

## Prolongation of Rat Skin and Cardiac Allograft Survival by Low Molecular Weight Heparin

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**Background.** We have previously reported that very low doses of low molecular weight heparin compounds (LMWH) inhibit a variety of T-cell-mediated reactions by down-regulation of TNF- $\alpha$  production. This study tested the efficacy of LMWH in organ transplantation.

**Methods.** Skin and heterotopic heart transplantations were performed between recipient Wistar rats and donor BN rats. Two doses of LMWH were given sc, 1 and 20  $\mu$ g, each in three protocols, with day of grafting as Day 0: (A) Daily: -1, 0, 1 . . . , (B) Late Weekly: -1, 6, 13 . . . , and (C) Early Weekly: -7, 0, 7 . . . . Doses and schedules were selected based on efficacy in autoimmune models. Skin graft rejection was defined by complete separation of the graft, and heart transplant rejection was defined as cessation of heartbeat.

**Results.** Treatment with 1  $\mu$ g (26.8  $\pm$  2.0 days) and 20  $\mu$ g (24.5  $\pm$  2.3 days) of LMWH using the Early Weekly protocol significantly prolonged skin allograft survival compared to controls (17.8  $\pm$  4.4 days),  $P < 0.001$  for both, whereas other protocols did not. Compared to controls (8.3  $\pm$  1.4 days), treatment with both 1 and 20  $\mu$ g of LMWH using all three protocols significantly prolonged cardiac allograft survival. The efficacy, however, varied considerably. Increase in graft survival ranged from 18% (1  $\mu$ g, Daily, 9.8  $\pm$  0.7 days,  $P = 0.02$ ) to more than twofold (20  $\mu$ g, Early Weekly, 20.8  $\pm$  5.5 days,  $P < 0.001$ ) according to the dose and schedule of LMWH.

**Conclusions.** Treatment with very low doses of non-anticoagulant LMWH preparations having anti-TNF- $\alpha$

activity significantly prolongs rat skin and cardiac allograft survival in a dose- and schedule-dependent manner. © 1999 Academic Press

**Key Words:** low molecular weight heparin; tumor necrosis factor  $\alpha$ ; cardiac transplantation; graft survival; rat.

### INTRODUCTION

Heparin has been noted to inhibit inflammatory reactions independent of its anticoagulant activity [1–14]. We have previously demonstrated that administration of very low doses of heparin compounds devoid of anticoagulant activity inhibits a variety of T-cell-mediated immune reactions such as delayed-type hypersensitivity and adjuvant arthritis in rats and mice [13, 14]. These immunosuppressive effects have been recently found to be related to down-regulation of TNF- $\alpha$  production by specific sulfated disaccharide molecules derived from heparin or from heparan sulfate [15, 16]. The present study was undertaken to evaluate the effects of LMWH compounds having anti-TNF- $\alpha$  activity in a model of organ transplantation.

### METHODS

**Animals.** Skin and cardiac transplantations were performed between male inbred Wistar rats (RT1<sup>m</sup>) weighing 350–500 g serving as recipients and male inbred BN rats (RT1<sup>n</sup>) serving as donors. The rats were obtained from the Animal Breeding Center of the Weizmann Institute of Science. All animals received humane care according to the *Guide for Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication Number 86-23, revised 1985).

**Skin allografting.** Full thickness skin grafting was performed as previously described [17]. One rat served as a donor to up to 10

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TABLE 1

## BN Skin Allograft Survival in Wistar Rats Treated with Low Molecular Weight Heparin

Heparin dose ( $\mu\text{g}$ )	Treatment protocol	n	Individual graft survival (days)	Mean graft survival (days)	P value
	Control	16	9, 13, 13, 15, 15, 16, 16, 17 17, 19, 19, 20, 23, 23, 24, 26	$17.8 \pm 4.4$	
20	LW	7	17, 20, 20, 20, 21, 24, 28	$21.4 \pm 3.3$	0.08
20	EW	6	24, 24, 28, 28, 28, 29	$26.8 \pm 2.0$	0.0001
20	D	7	10, 14, 14, 15, 17, 18, 20	$15.4 \pm 3.2$	0.23
1	LW	7	12, 13, 16, 16, 17, 19, 20	$16.1 \pm 2.9$	0.39
1	EW	6	20, 24, 24, 26, 26, 27	$24.5 \pm 2.3$	0.0023
1	D	6	11, 12, 15, 15, 18, 21	$15.3 \pm 3.7$	0.26

recipients. Day of grafting was Day 0. The day of rejection was defined as the day on which complete separation of the graft occurred.

**Cardiac transplantation.** Heterotopic cardiac transplantation was performed under pentobarbital anesthesia (50 mg/kg intraperitoneally), using the modified technique of Ono and Lindsey [18]. Day of grafting was Day 0. Graft survival was assessed by daily palpation. The day of rejection was defined as the day of cessation of heartbeat. Rejection was confirmed histologically. Technical failures within the first 72 h were excluded from the experimental groups.

**Heparin.** We used a LMWH produced by KabiVitrum (Sweden) batch number 38609, demonstrated to have anti-TNF- $\alpha$  activity as described [14, 15]. Average molecular weight was 5000 Da (range 1000–10,000 Da, with 65–78% of the molecules in the range of 3000–8000 Da). At human therapeutic doses the drug has an activity of 5000 anti-factor Xa units/0.2 ml with minimal effect on thrombin generation. However, at the dose range used in this study (adjusted for rat size), the drug has no measurable anticoagulant effect. Absolute bioavailability after subcutaneous injection in healthy human volunteers is  $87 \pm 6\%$ . The exact bioavailability after intraperitoneal injection in rats is unknown.

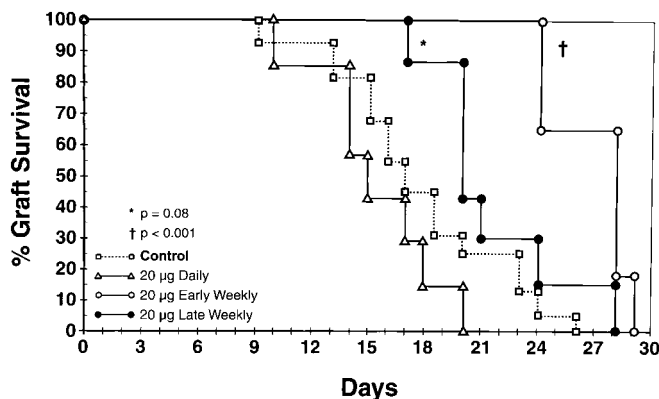
**Study design.** The LMWH was diluted in phosphate-buffered saline (PBS) to concentrations of 1 and 20  $\mu\text{g}/\text{ml}$  and these doses were injected subcutaneously each according to three different protocols with day of grafting as Day 0: (A) Daily: -1, 0, 1, 2 . . . , (B) Late Weekly: -1, 6, 13, 20 . . . , and (C) Early Weekly: -7, 0, 7, 14 . . . . Control animals were sham-injected with PBS. The dosages and frequency of administration were so selected based upon our previous experience with these protocols treating immune reactions [13, 14].

**Statistics.** Data are expressed as mean  $\pm$  standard deviation. The Kaplan-Meier method was used to compute actuarial graft survival. The log-rank test was used to compare survival curves with a P value of less than 0.05 considered significant.

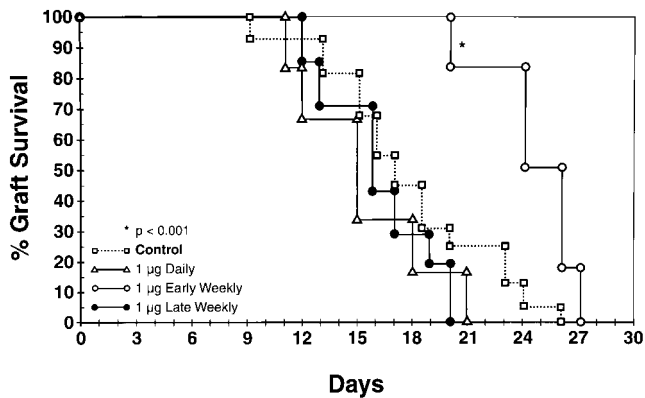
## RESULTS

**Low doses of LMWH prolong skin allograft survival (Table 1, Figs. 1 and 2).** Untreated Wistar rats rejected BN skin allografts within  $17.8 \pm 4.4$  days. Treatment with the Early Weekly protocol, in both 20- and 1- $\mu\text{g}$  dosages, resulted in a significant prolongation of graft survival ( $26.8 \pm 2.0$  days,  $P = 0.0001$ ; and  $24.5 \pm 2.3$  days,  $P = 0.0023$ ). A dose of 20  $\mu\text{g}$  using the Late Weekly protocol had some effect, but it was too small to reach significance ( $21.4 \pm 3.3$  days,  $P = 0.08$ ). Doses of 1  $\mu\text{g}$  administered using the Late Weekly protocol and the Daily Protocol using either dose were ineffective.

**Low doses of LMWH prolong cardiac allograft survival (Table 2, Figs. 3 and 4).** Untreated Wistar rats rejected BN cardiac allografts very consistently at  $8.3 \pm 1.4$  days. Treatment with both 1- and 20- $\mu\text{g}$  doses of heparin, using any of the three treatment protocols, resulted in a significant prolongation of cardiac allograft survival. However, the various treatment regimens showed markedly different efficacy. The 20- $\mu\text{g}$  dose was more effective than the 1- $\mu\text{g}$  dose. Of the treatment protocols, the Early Weekly protocol was the most effective. Thus, the most effective treatment regimen was 20  $\mu\text{g}$  of LMWH administered every 7 days, starting 1 week prior to transplantation. Using this regimen, a greater than twofold increase in graft survival was achieved ( $20.8 \pm 5.5$  days,  $P < 0.00001$  vs Control). The regimen that was the least effective was 1  $\mu\text{g}$  administered Daily. Using this regimen, only an 18% increase in graft survival was achieved ( $9.8 \pm 0.7$  days,  $P = 0.02$ , vs Control,  $P < 0.01$ , vs 20  $\mu\text{g}$  administered Early Weekly). Alterations in dosage or frequency of administration of LMWH were associated with a significant change in its potency (Table 2, Figs. 2 and 3).



**FIG. 1.** Prolongation of rat skin allograft survival by treatment of animals with 20  $\mu\text{g}$  of heparin. The most effective treatment is administration of the drug every 7 days starting 1 week prior to transplantation (Early Weekly protocol). Daily administration of the heparin is paradoxically ineffective.



**FIG. 2.** Prolongation of rat cardiac allograft survival by treatment of animals with 1  $\mu\text{g}$  of heparin. The Early Weekly protocol is the only effective treatment.

## DISCUSSION

The major finding in this study is that administration of very small doses of LMWH preparations to recipient animals results in a significant prolongation of skin and cardiac allograft survival. This effect is sensitive to changes of dose and schedule, which can cause a significant drop in drug potency. The immunosuppressive effect of the LMWH is unrelated to the anticoagulant properties. We have used LMWH preparations with low anticoagulant activity at a dose range that is much lower than that required for therapeutic anticoagulation or antithrombosis. Worthy of note was our finding that increasing the frequency of administration from weekly to daily led to a marked reduction in the clinical effect on allograft survival (Tables 1 and 2, Figs. 1–4). This further indicates that the mechanism of the effect of LMWH on allograft survival must be different from its anti-thrombotic effects, which are dependent on frequent administration of the LMWH. Indeed studies of the effects of defined heparin disaccharides on TNF- $\alpha$  production also

showed a loss of activity with too frequent administration [15, 16].

Allograft rejection is a T-cell-mediated immune reaction [19, 20]. Alloantigen-activated T lymphocytes penetrate the target organ, express enzymes, and produce an array of inflammatory cytokines resulting in activation of other immune cells and inflammatory cascades leading to graft destruction [19, 20]. Among the enzymes released by the activated T lymphocytes is heparanase [21]. Heparanase specifically attacks the glycosaminoglycan moiety of the subendothelial extracellular matrix (ECM) that lines blood vessels [22–24]. Expression of the heparanase results in degradation of the ECM, enabling T lymphocytes to penetrate blood vessel walls and reach the target organ [22–24].

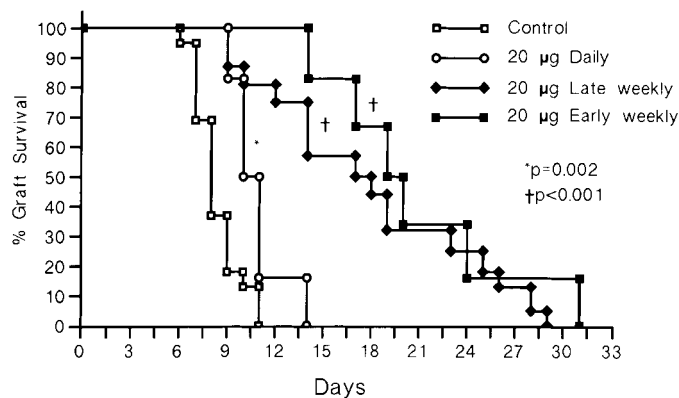
Lider and colleagues have shown that among the degradation products of ECM generated by heparanase is a trisulfated disaccharide that inhibits delayed-type hypersensitivity in mice [15]. This inhibition of T-cell-mediated response *in vivo* was associated with an inhibitory effect of the disaccharide on the biologically active TNF- $\alpha$ . Both the *in vivo* and *in vitro* effects of the disaccharide manifested a bell-shaped dose-response curve [15]. Thus, it appears that the enzyme heparanase has a dual action—it enhances inflammation by allowing penetration of T-cells to the target organ and at the same time it produces molecules with strong anti-TNF- $\alpha$  activity, creating a negative feedback loop [15]. Similar to the effect of mammalian ECM-generated disaccharide molecules, Cahalon and colleagues have recently shown that sulfated disaccharide molecules cleaved from commercial heparin preparations by heparin-degrading enzymes inhibit delayed-type hypersensitivity reaction and adjuvant arthritis when given in nanogram amounts. Both *in vitro* and *in vivo* effects were characterized by a bell-shaped dose-response curve [16].

LMWH preparations are products of chemical or enzymatic digestion of the standard heparin. They are a heterogeneous mixture of sulfated mucopolysaccharides with a molecular weight of approximately 5000 Da.

**TABLE 2**

### BN Cardiac Allograft Survival in Wistar Rats Treated with Low Molecular Weight Heparin

Heparin dose ( $\mu\text{g}$ )	Treatment protocol	<i>n</i>	Individual graft survival (days)	Mean graft survival (days)	<i>P</i> value
	Control	16	6, 7, 7, 7, 7, 8, 8, 8, 8, 8, 9, 9, 9, 10, 11, 11	8.3 $\pm$ 1.4	
20	LW	16	9, 9, 10, 12, 14, 14, 14, 17, 18, 19, 19, 23, 25, 26, 28, 29	17.9 $\pm$ 6.5	<0.0001
20	EW	6	14, 17, 19, 20, 24, 31	20.8 $\pm$ 5.5	<0.0001
20	D	6	9, 10, 10, 11, 11, 14	10.8 $\pm$ 1.6	0.002
1	LW	6	9, 9, 15, 15, 16, 19	13.8 $\pm$ 3.7	<0.0001
1	EW	6	10, 11, 14, 14, 14, 19	13.7 $\pm$ 2.9	<0.0001
1	D	6	9, 9, 10, 10, 10, 11	9.8 $\pm$ 0.7	0.02



**FIG. 3.** Prolongation of rat cardiac allograft survival by treatment of animals with 20  $\mu\text{g}$  of heparin. Similar to skin allografting, the most effective treatment protocol was heparin administration every 7 days, starting 1 week prior to transplantation (Early Weekly protocol).

When compared to standard heparin, LMWH preparations have longer half-life, higher bioavailability, and decreased side effects [25]. We suggest that the immunosuppressive effects of LMWH in the organ transplant model demonstrated in this study and by others [10–12] are related to both the inhibition of the activated T-lymphocyte membrane heparanase, rendering the cells incapable of crossing the subendothelial extracellular matrix and reaching the target organ, and the down-regulation of TNF- $\alpha$  production induced by nanogram amounts of sulfated disaccharide molecules that are present in the mixture of microgram amounts of commercial LMWH preparations. Different LMWH preparations may contain different amounts of anti-TNF- $\alpha$  disaccharide molecules, and therefore have different potency.

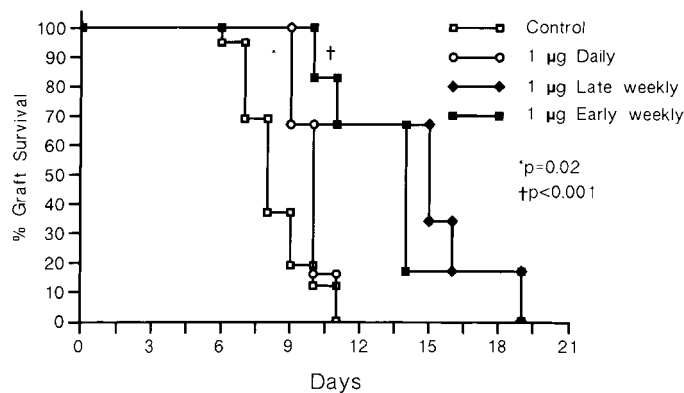
This study evaluated LMWH compounds demonstrated to have anti-TNF- $\alpha$  activity in other models [13–15]. However, it is possible that mechanisms other than TNF- $\alpha$  inhibition account for the effect of heparin on allograft survival in this study. Heparin compounds have been demonstrated to have multiple immunosuppressive effects unrelated to their anticoagulant activity. These include inhibition of natural killer cells, inhibition of T-helper activity as manifested in mixed lymphocytic reaction, inhibition of complement response, decreasing the cytopathic effect of sensitized spleen cells, inhibition of delayed-type hypersensitivity reaction, and inhibition of T-cell migration [1–12].

Similar to the bell-shape dose-response curves observed in delayed-type hypersensitivity reaction and adjuvant arthritis [13, 14], the effect of LMWH on skin and cardiac allograft rejection was highly dose- and schedule-sensitive. Alterations in regimen resulted in marked decrease in the drug potency. A similar dose-response relationship was reported by Lagodzinski and colleagues in a model of skin graft in mice [10]. The explanation for this phenomenon is not entirely clear

at present and must be further explored. Plausible mechanisms include desensitization of the receptors to the sulfated disaccharide molecules and interaction of the disaccharide molecules with different receptors when administered at high doses.

Although this study has focused on the effects of LMWH on acute allograft rejection, it is possible that LMWH compounds may also be important in chronic rejection. The hallmark of chronic heart-transplant rejection is accelerated graft arteriosclerosis [26]. Clauzel and colleagues recently reported that *in vivo* blockade of TNF- $\alpha$  after cardiac transplant inhibits acute coronary neointimal formation [27]. Aziz and colleagues have demonstrated that low molecular heparin administered in the usual therapeutic dosages enhances the immunosuppressive effect of cyclosporine on cardiac allograft rejection in rats [28]. They demonstrated a reduction in accelerated graft coronary disease, which was related to inhibition of smooth muscle cell migration [28]. It is possible that the inhibition of vascular smooth muscle proliferation and accelerated graft atherosclerosis observed by Aziz and colleagues were induced by disaccharide molecules having anti-TNF- $\alpha$  activity that could have been present in the crude mixture of the commercial heparin preparation they used.

In summary, this study demonstrates that treatment of animals with low doses of LMWH preparations devoid of anticoagulant activity prolongs skin and cardiac allograft survival. This effect may be related to inhibition of the T-lymphocyte heparanase (inhibiting T-cell migration) as well as down-regulation of TNF- $\alpha$  production, although other immunosuppressive effects of LMWH cannot be excluded. The immunosuppressive effect of LMWH is sensitive to alterations in dose and schedule of the drug. The mechanism of the latter phenomenon as well as the clinical implications must be further explored.



**FIG. 4.** Prolongation of rat cardiac allograft survival by treatment of animals with 1  $\mu\text{g}$  of heparin. The results with 1- $\mu\text{g}$  dose are clearly inferior to the 20- $\mu\text{g}$  dose.



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## REFERENCES

- Wood, R. M., and Bick, M. W. The effect of heparin on ocular tuberculin reaction. *Arch. Ophthalmol.* **61**: 709, 1959.
- Nelson, D. S. The effects of anticoagulants and other drugs on cellular and cutaneous reactions to antigen in guinea pigs with delayed type hypersensitivity. *Immunology* **9**: 219, 1965.
- Halpern, B., Milliez, P., Lague, G., Fray, A., and Morard, J. C. Protective action of heparin in experimental immune nephritis. *Nature* **205**: 257, 1965.
- Taylor, H. E., and Culling, C. F. A. Cytopathic effect of sensitized spleen cells on fibroblasts. The protective effect of heparin. *Lab. Invest.* **15**: 1960, 1966.
- Currie, G. A. Effect of heparin on mixed lymphocyte culture. *Nature* **215**: 164, 1967.
- Chelmicka-Szorc, E., and Arnason, B. G. Partial suppression of experimental allergic encephalomyelitis with heparin. *Arch. Neurol.* **27**: 153, 1972.
- Sy, M. S., Schneeberger, E., McCluskey, R., Greene, M. I., Rosenberg, R. D., and Benacerraf, B. Inhibition of delayed-type hypersensitivity by heparin depleted of anticoagulant activity. *Cell. Immunol.* **82**: 23, 1983.
- Frieri, M., and Metclafe, D. D. Analysis of the effect of mast cell granules on lymphocyte blastogenesis in the absence and presence of mitogens: Identification of heparin as a granule-associated suppressor factor. *J. Immunol.* **131**: 1942, 1983.
- Yamamoto, H., Fuyama, S., Arai, S., and Sendo, F. Inhibition of mouse natural killer cell cytotoxicity by heparin. *Cell. Immunol.* **96**: 409, 1985.
- Lagodzinski, Z., Gorski, A., and Wasik, M. Immunosuppressive action of low-dose heparin. Effect on skin allograft survival. *Transplantation* **50**: 714, 1990.
- Gorski, A., Gradowska, L. L., Nowaczyk, Wasik, M., and Lagodzinski, Z. New strategies of heparin treatment used to prolong allograft survival. *Transplant. Proc.* **23**: 2251, 1991.
- Gorski, A., Wasik, M., Nowaczyk, M., and Korczak-Kowalska, G. Immunomodulating activity of heparin. *FASEB J.* **5**: 2287, 1991.
- Lider, O., Baharav, E., Mekori, Y. A., Miller, T., Naparstek, Y., Vlodavsky, I., and Cohen, I. R. Suppression of experimental autoimmune diseases and prolongation of allograft survival by treatment of animals with low doses of heparins. *J. Clin. Invest.* **83**: 752, 1989.
- Lider, O., Mekory, Y. A., Miller, T., Bar-Tana, R., Vlodavsky, I., Baharav, E., Cohen, I. R., and Naparstek, Y. Inhibition of T lymphocyte heparanase by heparin prevents cell migration and cell-mediated immunity. *Eur. J. Immunol.* **20**: 493, 1990.
- Lider, O., Cahalon, L., Gilat, D., Hershkovitz, R., Siegel, D., Margalit, R., Shoseyov, O., and Cohen, I. R. A disaccharide that inhibits tumor necrosis factor  $\alpha$  is formed from the extracellular matrix by the enzyme heparanase. *Proc. Natl. Acad. Sci. USA* **92**: 5037, 1995.
- Cahalon, L., Lider, O., Schor, H., Avron, H., Gilat, D., Hershkovitz, R., Margalit, R., Eshel, A., Shoseyov, O., and Cohen, I. R. Heparin disaccharides inhibit tumor necrosis factor- $\alpha$  production by macrophages and arrest immune inflammation in rodents. *Int. Immunol.* **9**: 1517, 1997.
- Baharav, E., Lider, O., Margalit, M., and Cohen, I. R. A modified technique for experimental skin grafting. *J. Immunol. Methods* **90**: 1413, 1986.
- Ono, K., and Lindsey, E. S. Improved technique of heart transplantation in rats. *J. Thorac. Cardiovasc. Surg.* **45**: 225, 1969.
- Kirby, J. A., Reader, J. A., Greta, J. P., Parfett, G. J., and Pepper, J. R. Rat heterotopic heart transplantation: Quantification and analysis of cell mediated cytotoxicity. *Clin. Exp. Immunol.* **71**: 113, 1988.
- Forbes, R. D. C., Lowry, R. P., Darden, A. G., Gomersall, M., and Marghesco, D. M. Morphologic studies of acute rat cardiac allograft rejection across an isolated major histocompatibility complex class I (RT<sup>1</sup>A) disparity. *Transplantation* **45**: 943, 1988.
- Naparstek, Y., Cohen, I. R., Fuks, Z., and Vlodavsky, I. Activated T lymphocytes produce a matrix-degrading heparan sulphate endoglycosidase. *Nature* **310**: 241, 1984.
- Savion, N., Vlodavsky, I., and Fuks, Z. Interaction of T lymphocyte and macrophages with cultured vascular endothelial cells: Attachment, invasion and subsequent degradation of the subendothelial extracellular matrix. *J. Cell. Physiol.* **118**: 169, 1984.
- Matzner, Y., Bar-Ner, M., Yahalom, J., Ishai-Michaeli, R., Fuks, Z., and Vlodavsky, I. Degradation of heparan sulphate in the subendothelial extracellular matrix by a readily released heparanase from human neutrophils. Possible role in invasion through basement membranes. *J. Clin. Invest.* **76**: 1306, 1985.
- Friedman, R., Lider, O., Naparstek, Y., Fuks, Z., Vlodavsky, I., and Cohen, I. R. Soluble antigen induces T lymphocytes to secrete an endoglycosidase that degrades the heparan sulfate moiety of subendothelial extracellular matrix. *J. Cell. Physiol.* **130**: 85, 1987.
- Fareed, J., Kumar, A., Walenga, J. M., Emanuele, R. M., Williamson, K., and Hoppensteadt, D. Antithrombotic actions and pharmacokinetics of heparin fragments. *Nouv. Rev. Fr. Hématol.* **26**: 267, 1984.
- Shi, C., Lee, W. S., He, Q., Zhang, D., Fletcher, D. L., Jr., Newell, J. B., and Haber, E. Immunologic basis of transplant-associated arteriosclerosis. *Proc. Natl. Acad. Sci. USA* **93**: 4051, 1996.
- Clausel, N., Molossi, S., Sett, S., and Rabinovitch, M. In vivo blockade of tumor necrosis factor- $\alpha$  in cholesterol-fed rabbits after cardiac transplant inhibits acute coronary artery neointimal formation. *Circulation* **89**: 2768, 1994.
- Aziz, S., Tada, Y., Gordon, D., McDonald, T. O., Fareed, J., and Verrier, E. D. A reduction in accelerated graft coronary disease and an improvement in cardiac allograft survival using low molecular weight heparin in combination with cyclosporine. *J. Heart Lung Transplant* **12**: 634, 1993.