Intimal hyperplasia, the obstacle in bypass grafts

Toes, Gerrit Jan

CHAPTER 4

Inhibition of vein graft intimal and medial thickening by periadventitial application of a sulfated carbohydrate polymer

Gert-Jan Toes, Elliot S. Barnathan[2] , Hengyi Liu[3], John E. Tomaszewski[1], P.N. Raghunath[1], Robert Carone[1], Paul B.Weisz[2], Wim van Oeveren, Michael A. Golden[3]

Cardiothoracic Surgery Research Division, University Hospital Groningen, the Netherlands.
Departments of Pathology[1], Medicine[2], and Surgery[3], University Hospital Pennsylvania, Philadelphia, PA.

Journal of Vascular Surgery 1996;23:650-6

ABSTRACT

Purpose: The purpose of this study was to determine whether the wall thickening observed in vein grafts after they were placed into the arterial circulation could be inhibited by periadventitial delivery of an insoluble sulfated polymer of ß-cyclodextrin (P-CDS) capable of tightly binding heparin binding growth factors.

Methods: Thirty-four New Zealand White rabbits underwent implantation of reversed autologous jugular vein interposition grafts in the common carotid artery and were randomized to receive either 20 mg P-CDS (n=18) topically around the graft or no additional therapy (n=16). Before being killed at 28 days, animals were given bromodeoxyuridine to assess smooth muscle cell proliferation. Histomorphometric analyses were performed after perfusion fixation.

Results: Compared to controls, treatment with P-CDS was associated with reduced mean intimal thickness (24±3 vs 38± 4 μm ;mean SEM, $p<0.01$) and intimal area (0.25±0.03 vs 0.54±0.09 mm; $p<0.01$). There was also significantly less medial thickness in the P-CDS group (45±3 vs 63±3 μm, $p<0.001$). There was no significant difference in intimal or medial smooth muscle cell proliferation between P-CDS treated and control vein grafts at 28 days. The polymer persisted in the adventitia of all treated vein grafts with a mild foreign body reaction.

Conclusions: Periadventitial placement of, a novel, insoluble, sulfated carbohydrate polymer, inhibits intimal and medial thickening of vein bypass grafts in this model of vein grafting. The persistence of P-CDS in vivo for prolonged periods, and the ease of topical application of P-CDS during vascular bypasses may have important implications for future use in vascular surgery.

INTRODUCTION

Vein grafts inserted into the arterial circulation develop intimal and medial thickening soon after implantation. Although wall thickening may be an appropriate healing process of the vein to new conditions and forces in the arterial environment, it may also result in pathologic lumen narrowing finally resulting in vein graft failure. Vein graft wall thickening is considered to be a leading cause of late vein graft failure.[1,2] Recent studies demonstrate the pivotal role of growth factors in the formation of intimal thickening after arterial injury[3]; growth factors may also play an important role in the normal physiologic condition and repair mechanisms of the vessel wall. [4-6] The healing process of autologous vein grafts implanted into the arterial circulation is likely to be, at least in part, in response to growth factor- mediated stimulation. Therefore agents that interact or interfere with growth factor activity may be beneficial for controlling the wall thickening observed after vein grafts are placed in the arterial circulation.

Heparin has been shown to inhibit proliferation and migration in vitro and inhibits its intimal thickening in models of arterial injury [7,8] , although systemic continuous administration at higher levels is usually required. [9] However, heparin's action on smooth muscle cell growth appears independent of its anticoagulant function. [10] To avoid problems with systemic administration, local administration to the periadventitial space has also succesfully used to inhibit intimal thickening after arterial injury[11] but often requires implantation of delivery vehicle.

To provide periadventitial therapy without an additional delivery vehicle, we developed an insoluble, sulfated carbohydrate polymer of ß-cyclodextrin (P-CDS) that has several properties that make it an attractive candidate to prevent intimal thickening associated with vein graft arterilization: (1) it has no anticoagulant properties, (2) it is stable for prolonged periods when implanted periadventitially in animals wihout toxicity, (3) it has the capacity to tightly bind heparin-binding growth factors (HBGF), and (4) it inhibits its smooth muscle cell proliferation and migration in vitro.[12]

The purpose of this study was to determine whether periadventitial therapy with P-CDS would inhibit vein graft wall thickening after implantation in a well characterized rabbit model.

METHODS

Rabbit vein graft model. Thirty-four male New Zealand White rabbits (Hazelton Research Denver, PA) weighing 3.0 to 3.5 kg at the time of the operation were anesthetized by intramuscular injection with a solution composed of ketamine (50 mg/kg) and xylazine (7 mg/kg). The rabbits underwent vein bypass graft interposition in the left carotid artery. Surgery was performed under ster-

ile conditions. A ventral midline incision was made in the neck and the left jugular vein and common carotid artery were carefully exposed and dissected free from surrounding tissues. Heparin (1000 U; Sima Chemical Co., St. Louis, Mo.) was given intravenously. The flow through the artery was interrupted with microvascular clips and then cut 15 mm segment of the external jugular vein was excised, reversed, and interposed into the divided artery. Proximal and distal anastomoses were performed end to end by the use of interrupted 7-0 polyprolene sutures (Ethicon, Inc.,Somerville,N.Y.). After graft implantation the experimental group received P-CDS as described below. The skin was closed with 2-0 polyglactin suture.

Treatment group groups. Rabbits were randomized to treatment with P-CDS (n=18) or to control group (n=16) that underwent similar graft implantation and manipulation without polymer placement. P-CDS was provided as a dry, white powder by American Maize, Inc (Hammonton, Ind). With the vein graft in place, P-CDS was sprinkled topically to coat the graft. After placement of the dry polymer, three drops of normal saline solution were added to the treated area, making a gel-like substance to assure that the entire circumference of the vein graft and proximal and distal anastomoses had adequate contact with the polymer.

The P-CDS and saline solution mixture has a consistency much like wet sugar, and, because it has no architectural strength, it did not prevent the vein graft from distention of the vein graft. All animal care was complied with the "Principles of Laboratory Animal Care,, and the "Guide for the care and Use of Laboratory Animals,, (NIH Publication No.80-23, revised 1985).

Harvesting procedure. Four weeks after vein graft implantation, at 17, 9, and 1 hour before being killed, the animals received 3 doses (1ml/kg) of 30mg/ml 5-bromo-2'deoxy-uridine solution (Boehringer Mannhein, Indianapolis, Ind). The rabbits were anesthetized and intravenously injected with Evans blue dye (25 mg/kg) and heparin (1000 units). Laparotomy was performed for placement of an abdominal aortic perfusion catheter and an inferior vena cava drainage catheter. The animals were killed, and the vascular system was washed out with lactated Ringer's solution via a retrograde cannula in the thoracic aorta. Perfusion fixation was performed with 10% formalin in neutral 0.1 M phosphate buffer at a pressure of 100 mm Hg in the standard fashion, with the vein graft undisturbed in situ. After perfusion fixation, with the head, neck, and upper thorax fixed, the left common carotid artery with the vein graft, and segments of the right carotid artery and the right jugular vein were removed and immersion fixed for an additional 48 hours.

Structure. Samples of the midsection of the vein graft were used for histologic,

morphometric and immunohistochemical analyses. Specimens were embedded in paraffin, cut in 4 μm sections and stained with hematoxylin and eosin, Verhoeff van Gieson stain, or Richardson's stain. Morphometric measurements were obtained with computer based representation measuring. (Biometrics, Nashville,In.). The inner intimal boundary was the luminal surface, and the intima-media boundary was identified by the color gradient with the Verhoeff van Gieson stain and the Richardson stain.

The outer boundary was defined by the perivascular capillaries. The thickness of the vein graft wall compartments were determined at 8 separate, equally spaced sites along the circumference of the vein. These values were averaged. The vein graft wall circumference was measured, and the cross-sectional vein wall compartment areas were identified as the product of the perimeter and the corresponding compartment thickness. Alternatively, sections were used for immunohistochemistry to detect Brdu-positive cells as described previously.[13]

These sections were counterstained with hematoxylin-and-eosin. The number of Brdu-positive cells and the total number of cells per unit area in both the intima and media of control and P-CDS treated vein grafts were counted manually. An optical graticule was used, and nuclei were counted per unit area at eight even intervals around the circumference. The intimal and medial boundaries were determined by comparison the corresponding VVG stained sections. The BrdU intimal and medial labeling indexes were defined as the number of Brdu-labeled cells divided by the total number of cells in that compartment.

Statistical Analysis. All data are expressed as mean± standard error of the mean. A two tailed, unpaired Student's t-test was used to determine statistical significance. A P value greater than 0.05 was regarded as statistically significant.

RESULTS

There were two peri-operative and two late deaths in each of the P-CDS and control group. At harvest four vein grafts in the P-CDS group and five vein grafts in the control group were occluded. Therefore 10 patent P-CDS and 7 control vein grafts were available for further analysis and form the basis for the basis for all further analyses.

Periadventitial, topical application of the carbohydrate polymer, P-CDS, resulted in a significant reduction in intimal and medial thickness compared with controls. (Figure 1) P-CDS treatment reduced intimal thickening by 38% (24±3 vs 38±4 m, $P<0.01$) and medial thickening by 28% (45±3 vs 63±3 , $p<0.001$) (Figure 2).

There was also a significant inhibition of intimal area accumulation in the P-CDS treated grafts compared with controls (54% reduction) (Figure 3), whereas there was no significant reduction in medial area.

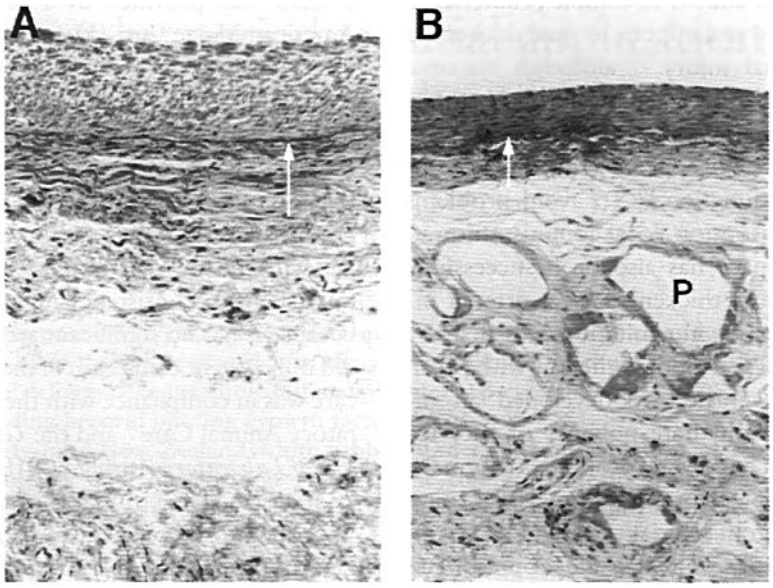

Fig.1. Inhibition of rabbit jugullar vein graft thickening by P-CDS. Tisue sections of vein grafts 28 days after implantation from control (A) and P-CDS- treated rabbits (B) were stained with Richardson's stain (trichrome). Sections are aligned along internal elastic laminae (white arrows) with lumen above and adventitia below. Periadventitial P-CDS particles (P) can be seen in adventitia with some tissue organisation around them. Original magnification x100.

The distribution of intimal thickening present in the vein grafts was somewhat variable. The P-CDS vein grafts showed more uniformity of intimal thickening. Four P-CDS experiments showed a striking reduction in intimal thickening, and five had moderate to low intimal thickening. However, one of the P-CDS grafts displayed severe intimal thickening.

In both groups the smooth muscle cells in the intima appeared to be arranged in an irregular pattern with extracellular matrix between the cells. The media of control and P-CDS-treated vein grafts underwent cocncentric thickening and was less cellular in nature than the intima. Verhoeff van Gieson staining demonstrated elastin in the intima of control and P-CDS-grafts, with a predominance of collagen but not elastin staining in the media.

The light microscopical appearances of the cells and the extracelluar matrix in P-CDS and control grafts showed some variability and no consistent qualitative differences at 28 days. There was no histologic evidence of toxic effects of P-CDS in terms of endothelial cell and smooth muscle cell structure or extracellular matrix appearance, and multinucleated giant cells were seen around the P-CDS particles, suggestive of a mild foreign body reaction. Evans blue staining revealed 100% confluent endothelial lining in all

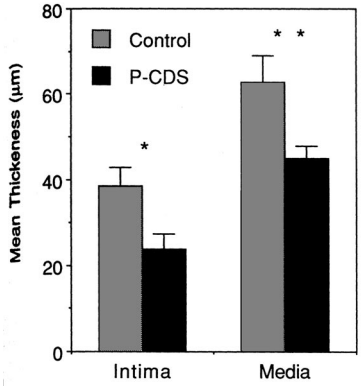

Fig. 2. P-CDS delivered locally inhibits vein graft intimal and medial thickening. Bar graph indicates mean thickness of intima and media of rabbit jugular vein interposition grafts, harvested at 28 days after implantation for control *(shaded bars)* and P-CDS-treated animals *(black bars)*. Values represent mean ± SEM. *Asteris*k represents $p < 0.01$; *double asterisk* represents $p < 0.001$.

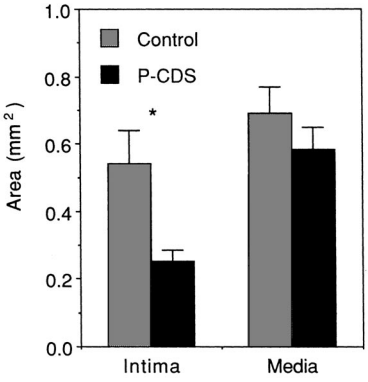

Fig. 3. P-CDS delivered locally inhibits intimal cross-sectional area accumulation. Bar graph indicates cross-sectional area of intima and media of rabbit jugular vein interposition grafts, harvested at 28 days after implantation for control *(shaded bars)* and P-CDS-treated animals *(black bars)*. Values represent mean ± SEM. *Asteris*k represents $p < 0.01$.

vein grafts.

Finally, we assessed the degree of smooth muscle cell proliferation at the time the animals were killed (28 days). At this time point, the overall degree of proliferation (~5%) was clearly higher than normal control jugular vein (~1%). However, there was no significant difference in the percentage of intimal and medial smooth muscle cell that were proliferating between P-CDS-treated and control vein grafts (Table I).

DISCUSSION

In this study we have demonstrated that periadventitial delivery of polymeric ß-cyclodextrin sulfate significantly reduces the development of wall thickening in experimental vein bypass grafts at 28 days. This is the first report of locally applied polymer, which itself both therapeutic and nontoxic to reduce experimental vein graft wall thickening.

Wall thickening develops in all vein grafts within a short period after implantation into the arterial circulation. Vein graft intimal hyperplasia has been assiocated with vein graft stenosis and is a leading cause of late vein graft failure.[1] Human autologous vein bypass graft studies [1,2] and experimental vein graft models [14,15] have shown that the healing process consists of vascular SMC undergoing an early proliferative phase followed by a synthetic phase resulting in a rapid intimal thickening. Smooth muscle cell proliferation and migration into the intima are consid-

	Control	P-CDS	p
Media	0.04 ± 0.008	0.05 ± 0.01	NS
Intima	0.06 ± 0.01	0.04 ± 0.006	NS

Table I. Cell proliferation indexes of the media and intima of vein grafts at 28 days.

ered as the major processes in the formation of vein graft intimal thickening.[5] It is postulated that the healing process after vein graft implantation is driven by growth factors derived from vein graft smooth muscle cells and endothelium cells. [3,5,6] This hypothesis is strengthened by the observation that in vein grafts in rabbits SMC and endothelium cells continue to proliferate in the absence of detectable endothelial denudation or platelet accumulation.[14]

It has been suggested that in particular heparin binding growth factors (HBGF) play a pivotal role in vessel remodelling after vascular injury.[16] It is known that heparin and related glycosaminoglycans bind avidly to these HBGF and may affect their function.[17]

Heparin, P-CDS, and other heparin-like compounds, such as of ß-cyclodextrin tetradecasulfate, inhibit SMC proliferation and migration in vitro and inhibit intimal thickening in animal models of arterial injury. [7,10,18,19] Systemic administration of heparin, however, has yielded conflicting results with respect to its effect on wall thickening in experimental vein grafts. Makhoul et al [16] reported that continuous intravenous administration of heparin significantly reduced intimal thickening in the rabbit carotid vein graft.[13]

Kohler et al [20] , using the same model of vein grafting, reported that heparin failed to reduce vein graft intimal thickening or SMC proliferation.[14] Conflicting data of the effect of systemic heparin on vein graft wall thickening were also reported in studies using rat vein graft models.[21,22] It is postulated that the mechanisms of intimal thickening between arterial injury model and vein graft model differ. Structural and functional differences between veins and arteries[1,20] may also explain why certain therapeutic regimens succeeded in reducing intimal hyperplasia in models of arterial injury but failed to reduce intimal hyperplasia in models of vein grafting. Therefore the results of successful reduction of intimal hyperplasia in arterial injury models can not directly be extrapolated to models of vein grafting.

Multiple agents have been studied for their effects on vein graft remodeling with variable success. [23-26] Mechanistically, blockade of growth factor function is an attractive option. Systemic administration of growth factor antagonists such as heparin may be harmful, since growth factors are widespread and not limited to the vasculature. Wound healing in general

could be impaired, presenting a problem after surgical procedures.[18] Furthermore, the usefulness of heparin is limited by its potential bleeding complications and its higher dose requirements for inhibiting SMC proliferation than the dose required for anticoagulation.

This stimulated the search for novel heparin mimics with the antiproliferative heparin characteristics of heparin but that could be applied locally. Local application of a pharmacologic compound may reduce its systemic effects and allow high concentrations at the target side. Monomeric ß-cyclodextrin tetradecasulfate (CDS) is a universal heparin analog, paralleling and exceeding the demonstrated cell modulating capabilities of heparin without significant anticoagulant activities.[19,27,28] We have previously shown that orally administered of this compound inhibits restenosis and intimal hyperplasia in a hypercholesterolemic rabbit angioplasty model.[18] The polymer from P-CDS has biologic and therapeutic activity in that it inhibits SMC proliferation and migration in vitro and inhibits the arterial wall response to injury.

In this study, the polymer was applied to the adventitial side of the vein graft and both anastomoses in the form of dry, fine powder. Hydration of the polymer yields a soft gel that has no architectural strength and does not interfere with vessel dilatation. After hydration the polymer offers an internal heparin-like surface capable of absorbing HBGF proteins like basis fibroblast growth factor (bFGF)[29], platelet-derived growth factors[30] or vascular endothelial growth factor.[31] Although the mechanism through which P-CDS reduces arterial and vein graft wall thickening is still unknown, we speculate that P-CDS may provide a sustained therapeutic effect by tightly binding and sequestering growth factors in the periadventitial space and that the cyclic structure of the carbohydrate polymer subunits would render it relatively resistant to degradation. Previous studies have demonstrated that the ability of P-CDS to tightly bind bFGF and prevent proliferation of rat SMC in vitro by bFGF[12,29], platelet derived growth factor-BB and epidermal growth factor.[12] Whether growth factors generated in the wound site are sequestered and prevented from gaining access to their cognate receptors remains to be investigated. However, wound response in general was not impaired by the presence of this polymer in this model. At 28 days after injury there was no significant difference in medial and intimal proliferation indexes between control and P-CDS treated vein grafts. This is concordant with a study by Schwartz et al.,[15] who reported that smooth muscle cell proliferation in this model of vein grafting appears to begin within 24 hours and to peak at 2 days but then returns to normal at 14 days.[15] Future studies are needed to determine whether P-CDS limits the early SMC proliferative burst soon after the implantation into the arterial circulation, which may in part account for the reduction in

intimal thickening.

In summary, this is the first report of locally applied novel heparin-like polymer without an additional delivery vehicle to reduce experimental vein graft wall thickening. The ease of local application of this polymer at the time of vascular surgery, especially at the anastomoses where intimal thickening is a particular problem, will facilitate its application in human vascular bypasses. Further investigations into its mechanism of action seem warranted to optimize its utility as a therapeutic agent.

References

1. Cox JL, Chiasson DA, Gotlieb AL. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences veins and arteries. Prog Cardiovasc Dis 1991; 34:45-68.
2. Kockx MM, Cambier BA, Bortier HE, De Meyer GR, Van Cauwelaert PA. The modulation of smooth muscle cell phenotype is an early event in human aorto-coronary saphenous vein graft. Virchow Archiv A Pathol Anat 1992; 420:155-162.
3. Reidy MA. Factors controlling smooth muscle cell proliferation. Arch Pathol Lab Med 1992;116:1276-80.
4. Ross R.The pathogenesis of atherosclerosis-an update. N Eng J Med 1986;8:488-500.
5. Bull DA, Hunter GC, Putman CW. Growth factors and the arterial wall: implications for vessel repair, atherosclerosis and intimal hyperplasia. Perspect Vasc Surg 1991;4:89-103.
6. Adoock GD. Implantation injury. J Vasc Surg 1989; 10:587-9.
7. Clowes AW, Karnovsky MJ. Suppression by heparin of smooth muscle cell proliferation in injured arteries. Nature 1977;265:625-6.
8. Clowes AW, Clowes MM. Kinetics of cellular proliferation after arterial injury-IV: heparin inhibits rat smooth muscle mitogenesis and migration. Circ Res 1986;58:839-45.
9. Edelman ER, Karnovsky MJ. Contrasting effects of the intermittent and continuous administration of heparin in experimental restenosis. Circulation 1994;89:770-6.
10. Casteliot JJ Jr, Choay J, Lormeau JC, Petitou M, Sache E, Karnosky MJ. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells-II: evidence for a pentasaccharide sequence that contains a 3-O-sulfate group. J Cell Biol 1986;102:1979-84.
11. Edelman ER, Adams DH, Karnovsky. Effect of controlled adventitial heparin delivery on smooth muscle cell proliferation following endothelial injury. Proc Natl Acad Sci U S A 1990;87:37773-7.
12. Bachinsky WB, Barnathan ES, Liu H, et al. Sustained inhibition of intimal thickening; in vitro and in vivo effects of polymeric ß-cyclodextrin sulfate. J Clin Invest 1995;96:2583-92.
13. Campana D, Coustan-Smith E, Janossy G. Double and triple staining methods for studying the proliferative activity of human B and T lymfoid cells. J Immunol Methods 1988;107:79-88.

14. Zwolak RM, Adams MC, Clowes AW. Kinetics of vein graft hyperplasia: assiocation with tangential stress. J Vasc Surg 1987; 5:126-136.
15. Schwartz LB, Pence JC, Kerns BJ , Ingehart JD, Mc Cann RL, Hagen PO. Kinetics of vein graft cell division and function. Surg Form XLVII 1991:362-365.
16. Makhoul RG, Davis WS, McCann RL, Hagen PO. Heparin decreases intimal hyperplasia in experimental vein graft. Circulation 1986;74:11-26.
17. Burgess WH, Maciag T. The heparin-binding (fibroblast) growth factor family of proteins. Ann Rev Biochem 1989;58:575-606.
18. Herrmann HC, Okada SS, Hozakowka E, et al. Inhibition of smooth muscle cell proliferation and experimental angioplasty restenosis by ß-cyclodextrin tetradecasulfate. Art and Thromb 1993;13:924-931.
19. Okada SS, Muttreja M, Hozakowska E, et al. Inhibition of smooth muscle cell proliferation and experimental angioplasty restenosis by ß-cyclodextrin tetradecasulfate. J Pharmacol Exp Ther 1995;273:948-54.
20. Kohler TR, Kirkman T, Clowes AW. Effect of heparin on adaption of vein grafts to arterial circulation. Arteriosclerosis 1989;9:523-528.
21. Norman PE, House AK. Heparin reduces the intimal hyperplasia seen in microvascular vein grafts. Aust N Z J Surg 1991; 61:942-948.
22. Cambria RP, Ivarsson BL, Fallon JT, Abbott WM. Heparin fails to suppress intimal proliferation in experimental vein grafts.Surgery 1992;111:424-429.
23. El-Sanadiki MN, Cross KS, Murray, et al. Reduction of intimal hyperplasia and enhanced reactivity of experimental vein grafts with verapamil treatment. Ann Surg 1990;212:87-96.
24. Calcagno D, Conte JV, Howell MH, Foegh ML. Peptide inhibition of neointimal hyperplasia in vein grafts. J Vasc 1991;13:475-9.
25. O'Donohoe MK, Schwartz LB, Radic ZS, et al. Chronic ACE inhibition reduces intimal hyperplasia in experimental vein grafts. Ann Surg 1991;214:727-32.
26. Nassey MF, Davies MG, Svendsen E, et al. Reduction of experimental vein graft intimal hyperplasia by ketanserin. J Surg Res 1993;54:530-8.
27. Folkman J, Weisz PB, Joullie MM, Li WW, Ewing WR. Control of angiogenesis with synthetic heparin substitutes. Science 1989;243:1490-3.
28. Weisz PB, Kumor K, Macarak EJ. Protection of erythrocytes against hemolytic agents by cyclodextrin slufate. Biochem Pharmacol 1993;45:1011-6
29. Shing Y, Folkman J, Weisz PB, Joullie MM, Ewing W. Affinity of fibroblast growth factors for by ß-cyclodextrin tetradecasulfate. Analyt Biochem 1990;185:108-11.
30. Raines EW, Ross R. Purification of human platelet-derived growth factor. Methods Enzymol 1985:109:749-73.
31. Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW. The vascular endothelial growth factor family of polypeptides. J Cell Biochem 1991;47:211-218.