Heparin Prevents Vascular Smooth Muscle Cell Progression through the G₁ Phase of the Cell Cycle*

(Received for publication, October 19, 1988)

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To gain insight into the mechanism of the antiproliferative effects of heparin on vascular smooth muscle cells (SMC), the influence of this glycosaminoglycan on cell cycle progression and the expression of the c-fos, c-myc, and c-myb proto-oncogenes and two other growth-regulated genes was examined. SMC, synchronized in phase of serum-deprivation protocol, enter S phase 12–16 h after serum stimulation. Pretreatment with heparin for 48 h blocked the induction of histone H3 RNA, an S phase-expressed product, and prevented cell replication. Thus, heparin prevents entry of cells into S phase. Conversely, heparin had essentially no effect on changes in expression of the c-fos and c-myc proto-oncogenes during the G₀ to G₁ transition. Normal increases in c-fos and c-myc RNA were observed 30 min and 2 h following serum addition, respectively. However, the increase in expression of the mRNA of the c-myb proto-oncogene and the mitochondrial ATP/ADP carrier protein, 2F1, which begins to occur 8 h following serum addition to SMC, was completely inhibited by heparin. Two-dimensional polyacrylamide gel electrophoresis of the products of a rabbit reticulocyte cell-free translation of RNA isolated at various times confirmed this temporal assessment of the effects of heparin. These results suggest that heparin does not inhibit cell proliferation by blocking the G₀ to G₁ transition. Rather, heparin may affect a critical event in the mid-G₁ phase of the cell cycle which is necessary for subsequent DNA synthesis.

Mesenchymal cell proliferation is under the positive control of peptide mitogens such as platelet-derived growth factor (PDGF), epidermal growth factor, and fibroblast growth factor. After binding to specific receptors on their target cells, these factors initiate a series of intracellular events which includes the temporal expression of distinct genes which appear as the cell prepares for DNA replication. Two genes which are induced promptly after serum or mitogen addition to growth-arrested cells, including SMC, are the c-fos and c-myc proto-oncogenes (1–7). Their homology to known transforming genetic elements (reviewed in Ref. 8) and direct effects on cellular proliferation (9–12) suggest that c-fos and c-myc control subsequent events which culminate in cell division. The c-myc proto-oncogene is homologous to the transforming gene of the avian myeloblastosis virus. While c-myc was originally thought to be expressed exclusively in cells of the hematopoietic lineage, Thompson et al. (13) observed c-myc RNA in chick embryo fibroblasts, and we have detected c-myc transcripts in bovine aortic SMC although at much lower levels of expression. The expression of c-myc is growth regulated. In quiescent fibroblasts and SMC, c-myc RNA levels are low but increase following serum addition during the mid- to late G₁ phase of the cell cycle. Another growth-regulated gene is 2F1, which codes for the mitochondrial ATP/ADP carrier protein (14). 2F1 was originally described as a G₁-specific gene; its expression increased 6 h following serum addition to quiescent cultures of temperature-sensitive baby hamster kidney cells (15).

The glycosaminoglycan, heparin, is a potent inhibitor of vascular SMC proliferation. Clowes and Karnovsky (16) and Guyton et al. (17) established that anticoagulantly active and nonanticoagulantly active heparin species suppress the growth of vascular SMC following in vivo damage to the endothelium of the rat carotid artery. Subsequent studies demonstrated that heparin and heparan sulfate moieties produced by postconfluent cultures of bovine aortic SMC (18) and endothelial cells (19) inhibit the growth of confluent SMC in vitro. Since aberrant SMC growth within the intimal tissues of the arterial wall may play a major role in the development of the atherosclerotic plaque (20), heparin-like moieties could be potentially useful for the prevention of atheroma formation. Moreover, these data suggest that the normal function of heparan sulfate species present within the arterial wall is to maintain medial SMC in the quiescent growth state by opposing the action of vascular mitogens.

In culture, SMC treated with heparin are arrested in the G₀/G₁ phase of the growth cycle (21). Growth inhibition is not simply due to the complexing of serum mitogens or essential nutrients such as PDGF and low density lipoproteins by the anionic mucopolysaccharide (22), rather heparin apparently transmits its antiproliferative effects after binding to specific sites on the cell surface (23). In the present paper, the possibility that heparin attenuates gene expression in

* This work was supported by National Institutes of Health Grant HL 33014 and National Foundation of Cancer Research Grant 424 (to R. D. R.), National Institutes of Health Grants HL 13962 and CA 36355 (to G. E. S.), and Postdoctoral Fellowship NIH HL 07116 (to C. F. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: PDGF, platelet-derived growth factor; CS, calf serum; DMEM, Dulbecco’s modification of Eagle’s minimal essential medium; PDS, plasma-derived serum; SDS, sodium dodecyl sulfate; SMC, smooth muscle cells; PAGE, polyacrylamide gel electrophoresis.

2 M. Kindy, K. Brown, and G. E. Sonenshein, submitted for publication.
Heparin Prevents G1 Progression of Smooth Muscle Cells

Heparin Prevents Entry into S Phase—To measure the effect of heparin on SMC proliferation, cells were grown arrested by a serum-deprivation protocol (Fig. 1). Following exposure to 10% CS-DME on day 4 (arrow), control cultures began exponential growth and eventually underwent approximately three population doublings before reaching confluence (day 8). The first cell cycle following addition of serum to the cultures of growth-arrested control SMC appeared prolonged, requiring greater than 24 h. Cell number determinations, performed at intervals during the 48 h immediately following serum addition to the quiescent cultures, indicated that the initial cell doubling required approximately 40 h (data not shown).

To determine when S phase occurred during the first cell cycle, [3H]thymidine incorporation into DNA was measured at varying times after the cultures were released from growth arrest. As shown in Fig. 2A, DNA synthesis began 10–15 h following serum addition, and incorporation was greatest 28 h later. As another measure of S phase progression, the mRNA levels of an S phase-specific transcript, histone H3, were measured (Fig. 2B). Total RNA was isolated at the times

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**RESULTS**

Heparin inhibits the proliferation of vascular smooth muscle cells. SMC were placed in 0.1% PDS and exposed to heparin (100 μg/ml) as described under "Materials and Methods." On day 4 (arrow), the cultures were removed from the growth-arrest medium by the addition of 10% CS-DME. Cell numbers were determined each day in duplicate until the control cultures had reached confluence.* 0.1% PDS only; O, 10% CS-DME; ©, 10% CS-DME + heparin.

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**FIG. 1.** Heparin inhibits the proliferation of vascular smooth muscle cells. SMC were placed in 0.1% PDS and exposed to heparin (100 μg/ml) as described under "Materials and Methods." On day 4 (arrow), the cultures were removed from the growth-arrest medium by the addition of 10% CS-DME. Cell numbers were determined each day in duplicate until the control cultures had reached confluence.* 0.1% PDS only; ©, 10% CS-DME; ©, 10% CS-DME + heparin.

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**FIG. 2.** Median cell number (closed circles) and total RNA content (open circles) of control cultures (open circles) and SMC exposed to heparin (100 μg/ml) (closed circles). Results are representative of three experiments.
Heparin Prevents GI Progression

**Fig. 2.** Heparin prevents entry into S phase of serum-stimulated smooth muscle cells. A, kinetics of DNA synthesis. The incorporation of [methyl-3H]thymidine into SMC cultures was determined at the times indicated as described under "Materials and Methods." The results are presented as the percentage cpm relative to the maximal cpm obtained at the 28-h time point. B, appearance of histone H3 mRNA. Total RNA, isolated at the indicated times following addition of 10% CS-DME to quiescent cultures, was subjected to Northern blot analysis for histone H3 RNA (15 μg/lane). C, effect of heparin on histone H3 mRNA expression. Total RNA was isolated from control and heparin-treated cultures between 4 and 16 h after serum addition and Northern blotted for histone H3 (15 μg/lane).

indicated and subjected to Northern blot analysis using a 32P-labeled probe specific for H3. A noticeable increase in H3 occurred between 10 and 15 h post-serum addition, and H3 levels were maximal 20–25 h later. By 35 h, H3 levels had nearly returned to those observed in quiescent cultures. Thus, under the conditions employed in these studies, growth-arrested SMC enter S phase approximately 10–15 h after serum exposure and require 40 h to traverse the initial cell cycle.

The effect of heparin on SMC proliferation is illustrated in Fig. 1. Exposure of SMC to heparin during the 48-h period prior to serum addition as well as during the subsequent 4-day growth period suppressed the final cell density on day 8 by 97%. The extent of heparin inhibition on day 6, where the serum-treated control cultures had undergone approximately one cell doubling, equaled 92%. The effect of heparin on histone H3 levels is shown in Fig. 2C. Under conditions where cell growth was inhibited, the glycosaminoglycan completely suppressed the increase in H3 observed in control cultures at 12 and 16 h. Thus, heparin blocks the initial cell cycle traverse of SMC and inhibits serum growth factor action prior to entry of the cells into S phase.

**Heparin Does Not Inhibit Changes in Gene Expression during the G0 to G1 Transition**—Since the addition of serum to growth-arrested SMC results in the rapid induction of c-fos and c-myc RNA (4), we determined whether heparin could attenuate the serum-induced increase in these two oncogenes. As shown in Fig. 3, the levels of c-fos RNA were nondetectable in quiescent cultures (lane 1) and increased dramatically 30 min after serum addition (lane 2). Heparin, under conditions where it inhibited subsequent cell growth, had no effect on the induction of c-fos RNA (lane 3).

The effect of heparin was established on the expression of c-myc RNA 2 h following serum addition to the growth-arrested cultures (Fig. 3). Levels of c-myc RNA in serum-stimulated SMC (lane 2) were 4-fold higher than the basal amount present in quiescent cells (lane 1). Heparin had no effect on the basal levels of c-myc RNA found in quiescent cultures (data not shown). The expression of c-myc transcripts in serum-treated SMC which had been exposed to heparin (lane 3) also increased sharply relative to that observed in the quiescent cultures, but the amounts appeared slightly lower than those seen in the cultures exposed to serum only. The results of this and two duplicate experiments were quantitated at various exposures of the autoradiograms. The levels of c-myc RNA in the heparin-treated cells were 18, 2, and 13% below the values obtained in control cells. Thus, growth-inhibitory concentrations of heparin appear to have no significant effect on the initial serum-induced appearance of c-myc RNA.

To assess further whether heparin affected gene expression during the G0 to G1 transition, the products of rabbit reticulocyte cell-free translation reactions directed with RNA from quiescent as well as control and heparin-treated SMC which had been exposed to serum for 2 h were examined by two-dimensional PAGE. Exposure of control SMC to serum resulted in the appearance of several new translation products (arrowheads, Fig. 4B) which were either diminished or absent in quiescent cells (Fig. 4A). A pattern similar to that of the serum-treated control SMC was observed in the heparin-treated SMC (Fig. 4C). These data indicate that the induction of major new translationally active species which occurs during the G0 to G1 transition is unaffected by heparin.

**Heparin Inhibits the Induction of c-myb and 2F1 RNA during Mid-G1**—The expression of c-myb increases sharply in the mid-G1 phase of the cell cycle of serum-stimulated SMC. To determine the effect of heparin on c-myb induction, total RNA was isolated from heparin-treated and control cultures at varying times after serum addition. As shown in Fig. 5, c-myb RNA was induced between 4 and 8 h following serum addition to control cultures, reached maximal levels at 12 h, and declined slightly by 16 h. In heparin-treated SMC, however, serum failed to induce c-myb at 8 h or at any time later. Thus, heparin inhibits the increase in c-myb gene expression, and the block was maintained as late as 16 h after serum addition.

To establish whether heparin modulated the expression of other growth-regulated genes induced during mid-G1, the effect of the glycosaminoglycan on the expression of the mitochondrial ATP/ADP translocase, 2F1, was examined. As shown in Fig. 5, 2F1 RNA levels increased 8 h following serum addition to quiescent SMC and were maximal (3-5-fold increase) after 12–16 h. When cultures were treated with heparin, the basal levels of 2F1 RNA (4-h time point) were normal; however, the induction of 2F1 RNA observed at 8 h...
Heparin Prevents GI Progression of Smooth Muscle Cells

**FIG. 4.** Heparin does not affect the levels of most translatable RNA species during the G0 to G1 transition. Total RNA, isolated from 2-h serum-stimulated control and heparin-treated cultures as well as from quiescent cultures, was used to direct protein synthesis in a rabbit reticulocyte lysate system. The products were analyzed by two-dimensional PAGE and autoradiography. Mr, migration in the second dimension (SDS-PAGE). The position of actin is indicated by A. The major spots which have changed due to heparin treatment at the 16-h time point are indicated by arrowheads. The assignments were made when marked differences existed in intensity between corresponding spots in the different gels and are relative to other spots which had undergone minimal changes between experiments. The smear immediately below actin seen in c was not observed in other experiments using the same preparation of RNA. a, quiescent SMC; b, 10% CS-DME + heparin treatment; c, 16 h, 10% CS-DME only; d, 16 h, 10% CS-DME + heparin treatment.

**FIG. 5.** Heparin inhibits c-myb and 2F1 RNA induction in the mid-G1 phase of the cell cycle. Total RNA was isolated from control and heparin-treated cultures at varying times after serum addition, subjected to Northern blotting, and hybridized with probes specific for c-myb (15 µg/lane) and 2F1 (10 µg/lane).

| hours | 4 | 8 | 12 | 16 |
|-------|---|---|----|----|
| heparin | - | + | - | - |
| c-myb |  |
| 2F1  |  |

was blocked, and no increases were observed subsequently. These data indicate that heparin inhibits the increase in RNA of at least two cell cycle-specific genes which are induced by serum in mid-G1.

**FIG. 6.** Heparin does not affect the levels of most translatable RNA species until early S phase. Total RNA, isolated from serum-stimulated control and heparin-treated cultures at 8 and 16 h post-serum addition, was used to direct protein synthesis in a rabbit reticulocyte lysate system. The products were analyzed by two-dimensional PAGE and autoradiography. Mr, migration in the second dimension (SDS-PAGE). The position of actin is indicated by A. The major spots which have changed due to heparin treatment at the 16-h time point are indicated by arrowheads. The assignments were made when marked differences existed in intensity between corresponding spots in the different gels and are relative to other spots which had undergone minimal changes between experiments. The smear immediately below actin seen in c was not observed in other experiments using the same preparation of RNA. a, 8 h, 10% CS-DME only; b, 8 h, 10% CS-DME + heparin treatment; c, 16 h, 10% CS-DME only; d, 16 h, 10% CS-DME + heparin treatment.

Heparin Blocks the Increase in the Major Inducible RNA Species during Late G1 and Early S Phase—To address whether heparin affects the induction of the majority of the new translatable RNA species induced during mid-G1 and later during the cell cycle, cell-free translation assays were performed using RNA isolated at 8 and 16 h post-serum addition. Products directed with total RNA from serum-treated control and heparin-exposed SMC were examined by two-dimensional PAGE (Fig. 6). As expected, a marked difference in the profiles is seen with control cultures at 8 h post-serum addition relative to that observed in quiescent and 2-h serum-treated cells (compare Fig. 6a with Fig. 4). Moreover, the newly induced RNA species were also present in 8-h cultures which had been exposed to heparin (Fig. 6b). Thus, heparin does not have an effect on the expression of most new RNA species at 8 h post-serum addition. When the effects of heparin on expression of translatable RNAs were analyzed at 16 h, a different result was seen. In this case, several of the spots present in the 16-h control cultures were either absent or greatly diminished in the heparin-treated cultures (arrowheads, Fig. 6, c and d). Interestingly, many of these same spots were present in the heparin-treated profile at 8 h (Fig. 6b). The arrowhead at the top left of Fig. 6d indicates a spot which has increased after heparin treatment. In summary, heparin significantly inhibits serum-induced gene expression during late G1/early S phase.

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**Heparin Prevents GI Progression of Smooth Muscle Cells**

6993
Heparin Prevents G1 Progression of Smooth Muscle Cells

Under conditions where vascular SMC proliferation is attenuated by heparin, cells exit the G0 phase but progress through mid- to late G1, and the cells fail to enter S phase. Heparin treatment inhibits the normal induction of histone mRNA at 12–16 h post-serum addition. In sharp contrast to the effects on histone gene expression, however, heparin has no effect upon the serum-induced increase in the RNA levels of the proto-oncogenes, c-fos and c-myc; the induction of these two oncogenes appears to be intimately linked with the G0 to G1 transition (1–7). These data suggest that heparin does not exert its growth-inhibitory effect at this point in the cell cycle. This conclusion is strengthened by the finding that the two-dimensional gels of translated RNA from 2-h serum-stimulated cells exhibited virtually identical patterns in both control and heparin-treated cultures when compared with cells in quiescence. It should be noted, however, that heparin could affect a key event during the G0 to G1 transition that went undetected by these methods. Heparin suppressed the initial increase in c-myb RNA induction and moreover prevented any subsequent increase even as late as 16 h following serum treatment. In addition, heparin inhibited the induction of 2F1 RNA which was stimulated by serum with a temporal pattern similar to that of c-myb. These data place the first detectable effect of heparin on growth-related gene expression during the mid-G1 phase of the cell cycle and furthermore imply that heparin elicits a permanent block in G0, or severely retards cell cycle progression to S phase.

Although heparin inhibited the induction of c-myb and 2F1 RNA during mid-G1, we could not detect any effect of the glycosaminoglycan on the majority of the translatable RNA species at this time. The failure to observe any differences by the cell-free translation assays at the 6-h time point may reflect the lower sensitivity of this method to detect changes in relatively minor species. However, extensive differences in the two-dimensional patterns between control and heparin-treated cells at 16 h were noted. Thus, heparin appears to have delivered its "negative" growth signal to the cells by this time. The results indicate that inhibition of c-myb and 2F1 induction precedes the observed overall changes in RNA expression associated with the failure to enter S phase. These data raise the possibility that heparin transmits its antiproliferative effects by inhibiting the induction of critical RNA species during mid-G1. If elevated expression of the affected RNA species is required for subsequent cell cycle progression, then failure to induce the appropriate gene(s) during mid-G1 would lead to growth inhibition. In this context, it is interesting to note that c-myb is induced by a post-transcriptional process which apparently involves mRNA stabilization (13); perhaps heparin interferes with this process. Given that SMC possess high affinity binding sites for heparin (23), it is conceivable that cell surface-bound heparin generates its own inhibitory signal or alternatively blocks a crucial mitogen-derived signal normally operative during mid-G1. However, since SMC are also capable of internalizing this glycosaminoglycan (23), we cannot dismiss the notion that heparin transmits its suppressive effect directly within the cell.

The factor which accounts for the bulk of the SMC growth-promoting activity in serum and the mitogen whose effects heparin most likely opposes are PDGF. PDGF is not only released in high concentrations from activated platelets (32) but also is produced by endothelial cells (33), macrophages (34), as well as SMC (35). Moreover, in various animal models, SMC growth is a platelet-dependent process (36, 37). These findings together with the knowledge that the mRNA levels for the B chain of PDGF are elevated in atherosclerotic plaque tissue (38) suggest that PDGF contributes to the deregulated SMC growth which accompanies atherogenesis (20). We have established previously that while heparin has no effect on PDGF binding, the glycosaminoglycan markedly inhibits the mitogenic response of SMC to this same factor (39). Taken together with the present data, these results suggest that heparin blocks PDGF function not by preventing exit from G0, but by suppressing events during mid-G1 which were catalyzed by prior exposure to PDGF. Thus, exogenous heparin-like moieties or growth-inhibitory heparan sulfate species present within the arterial wall (18, 19) could conceivably modulate the SMC mitogenic response to PDGF or other mitogens which may be present within the vasculature. We should note that epidermal growth factor, which functions as a mid-G1 progression factor (40) as well as a competence factor (41), suppresses the growth-inhibitory effects of heparin toward SMC in a time-dependent manner (39, 42). Thus, heparin may govern a step in mid-G1, which affects multiple mitogenic pathways rather than a single pathway.

Acknowledgments—We would like to thank P. Leder, I. Verma, R. Baserga, T. Gonda, and W. Mannovz for generously providing cloned genes used in these studies and H. Youssoufian for excellent technical assistance.

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Heparin Prevents GI Progression of Smooth Muscle Cells

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