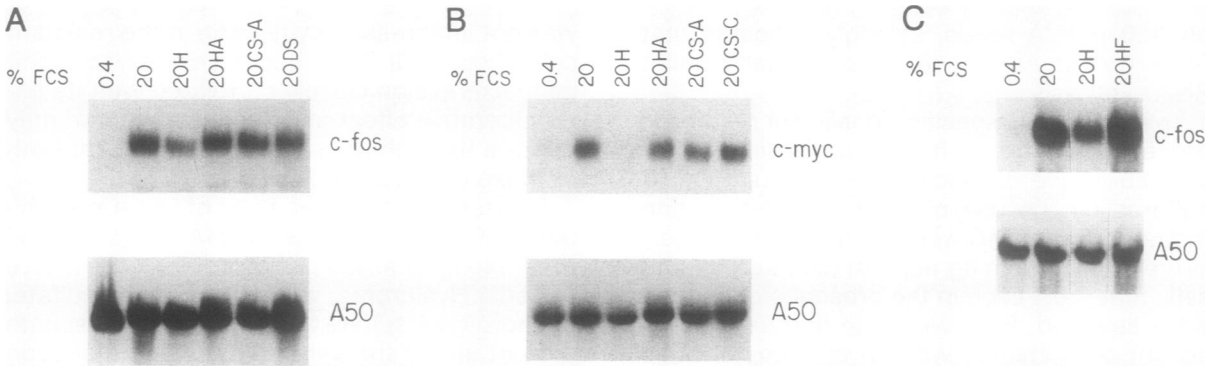

Figure 3. **Serum-stimulated induction of *c-fos* mRNA and *c-myc* mRNA is inhibited by heparin but not by other glycosaminoglycans or by an inactive heparin fragment.** Growth-arrested rat VSMC were exposed to medium containing 20% FCS or 20% FCS plus heparin (H), hyaluronic acid (HA), chondroitin 4-sulfate (CS-A), dermatan sulfate (DS), or chondroitin 6-sulfate (CS-C). The concentration was 200 μg/ml for all glycosaminoglycans. (A) Total RNA was isolated after 30 min and levels of *c-fos* and A50 mRNA were determined by Northern analysis. (B) Total RNA was isolated after 2 h and levels of *c-myc* and A50 mRNA were determined. (C) Growth-arrested rat VSMC were stimulated with medium containing 20% FCS or 20% FCS plus heparin (H, 200 μg/ml) or a hexasaccharide fragment (HF, 200 μg/ml). RNA was isolated after 30 min and *c-fos* and A50 mRNA levels were determined by Northern analysis.

midine incorporation after 72 h showed native heparin inhibited DNA synthesis in rat VSMC 42.8% ± 12%, whereas the hexasaccharide fragment repressed DNA synthesis only 7.2% ± 5.4%.

We have recently shown that heparin inhibits the proliferation of calf VSMC in response to TPA and basic fibroblast growth factor (bFGF), but not EGF (Castellot *et al.,* 1989). Previous studies have also shown EGF-stimulated responses to be relatively unaffected by heparin in cervical epithelial cells, VSMC, and Balb/c 3T3 cells (Wright *et al.,* 1985, 1989b; Reilly *et al.,* 1987). To test the specificity of heparin's inhibitory actions at the molecular level, we measured heparin's effect on *c-fos* mRNA induction in rat and calf VSMC in response to TPA, EGF, and bFGF. All the mitogens tested stimulated *c-fos* mRNA accumulation in rat and calf VSMC (Figure 4). Treatment of rat VSMC with either TPA or EGF does not stimulate their proliferation (Castellot *et al.,* 1989), indicating that *c-fos* induction alone is not sufficient for a mitogenic response. In both calf and rat VSMC, heparin inhibited the expression of *c-fos* mRNA in response to TPA and bFGF, but was not effective in suppressing *c-fos* mRNA induction in response to EGF (Figure 4).

To investigate the mechanism of heparin's inhibition of gene expression, we determined heparin's efficacy in the presence of metabolic inhibitors. First, we investigated whether heparin can inhibit *c-fos* mRNA accumulation in the presence of cycloheximide, a protein synthesis inhibitor. Quiescent rat and calf VSMC were treated with cycloheximide or stimulated with serum and cycloheximide in the presence or absence of heparin, RNA was isolated after 30 min, and *c-fos* levels were determined by Northern analysis and quantitated by densitometry (Figure 5). Heparin completely suppressed induction of *c-fos* mRNA by cycloheximide alone and inhibited the superinduction of *c-fos* mRNA in response to serum plus cycloheximide to the same extent as heparin inhibited *c-fos* mRNA induction by serum alone. Quantitation of [^{3}H]leucine incorporation in rat VSMC treated with 20% serum plus or minus cycloheximide showed that cycloheximide inhibited protein synthesis by 89% ± 9%. These experiments indicate heparin has a direct effect on the regulation of gene expression, independent of ongoing protein synthesis.

To study whether heparin inhibits *c-fos* induction at the transcriptional or posttranscriptional level, we have used the transcriptional inhibitor actinomycin D. VSMC were stimulated with media containing 15% FCS and were exposed to actinomycin D 15 min later, and RNA was isolated 20 min later. Heparin was added

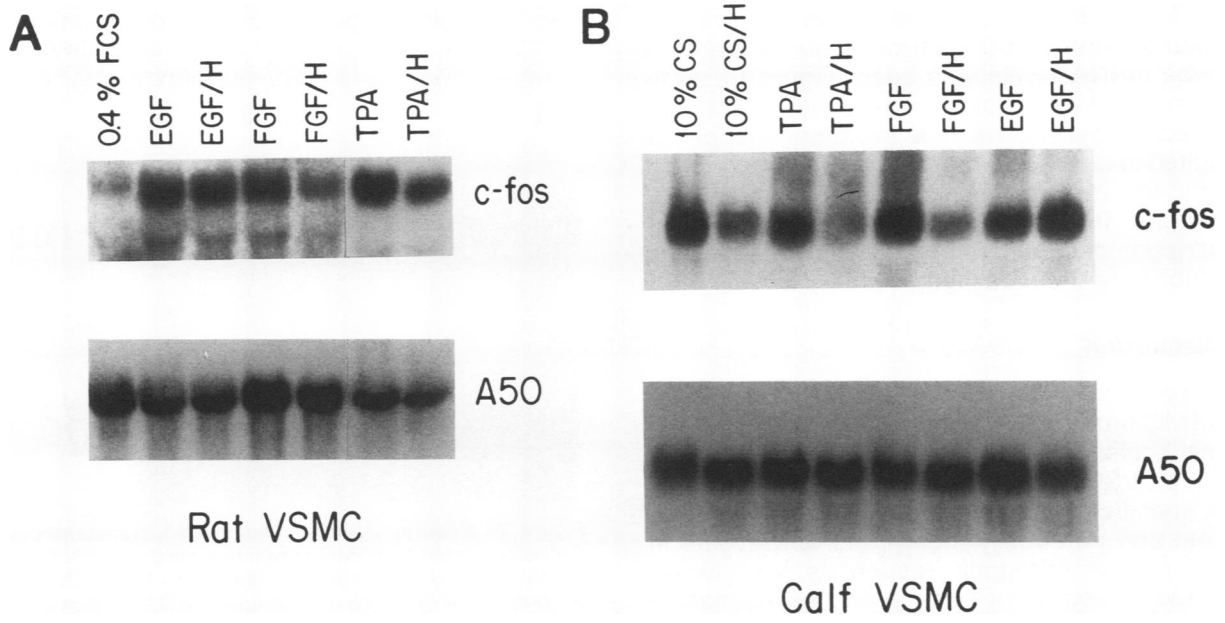

Figure 4. **TPA and bFGF but not EGF induction of *c-fos* mRNA is inhibited by heparin in rat and calf VSMC.** Growth-arrested rat (A) or calf (B) VSMC were stimulated with EGF (10 ng/ml), bFGF (2 ng/ml), or TPA (50 ng/ml), in appropriate basal medium containing 0.5% plasma-derived serum in the presence or absence of heparin (200 μg/ml). Total RNA was isolated after 30 min and *c-fos* and A50 mRNA levels were determined by Northern analysis.

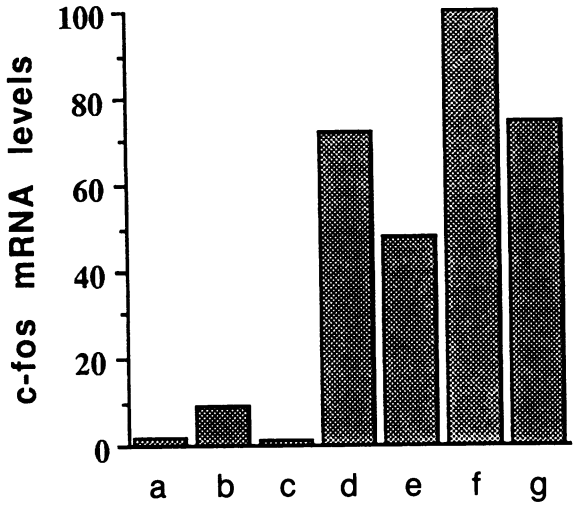

Figure 5. **Heparin inhibits *c-fos* mRNA induction independent of protein synthesis.** Growth-arrested rat and calf VSMC were (a) left untreated, or stimulated with (b) cycloheximide (5 μg/ml), (c) cycloheximide plus heparin (200 μg/ml), (d) serum, (e) serum plus heparin, (f) serum plus cycloheximide, (g) serum plus cycloheximide and heparin. After 30 min total RNA was isolated and *c-fos* and A50 mRNA levels were determined by Northern analysis. *c-fos* and A50 mRNA levels were quantitated by densitometry and *c-fos* levels were normalized to A50 mRNA.

either with the initial serum stimulation or at 15 min with the actinomycin D. If heparin was present during the initial 15 min while transcription was proceeding, *c-fos* mRNA levels were inhibited. However, if heparin was only present after actinomycin D treatment, no inhibition of *c-fos* mRNA levels was seen (Figure 6). Actinomycin D was shown to be active, as addition of actinomycin D with the initial stimulation resulted in complete inhibition of the transcription of *c-fos* mRNA. These experiments strongly suggest that heparin acts to inhibit the transcription of *c-fos* mRNA but does not affect the stability of the message.

Discussion

Heparin strongly inhibits the proliferation of VSMC both in vivo and in vitro. The molecular mechanisms through which these compounds inhibit VSMC growth is unknown. As changes in specific gene expression are among the earliest and potentially most important events in the cellular mitogenic responses, we investigated the effect of heparin on the expression of several immediate-early genes in rat and calf VSMC and examined the role inhibition of gene expression has in heparin's growth-inhibitory actions. VSMC were used in the present investigations because of their importance as potential physiological target cells for heparin/heparan sulfates and their intrinsic role in atherosclerosis. Our experiments show that in VSMC heparin inhibited the induction of *c-fos* and *c-myc* and, to a lesser extent, thrombospondin mRNA accumulation. The relatively slight inhibition by heparin of total RNA synthesis (<12%) was insufficient to explain the dramatic inhibition of *c-myc* and *c-fos* mRNA. Time course studies showed heparin was not acting merely to delay the induction of *c-fos* or *c-myc* mRNA.

The role that inhibition of protooncogene mRNA induction plays in heparin's antiproliferative effects is a critical question. Other growth inhibitors repress immediate-early genes in certain cell types. For example, interferons have been shown to inhibit *c-myc* mRNA levels in fibroblast and Daudi lymphoma cells (Knight *et al.*, 1985; Pietenpol *et al.*, 1989); and *c-fos*, *c-myc*, and ornithine decarboxylase mRNA expression in Balb/c 3T3 cells (Einat *et al.*, 1985). Transforming growth factor β suppresses proliferation and *c-myc* and JE but not *c-fos* mRNA levels in endothelial cells (Takehara *et al.*, 1987). Previously, we have shown that heparin inhibits *c-fos* and *c-myc* mRNA levels in Balb/c 3T3 cells, but we did not examine whether this effect was related to the inhibition of cell growth (Wright *et al.*, 1989b). In this report, the involvement of prootoncogene mRNA inhibition in heparin's antiproliferative effects was strongly suggested by the observations

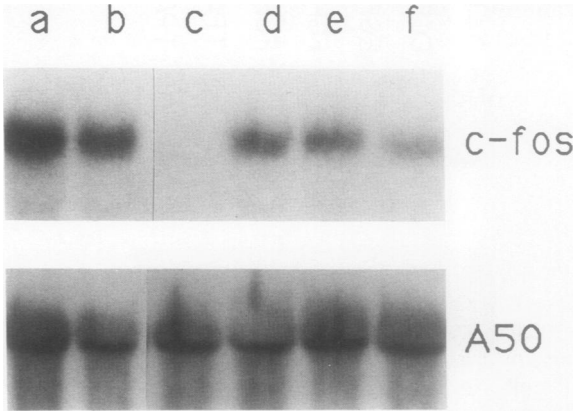

Figure 6. **Heparin inhibits *c-fos* mRNA transcription.** Growth-arrested rat VSMC were treated with (a) 15% FCS, (b) 15% FCS plus heparin (200 μg/ml), (c) 15% FCS plus actinomycin D (5 μg/ml) at time 0, (d) 15% FCS plus actinomycin D at 15 min, (e) 15% FCS plus actinomycin D and heparin both at 15 min, and (f) 15% FCS and heparin at time 0 and actinomycin D at 15 min. Total RNA was isolated at 35 min and *c-fos* and A50 mRNA levels were determined by Northern analysis.

that 1) *c-fos* mRNA induction was not suppressed in VSMC cultures resistant to heparin's antiproliferative activity; 2) nonantiproliferative glycosaminoglycans did not suppress *c-fos* or *c-myc* mRNA induction; 3) a hexasaccharide heparin fragment with low antiproliferative activity did not inhibit *c-fos* mRNA induction; and 4) heparin did not inhibit EGF-stimulated mitogenesis and was also unable to suppress EGF-induced *c-fos* mRNA accumulation.

Although we have shown that heparin significantly suppressed *c-fos* and *c-myc* mRNA levels, it was recently reported (Reilly *et al.,* 1989) that heparin inhibited *c-myb* protooncogene mRNA induction but did not greatly suppress *c-fos* and *c-myc* mRNA levels. Variables such as cell density, depth of quiescence, effective heparin concentration, and heparin treatment protocols may account for the different results observed and remain to be investigated. Inhibition of gene expression cannot be the sole mechanism by which heparin inhibits cell growth, because heparin can be added in late G_1 and still retain much of its antiproliferative activity (Castellot *et al.,* 1985, 1989). Our experiments indicate that heparin may have multiple target sites in VSMC, with actions both at the G_0/G_1 and the G_1/S transitions.

Several experiments were performed to begin to elucidate the mechanism by which heparin inhibits gene expression. First, heparin inhibited *c-fos* mRNA induction in the presence of cycloheximide, demonstrating that heparin did not require synthesis of a protein product but directly suppressed *c-fos* mRNA induction. As seen in other systems, cycloheximide by itself induced *c-fos* mRNA and, in concert with serum, caused a superinduction of *c-fos* mRNA. Cycloheximide induction of *c-fos* mRNA in Balb/c cells was shown to act by increasing transcription of *c-fos* through the *c-fos* serum response enhancer (Subramaniam *et al.,* 1989). Heparin's suppression of cycloheximide induction of *c-fos* mRNA may act by inhibition of this pathway. Second, actinomycin D experiments provide evidence that heparin does not affect the stability of *c-fos* message but rather acts to inhibit its transcription.

Finally, heparin was shown to inhibit *c-fos* mRNA induction in TPA- or bFGF-stimulated, but not EGF-stimulated, rat and calf VSMC. This correlates with studies showing that heparin inhibits growth of calf VSMC in response to TPA but not EGF (Reilly *et al.,* 1987; Castellot *et al.,* 1989) and studies in Balb/c 3T3 cells, which found that heparin inhibits TPA- but not EGF-mediated mitogenesis, changes in cellular morphology, and protooncogene induction (Wright *et al.,* 1989b). Because TPA directly activates PKC (Nishizuka, 1986) and bFGF also activates a PKC-mediated pathway (Tsuda *et al.,* 1985; Presta *et al.,* 1989)—whereas EGF may act through a PKC-independent second-messenger pathway (Ran *et al.,* 1986; Blackshear *et al.,* 1987; Kerr *et al.,* 1988)—these results suggest that heparin may selectively inhibit a PKC-dependent pathway for protooncogene induction.

It is important to determine the molecular basis for heparin inhibition of VSMC growth because heparin/heparan sulfate may play a physiological role in regulating VSMC growth in vivo. These experiments suggest inhibition of gene expression may be one mechanism used by heparin to inhibit cell proliferation. Heparin may act by blocking a PKC-dependent pathway for the induction of the transcription of genes important for VSMC proliferation.

Methods

Cell culture

Rat aortic SMC from Sprague-Dawley rats (Charles River, Wellesley, MA, CD strain) were isolated, cultured, and characterized as previously described (Hoover *et al.,* 1980; Castellot *et al.,* 1982). Briefly, the abdominal aorta was harvested and the adventitia removed under a dissecting microscope. The aorta was cut longitudinally, and pieces of media were carefully stripped from the vessel wall and placed under a small stainless steel mesh screen in 60-mm tissue culture dishes. Within 1–2 wk, SMC migrate from the explants; they were capable of being subcultured ~1 wk after the first appearance of cells. They were grown in RPMI-1640 medium containing 20% FCS. Calf aortic SMC were isolated from explants of bovine aortas as described (Ross, 1971). They were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS). VSMC were growth-arrested by culturing cells for 48 or 72 h in 0.4% FCS in RPMI medium (rat) or 0.1% CS in DMEM (calf).

Heparin-resistant rat VSMC were isolated by continuous passage of VSMC in medium containing heparin (200 μg/ml). Clones showing outgrowth in the presence of heparin were isolated and passaged. Resistant cell lines were established after ~10 passages, and heparin resistance appears to be a stable phenotype.

Cells were cultured in a humidified incubator at 37°C and 5% CO_2 atmosphere and were used from the 4th through 10th passage with the exception of the resistant cells as noted above. SMC phenotypes were evidenced by the presence of 1) numerous myofilament bundles in the cytoplasm, 2) numerous vesicles near the plasma membrane, 3) muscle cell–specific actin as demonstrated by immunocytochemistry, and 4) the characteristic "hill and valley" appearance of confluent cultures.

Metabolic assays

DNA and RNA and protein synthesis were determined by measuring the incorporation of [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine, respectively, into trichloroacetic acid (TCA)-precipitable material. VSMC, plated in 16-mm multiwells and growth arrested, were exposed to normal growth medium

or medium containing 200 μg/ml heparin to which 2 μCi/ml of [^{3}H]thymidine or [^{3}H]uridine or 1 μCi/ml of [^{3}H]leucine had been added. After labeling for the specified time, the cells were washed three times with phosphate-buffered saline and 1 ml 10% TCA, and TCA-precipitated material was extracted in 0.1 N NaOH, 1% sodium dodecyl sulfate (SDS) at 37°C overnight. Tritium content was measured by liquid scintillation counting. Percent inhibition of DNA or RNA synthesis by heparin was calculated as [1 − (CPM in cells treated with 20% serum plus heparin/CPM in cells treated with 20% serum)] × 100.

RNA isolation and Northern analysis

Total RNA was isolated by extraction of cells in guanidine thiocyanate and phenol/chloroform (Chomczmski and Sacchi, 1987). After isolation RNA was ethanol precipitated, dissolved in 1 mM EDTA, and stored at −20°C. Quantitation and purity was assessed by absorbance at 260 and 280 nm. RNA (20 μg) samples were separated on a 1% agarose, 1.1 M formaldehyde gel and transferred by blotting onto nitrocellulose membranes (Meinkoth and Wahl, 1984). Equivalent loading of RNA samples was confirmed by ethidium bromide staining of the ribosomal bands. The nitrocellulose membranes were baked, prehybridized, and hybridized with [^{32}P]-labeled DNA probes (10^6 CPM/ml). Hybridization conditions were 5× Denhardt's solution, 50% formimide, 50 mM tris(hydroxymethyl)aminomethane Cl (Tris.Cl) (pH 7.5), 800 mM NaCl, 0.1% pyrophosphate, 10% dextran sulfate, 75 μg/ml fish sperm DNA and 0.25% SDS. Probes were labeled with [^{32}P]dCTP by random primer extension (10^9 CPM/μg DNA). The probes used were a *c-fos* probe generated from a *v-fos* clone (Dr. I. Verma, Salk Institute); a murine *c-myc* cDNA clone (Dr. K. Marcu, State University of New York, Stony Brook); a rat A50 cDNA clone (Dr. B. Nadal-Ginard, Boston Children's Hospital); a rat thrombospondin cDNA clone (Dr. J. Lawler, St. Elizabeth's Hospital of Boston). Where indicated, *c-fos*, *c-myc*, or thrombospondin mRNA levels were quantitated by densitometry of Northern blot signals and normalized to the constitutively expressed A50 mRNA signal from the same lane.

Materials

Heparin used for these studies was the sodium salt derived from porcine mucosa with a molecular weight of 12 000 to 18 000 and was obtained from the Institute Choay (Paris, France). The hexasaccharide fragment used is a nitrous acid–derived fragment of heparin and has an average molecular weight of ~2000 (Wright *et al.*, 1989a). Disposable tissue culture dishes were purchased from Falcon (Oxnard, CA) and media and serum were from GIBCO (Grand Island, NY) and Flow Laboratories (McLean, VA). Plasma-derived serum was prepared from human donor blood as previously described (Pledger *et al.*, 1977). Radioisotopes were obtained from New England Nuclear (Boston, MA). TPA, cycloheximide, and actinomycin D were purchased from Sigma Chemical (St. Louis, MO). EGF (receptor grade) was obtained from Collaborative Research (Bedford, MA), and recombinant bFGF was generously supplied by California Biotechnology (Mountainview, CA).

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