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Inhibition of Vein Graft Intimal Proliferative Lesions in the Rat by Heparin

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The authors investigated the effect of heparin on the development of myointimal proliferative lesions in a rat vein graft model. Intimal thickening in this model was most pronounced in the anastomotic regions, and was composed principally of vascular smooth muscle cells, as identified by immunocytochemistry with anti-muscle actin antibody, HHF-35. Medial thickening was less cellular, and evenly distributed throughout the grafts. Continuous, intravenous infusion of whole heparin at 0.3 mg/kg/hr effectively inhibited the development of myointimal proliferative lesions, although with no effect on medial thickening. The authors suggest that heparin, through its antiproliferative activity for vascular smooth muscle cells, may have a potentially important pharmacologic role in preventing vein graft failure, which most commonly results from the development of myointimal proliferative lesions. (Am J Pathol 1991, 139:581–587)

Vascular bypass procedures employing vein grafts as conduits are among the most commonly performed surgical procedures. In 1985 alone, over 70,000 peripheral vascular and 150,000 aortocoronary bypass procedures were performed.[1,2] The leading cause of vein graft failure after the first postoperative month is proliferative, occlusive change within the graft itself.[3–6] The nature of these vein graft occlusive changes varies from pure fibrous intimal hyperplasia to true lipid-laden atherosclerosis, but in all descriptions, vascular smooth muscle cells (VSMC) are the predominant cell type in these lesions.[7–9] In some studies, the progression of native arterial atherosclerosis was thought to be the leading cause of graft failure after 2 years, but even then, myointimal proliferative lesions remained important causes of graft failure.[10,11] Both peripheral and aortocoronary vein grafts are subject to significant rates of failure. Although estimates of late graft failures vary with position of the graft as well as from series to series, it is generally accepted that 30% to 60% of grafts fail by 10 years.[12,13] Thus vein graft occlusive lesions are a matter of enormous clinical impact, for which no effective means of prevention has been developed.

This laboratory first noted that heparin effectively abolished the myointimal proliferative response that follows endothelial denudation in the rat carotid artery.[14] Heparin treatment did not affect the number of circulating or deposited platelets, nor the rate of re-endothelialization. We further demonstrated that both nonanticoagulant and anticoagulant fractions of heparin were effective in blocking VSMC proliferation.[15] Finally heparin was found to be strongly antiproliferative for VSMC in culture, thus corroborating the *in vivo* evidence.[16] These observations suggested a pharmacologic role for heparin in vein grafts, where intimal VSMC proliferation is the leading cause of late graft failure.

Kohler et al[17] investigated the heparin effect in a rabbit vein graft model, noting an inhibition of intimal proliferation at 2 weeks, but no significant effect at 4 weeks or beyond.[17] We investigated the effect of heparin on the development of myointimal proliferative lesions in a rat vein graft model, where the epigastric vein is interposed into the divided common femoral artery. In this model, myointimal proliferation is rapid in the first 30 days, and progresses steadily thereafter. Medial thickening occurs as well, although the medial thickening is much less cellular than the intimal proliferative lesions. In this study, we found that heparin therapy effectively inhibits the early development of vein graft myointimal proliferative lesions, although having no effect on medial thickening.

Materials and Methods

Vein Graft

Male Sprague-Dawley rats weighing from 300 to 350 g were anesthetized with an intraperitoneal injection of so-

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dium pentobarbital at 50 mg/kg. The right groin was shaved, prepared aseptically, and opened through a vertical incision. Under microscope magnification (6 to 18×), the common femoral artery (CFA) and epigastric vein (EV) were isolated and the adventitia dissected free from the central portion of the CFA as well as from either end of a 1-cm segment of EV. A constant profunda branch of the CFA was coagulated with diathermy and divided. A double-anastomosis clamp (Weck Instruments, Research Triangle Park, NC) was placed on the CFA and a 3- to 4-mm segment of artery resected. The ends of the arteries were gently distended with a microforceps and the ends flushed with a sterile solution of Hank's Hepes with heparin (100 units/ml) (Elkins Sinn, Cherry Hill, NJ). One centimeter of epigastric vein was then harvested, the graft ends dilated with forceps, and the graft gently flushed. The proximal and distal anastomoses were then fashioned with 10–0 nylon using an open-interrupted technique. Between 8 and 10 sutures were needed for each anastomosis. Once the anastomoses were completed, the distal clamp was released and the graft backperfused with blood for approximately 1 minute. The proximal clamp was then released and any anastomotic leak was treated with gentle pressure. Once adequate hemostasis was achieved, the wound was irrigated and closed with a single layer of running 4–0 nylon suture. Graft ischemic time, from harvest to restitution of flow, was standardized to 50 minutes.

Heparin Delivery

Whole, anticoagulant heparin (Instituit Choay, Paris, France) was administered intravenously at a dose of 0.3 mg/kg-hour using an implantable osmotic pump (Alza Corp., Palo Alto, CA) placed at the time of surgery. These osmotic pumps reliably deliver at a predictable rate over a period of 28 days. Pumps for experimental animals ($n = 9$) were loaded with heparin, whereas controls ($n = 9$) were loaded with sterile carrier alone (lactated Ringer's solution). Pumps were placed in a beaker of lactated Ringer's solution at 37°C for 48 hours to assure that uniform pumping had begun, and that the attached silastic catheters were filled by the pump. On the completion of the vein graft procedure, these pumps were implanted in a subcutaneous pocket over the dorsum of the rat's chest. The silastic tubing was lead from the pump through a subcutaneous tunnel, to the ventral neck, and placed in the right jugular vein using a cut-down technique. The surgeon was blind to the treatment group of any animal during the vein graft procedure and pump placement. Satisfactory drug delivery was confirmed at time of harvest by determination of pump residual volume, intravenous catheter position and patency, as well as satisfactory elevation of clotting time in experimental animals.

Clotting Time Determination

Clotting times were determined in all animals at 2 and 4 weeks. Rats were anesthetized with inhalational ether and tail bled into 0.6 mm inner diameter capillary tubes. The tubes were broken every 30 seconds until a strand of clot was seen to form between the broken ends. The interval between filling of the tube and the first appearance of a clot strand was recorded as the clotting time.

Harvest

At 30 days, animals received Evans Bloc dye, 60 mg/kg and 1 hour later were anesthetized with intraperitoneal sodium pentobarbital, 50 mg/kg. Hematocrits and clotting times were determined on tail blood drawn under ether anesthesia before deep anesthetic induction. The chest was opened and the animal perfused clear with lactated Ringer's solution by a 20-gauge needle in the left ventricular apex at a pressure of 90 cm H_2O. The inferior vena cava was transected in the chest to allow for exsanguination. Once the caval effluent was clear, the animals were perfusion fixed with 200 ml of a modification of Ito-Karnovsky fixative (final concentration: 4% paraformaldehyde, 0.05% glutaraldehyde, 15% saturated picric acid vol/vol).[18] The groin then was opened and the graft dissected out microsurgically. Graft patency was determined by satisfactory filling of the graft and femoral vein with fixative. The graft was divided into segments, (proximal anastomosis, midgraft, distal anastomosis) and further immersion fixation carried out at 4°C for 1 hour.

Histology and Morphometry

After fixation, specimens were extensively washed in 0.1 mol/l (molar) phosphate buffer at 4°C and stored under the same conditions until paraffin embedded. Five-micron sections were taken from the proximal anastomosis, midgraft, and distal anastomosis, and stained with Verhoeff's stain for elastin or hematoxylin and eosin (H & E). Ten semiserial sections, taken from the central portion of each of these segments, were examined, and the section with the most apparent myointimal proliferation was selected for measurement. All selections and measurements were carried out with the observer blind to the treatment group. The characteristic dotted line appearance of the epigastric vein internal elastic lamina as well as the bold internal elastic lamina of the adjacent arterial segments were easily identifiable with this stain. Slides

were projected onto a digitizing pad with a Leitz projecting microscope. The areas bound by the lumen, internal elastic lamina, and outer medial boundary (defined as the region where adventitial blood vessels were seen) then were measured and the intimal areas and medial areas calculated.

Nuclear Counting

Hematoxylin-and-eosin–stained slides were employed to determine mean cell number per unit area in both the media and the intima of control and heparin-treated vein grafts. An optical graticule was used and nuclei were counted per unit area at intervals of 45 degrees around the circumference of the vessel. An average of 6.7×10^5 $\mu 2$ was sampled for the intimal measurements per slide, and 10.7×10^5 $\mu 2$ for the medial measurements. Intimal and medial boundaries were determined by comparison with serial sections stained for elastin. All measurements were made with the observer blind to the treatment group.

Immunocytochemistry

Sections were deparaffinized in xylenes, rehydrated through graded alcohols, and endogenous peroxidase activity quenched with 0.6% hydrogen peroxide/methanol solution for 20 minutes at room temperature. Sections were washed in buffer and incubated with a 1:20 dilution of normal horse serum for 30 minutes at 37°C. The slides were then incubated for 1 hour at 37°C with a 1:5000 dilution of monoclonal antibody (MAb) HHF-35 (Enzo-Biochem, New York, NY), which reacts specifically with muscle actin. The slides were then washed in buffer and incubated with a 1:150 dilution of biotinylated, polyclonal horse anti-mouse IgG. Biotinylated secondary antibody was localized using the Vectastain (avidin-biotin complex) peroxidase method (Vector, Burlingame, CA), employing 3,3′ diaminobenzidine as the chromogen. Methyl green was employed as the counterstain. Specificity and sensitivity were tested with known positive controls (femoral artery), and negative controls in which the primary antibody was left out of the first incubation.

Statistics

All results were analyzed by Student's *t*-test.[19] Probabilities of 5% or less were interpreted as statistically significant. Mean values in the text and figures are plus or minus the standard error.

Results

Morphology

Previous experiments using scanning electron microscopy, monoclonal immunocytochemistry, and routine histologic staining in this model have shown near total endothelial denudation by 12 hours after vein grafting, except for sparing of islands of endothelium in the midgraft.[20] Platelets and inflammatory cells carpet the denuded intimal surface, which demonstrates abnormal permeability to albumin-bound Evans blue dye. These changes are accompanied by a transmural inflammatory cell infiltrate, with monocytes/macrophages predominant by 24 hours. Rapid intimal and medial thickening occur in the grafts in the first 30 days. Vascular smooth muscle cells are the predominant cell type in both intimal and medial thickenings, although the intimal thickenings are quite a bit more cellular than their medial counterparts. Endothelial cell healing, with return to normal macromolecular impermeability, has occurred by 30 days, and the inflammatory infiltrate is greatly diminished by this time as well.

In the current series of experiments, untreated control vein grafts underwent similarly rapid intimal thickening. These lesions were very cellular in nature, often eccentric, and most pronounced in the perianastomotic regions of the grafts. The media of untreated control grafts underwent concentric thickening, which was evenly distributed throughout the length of the graft, and less cellular in nature.

Immunocytochemistry

We carried out immunocytochemistry with MAb HHF-35, which is specific for smooth muscle actin. The intimal proliferative lesions at 30 days showed strongly positive, cell-associated staining, indicating the predominance of vascular smooth muscle cells in these lesions. The medial thickening was similarly HHF-35 positive, although the medial was less cellular, and stained less intensely (Figure 1).

Morphometry

Heparin therapy resulted in a striking inhibition of myointimal proliferation (Figure 2). The intimal areas of heparin-treated vein grafts, as determined by digital planimetry, were markedly reduced compared with untreated controls. The differences were most pronounced at the proximal anastomosis and midgraft (Figure 3). Finally heparin

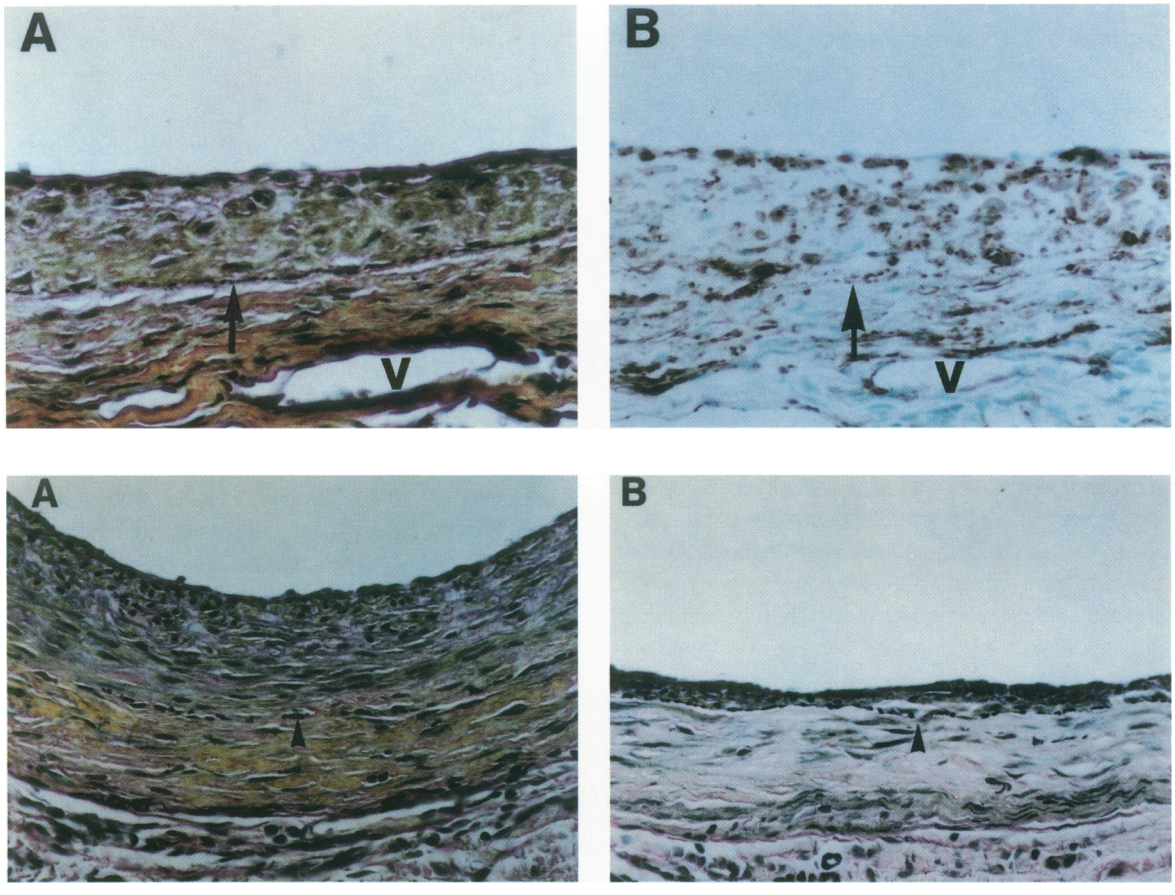

Figure 1. *(Top)* **A:** *Appearance of 30-day vein graft, section taken from mid-graft. Verhoeff elastin, ×160.* **B:** *Serial section taken distal to* **A,** *stained by immunoperoxidase with muscle actin-specific antibody HHF-35. Most intimal and medial cells are vascular smooth muscle cells. Arrow indicates internal elastic lamina. "V" overlies adventitial blood vessel. Methyl green counterstain, ×160.*
Figure 2. *(Bottom) Appearance of 30-day vein graft myointimal proliferative lesion at the distal anastamosis.* **A:** *Control rat; arrow indicates the internal elastic lamina.* **B:** *Rat given continuous intravenous heparin at 0.3 mg/kg/hr; arrow indicates internal elastic lamina. Verhoeff elastin, ×200.*

therapy had no effect on the medial thickening that occurs in these grafts (Figure 4).

Clotting Time

The mean clotting time for control animals (n = 9) was about 3 minutes at 2 weeks and at 4 weeks. In contrast to this, heparin-treated animals (n = 9) had mean clotting times elevated over 2.5 times control values (Figure 5).

Nuclear Counting

Control vein graft intima were markedly more cellular than graft media, with mean cell densities in the intima of 76 ± 4 cells per 1 × 10^5 μ2, and 33 ± 2 cells per 1 × 10^5 μ2 in the media. Heparin therapy had no effect on cell densities in either compartment: intimal cell density 70 ± 7

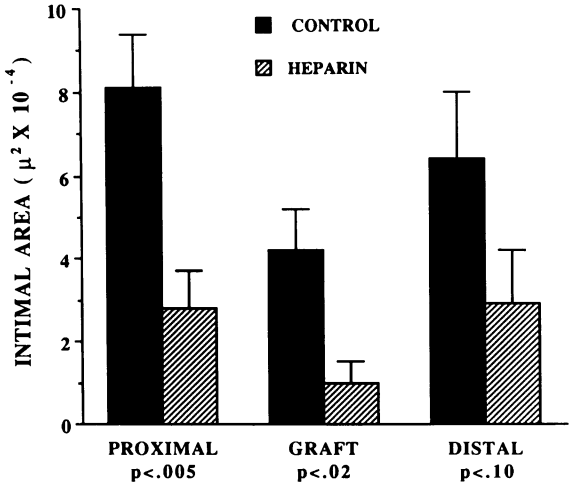

Figure 3. *Intimal areas from control and heparin-treated grafts were determined at 30 days by planimetry on random sections taken from the proximal anastamosis, mid-graft, and distal anastamosis. Values are expressed as mean ± SEM.*

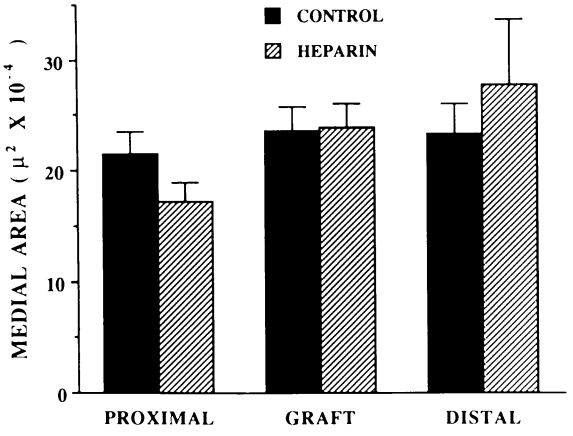

Figure 4. *Medial areas from control and heparin-treated grafts were determined at 30 days by planimetry on random sections taken from the proximal anastamosis, mid-graft, and distal anastamosis. Values are expressed as mean ± SEM.*

cells per 1×10^5 μ2, medial cell density 37 ± 2 cells per 1×10^5 μ2. Finally despite careful examination of all H & E-stained sections, no hyperploid nuclei could be clearly identified.

Discussion

Myointimal proliferative lesions within vein grafts are the leading cause of graft failure after the first postoperative month.[3-6] Failures in the first postoperative month are nearly always due to technical errors.[11] We have shown that heparin therapy effectively inhibited the early development of vein graft myointimal proliferative lesions in this model. We suggest that these results are due to heparin's antiproliferative effect for vascular smooth muscle cells (VSMC). This laboratory demonstrated that heparin almost completely abolishes the myointimal proliferation that follows endothelial cell injury in the rat carotid artery.[14] In these experiments, neither the number of circulating or deposited platelets was affected, nor the rate of re-endothelialization. Further work with nonanticoagulant heparin fractions showed that heparin's antiproliferative effect was independent of its anti-thrombin activity.[15] *In vitro* studies demonstrated that heparin is antiproliferative for VSMC in culture, with relative specificity, although heparin is also effectively antiproliferative for rat cervical epithelial cells and glomerular mesangial cells.[16,21,22] Other glycosaminoglycans do not inhibit VSMC growth.

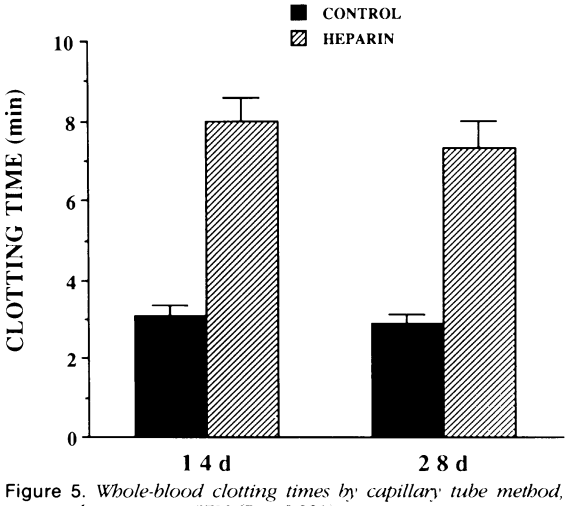

Figure 5. *Whole-blood clotting times by capillary tube method, expressed as mean ± SEM ($P < 0.001$).*

We have no ready explanation for why the distal anastomosis in control grafts had a more variable, and less marked, myointimal proliferative lesion than was seen in the proximal anastomosis in controls. Perhaps as a result of this, there was a less pronounced difference between heparin-treated and control intimal areas at the distal anastomosis. It is interesting to note, however, that heparin therapy resulted in the same degree of myointimal proliferation at both the proximal and distal anastomoses.

It should be noted that Kohler et al found that heparin therapy resulted in a reduction in myointimal proliferation at 2 weeks, but no difference at 4 weeks or beyond.[17] This may be partly due to differences between the vein graft models employed. Kohler performed jugular vein to carotid artery interposition grafts in rabbits. In addition to the species difference, there is more of a size discrepancy between graft and native artery, with the potential for flow disturbance across the anastomosis. In epigastric vein to femoral artery grafts, as we performed, there is nearly a perfect-sized match between the initially smaller epigastric vein, now under arterial pressure as a graft, and the native femoral artery. Furthermore, in our hands, Choay whole heparin is more effective in *in vitro* inhibition of VSMC proliferation, than Sigma type II heparin, as Kohler and co-workers employed. Finally, Kohler et al assessed adequate heparin delivery by elevation of the clotting time at 24 hours, and pump residual volume determination. We believe that persistent clotting time elevation (with determinations at 2 and 4 weeks), coupled with pump residual volumes, is a better criterion for confirmation of intravenous drug delivery, as it rules out the possibility of catheter failure after 24 hours (by clotting or kinking) with subsequent, less effective subcutaneous delivery of drug.

Heparin had no effect on medial thickening, which is in agreement with Kohler et al. We demonstrated that vein graft media are less cellular than their intimal counterparts. Furthermore heparin therapy had no effect on intimal or medial cell density. This indicates the inhibition of thickening by heparin is not due to diminished matrix secretion, but rather to a decrease in the total number of

cells, and that the inhibition is specific for the intima. Clowes and Clowes[24] showed that heparin therapy after arterial injury actually increases intimal area from 14 days to 28 days after injury. This increase was accounted for by increased connective tissue deposition, presumably by the heparin-treated intimal smooth muscle cells.[24] Furthermore Wight et al[25] have recently reported that heparin therapy after arterial injury results in a definite shift in extracellular matrix secretion, from elastin and collagen predominance to proteoglycan predominance. The observation that medial thickening is unaffected by heparin therapy may be of some clinical importance, because treated vein grafts may benefit by the lack of intrusion into the lumen of a myointimal proliferative mass, without being subject to potential aneurysm formation from excessive medial thinning. Finally although vascular smooth muscle cell hypertrophy as evidenced by hyperploid nuclei is the mechanism underlying increased smooth muscle cell mass in the vascular response to hypertension, no hyperploid nuclei were observed by light microscopy in our vein graft intima or media.[26]

Our characterization studies of this vein graft model have identified several factors that may be responsible for the myoproliferative response. In the first 30 days, these grafts undergo several acute changes, including endothelial denudation, platelet adherence and degranulation, abnormal macromolecular permeability, and leukocyte infiltration, with monocytes/macrophages predominant. All of these changes are reversed or greatly diminished by 30 days. In contrast, other more chronic factors of potential importance in vein graft proliferation also have been identified. These include exposure of a vascular conduit to increased wall tension as well as compliance mismatch between artery and vein at the anastomoses.[27,28] Our observations suggest that the development of intimal and medial thickening is maximal in the first 30 days after grafting, when endothelial injury, platelet deposition/degranulation, abnormal permeability, and leukocyte infiltration are maximal. We have shown that heparin therapy during this period effectively inhibited the early myointimal proliferative response. It remains to be demonstrated that a finite course of therapy also given during this period of maximal stimulus for vascular smooth muscle cells to proliferate, will have similar long-term results. Furthermore work in this laboratory has shown that the antiproliferative effect of heparin is independent of its anticoagulant activity. Nonanticoagulant fractions of heparin have been developed that are highly antiproliferative in arterial injury systems, but remain to be tested in the vein graft model. The results of these experiments will help determine the clinical usefulness of heparin or heparin-derivatives for preventing vein graft failure, a matter of enormous potential impact.

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