Studies on the Mechanism of Action of Salicylates III. Effect of Vitamin A on the Wound Healing Retardation Action of Aspirin

By K. H. LEE

Vitamin A and vitamin E reverse the skin-wound healing retardation action of aspirin in rats. Vitamin A alone promotes healing and it also increases acid mucopolysaccharide synthesis in the granulation tissue. The possible mechanisms of action of vitamin A and aspirin on wound healing are discussed.

IN A PREVIOUS PAPER (1), it was shown that aspirin, like cortisone (2, 3), reduces the tensile strength of healing skin wound in rats. Lysosomal enzymes are believed to be involved in inflammation (4). Since aspirin protects lysosomal membrane (5), it was proposed that aspirin retarded healing by its anti-inflammatory action. It is well-known that vitamin A, vitamin E, and a few other fat-soluble compounds can accelerate the release of lysosomal enzymes (6–8). In the present study it was found that both vitamin A and vitamin E reversed the inhibitory action of aspirin on wound healing in rats. It was found that, in the absence of aspirin, vitamin A promoted wound healing.

Mucopolysaccharide formation in the very early phase of wound healing was noticed in many laboratories (9–12). The role of mucopolysaccharides in the formation of connective tissue or collagen has been of interest for many years (12–14). Aspirin and other anti-inflammatory agents inhibited the biosynthesis of mucopolysaccharide and sulfate uptake for chondroitin sulfate synthesis by cartilage slices (15, 16). Vitamin A, on the other hand, increased sulfate incorporation into chrondritin sulfate (17) and increased mucopolysaccharide synthesis (18, 19). In this paper the effect of vitamin A on mucopolysaccharide content of the granulation tissues in rats is reported. The possible mechanisms of action of aspirin and vitamin A on healing are discussed.

EXPERIMENTAL

Wound Procedure—The wound procedure for tensile strength study was essentially the same as described before (1). For mucopolysaccharide study, the wounding technique described by Bentley (12) was followed. Sprague-Dawley rats of either sex, weighing 200–220 Gm., were anesthetized with ethyl ether in an open mask. The hair on the back was depilitated with an electric clipper. One circular piece of skin 5 cm. (2 in.) in diameter, was removed. The wound was left undressed. On the seventh day after wounding, each rat was decapitated and the granulation tissue of the wound was carefully dissected. There were 10 rats in each group. The granulation tissue of all 10 rats of the same group was pooled together.

The body weight of each rat was taken before inflicting the wound and before sacrificing the animal.

Mucopolysaccharide Separation—The mucopolysaccharide separation method described by Bentley (14) was followed with some modification. The granulation tissue was homogenized in a Virtis homogenizer at 45,000 r.p.m. in phosphate buffer pH 8. For every gram of granulation tissue, 5 ml. of buffer was added. Three volumes of boiling acetone was added to the homogenate with stirring. After the precipitate was settled, the supernatant was carefully removed by suction. The acetone extraction was repeated two more times. The fat-free precipitate was separated by suction filtration. The precipitate was washed once with dry ether and then stored *in vacuo* over paraffin wax and phosphorus pentoxide. Three aliquots of the dried fat-free precipitate were digested separately with twice-crystallized papain as described by Antonopoulos *et al.* (20) at 65° for 8 hr. After digestion, the mixture of each sample was centrifuged separately in a Sorvall SS-1 superspeed centrifuge at 10,000× g for 30 min. Aliquot amount of the clear supernatant in each centrifuge tube was dialyzed salt-free in a cellophane tube. Mucopolysaccharide was precipitated from the clear dialysate by the addition of ethanol to a concentration of 80% together with trace amounts of sodium acetate. The precipitate was collected in a centrifuge tube by centrifugation and then redissolved in a small amount of water and reprecipitated by the addition of a small amount of cetylpyridinium chloride (CPC) (21). The CPC-mucopolysaccharide precipitate (CPC-MPS) was redissolved in 60% *n*-propanol and again precipitated by the addition of ethanol and sodium acetate. The precipitate was washed in absolute ethanol, dry ether, and dried *in vacuo* over paraffin wax and phosphorus pentoxide. The dried precipitate was weighed.

Administration of Drugs—Aspirin was administered essentially the same way as described before (1). Crystalline vitamin A acetate (Calif. Corp. Biochem. Research, Los Angeles) was dissolved in cottonseed oil so that each ml. contained 15,000 I.U. One-tenth of a milliliter of this oil containing vitamin A was injected intraperitoneally into each rat on the first, third, and fifth day after inflecting the wound. To the controls, similar amounts of cottonseed oil without vitamin A were given in the same manner. *dl*-Alpha-tocopherol NF (N.B.C., Ohio) was also dissolved in cottonseed oil, so that each milliliter contained 500 I.U. One milliliter of this oil was injected intraperitoneally into each rat on the

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TABLE I—TENSILE STRENGTH OF HEALING WOUNDS

Group	No. of Animals	Aspirin Fed, mg./Kg.	Vit. A[a] —Days after	Vit. E[b] Wounding, i.p.—	Tensile Strength, Gm.	Percent Control
I[c]	21	—	—	—	438 ± 17.7	100
II[c]	13	150	—	—	262 ± 8.7	60
III	9	150	1, 3, 5	—	338 ± 22.7	77
IV	11	150	0, 2, 4, 6	—	325 ± 17.6	74
V		150	—	1, 3, 5	294 ± 14.8	67
VI[c]	12	75	—	—	343 ± 21.0	78
VII	10	75	0, 2, 4, 6	—	447 ± 14.0	100
VIII	18	—	0, 2, 4, 6	—	490 ± 11.0	112

[a] 3,000 I.U./Kg. [b] 1,000 I.U./Kg. [c] Reported before (1).

first, third, and fifth day after wounding. One milliliter of plain cottonseed oil was injected into each of the controls. For the effect of vitamin A on the mucopolysaccharide formation in the granulation tissue studies, the experimental rats received only vitamin A (3,000 I.U./Kg.) intraperitoneally on the first, third, and fifth day after wounding. The controls received cottonseed oil. All of the rats were fed on standard rat feed prepared by Feedstuffs Processing Company, San Francisco, Calif.

Tensile Strength Measurement—The tensile strength of the healing skin wound was measured with the laboratory made centiometer as described earlier (1).

RESULTS

In Table I it is shown that rats receiving an oral dose of 150 mg./Kg. of aspirin daily, starting 1 day before wounding, have an average tensile strength of skin wound of 262 ± 8.7 Gm. on the seventh day which is about 60% of that of the control. When 1,500 I.U./rat (3,000 I.U./Kg.) of vitamin A was given intraperitoneally to rats treated under essentially the same conditions as described above, on the first, third, and fifth day after wounding, the average tensile strength was increased to 338 ± 22.7 Gm., or 77% of the control (Table I, Group III). The increase in tensile strength is significant ($p < 0.001$). When vitamin A was given on the day of wounding and also on the second, fourth, and sixth day after wounding, the average tensile strength was increased to 325.5 ± 17.6 Gm. (Group IV). The improvement was about the same as that of the rats of Group III.

When the dose of aspirin was reduced to 75 mg./Kg. the average tensile strength of the skin wound was 343 ± 21 Gm. or 78% of the control (Group VI). When vitamin A was given to rats receiving 75 mg. of aspirin daily, the average tensile strength was increased to 447 ± 14.0 Gm. (Group VII). Group VII had no significant difference from the control. However, if vitamin A alone was given to rats receiving no aspirin, the average tensile strength was increased to 490 ± 11.0 Gm. or 112% of the control.

When vitamin E (1,000 I.U./Kg.) was given to rats receiving 75 mg. of aspirin daily on the day of wounding and also on the second, fourth, and sixth day after wounding, the average tensile strength on the seventh day after wounding was 294 ± 14.8 Gm. (Group V). Vitamin A significantly reversed the retardation of wound healing action of aspirin.

The results of the studies on mucopolysaccharide formation in granulation tissue of the wound are summarized in Table II. There were 10 rats in each group and at the end of the experiment, in all cases except one, only 8 rats survived. The first 2 days after operation were critical. Rats which survived the first 2 days usually survived to the end of the experimental period. The average amount of CPS-MPS in the granulating tissue of the wound inflicted on the rats receiving vitamin A was 14.10 ± 4.3 mg./rat. The average amount of CPC-MPS of the control was 8.92 ± 1.26 mg./rat. The difference is significant. The Student test was used for evaluating significance in all cases.

TABLE II—EFFECT OF VITAMIN A ON MUCOPOLYSACCHARIDE FORMATION IN GRANULATION TISSUE

Group	No. of Rats	Vit. A, 1,500 I.U./Kg., i.p.	CPC-MPS,[a] mg./Rat	Average
I	8	...	9.25	
II	7	...	9.93	
III	8	...	7.60	
				8.92 ± 1.26
IV	8	+	16.60	
V	8	+	18.74	
VI	8	+	11.67	
VII	8	+	9.42	
				14.10 ± 4.30

[a] Average of triplicate determinations for each group.

DISCUSSION

In a previous study (1), it was proposed that the wound healing retardation action of aspirin is due to its lysosomal membrane protection activity. This suggestion is further supported by the present study. Vitamin A and vitamin E are known to be able to release lysosomal enzymes (22). In the present study, it has been shown that vitamin A, as well as vitamin E, indeed, reversed the wound healing retardation action of aspirin.

Within 6 hr. and increasing for 4 days after wounding, mucopolysaccharide can be demonstrated in the ground substance of the regenerating tissue (23). The direct relation between mucopolysaccharide and collagen formation has been of interest for many years (24). Highberger *et al.* (25), from *in vitro* studies, have shown that a dialyzed acid solution of collagen can be made to precipitate into collagen fiber in the presence of an acid polysaccharide such as chrondroitin sulfuric acid. The collagen fiber formed looked essentially like the natural fiber under electron microscope. The soluble procollagenous substance requires mucopolysaccharide as a template to form insoluble collagen fiber (23). Collagen provides tensile strength to a wound.

Aspirin and a few other anti-inflammatory agents, which inhibited the metabolism of cartilage and other connective tissues *in vitro*, also inhibited the biosynthesis of polysaccharide sulfates by rat

rib cartilage *in vivo* (15). Aspirin (16) also decreased sulfate exchange of chondroitin sulfuric acid.

Wolf and his co-workers (18, 19) have demonstrated the role of vitamin A in the synthesis of mucopolysaccharide in rat colon segments. Recently, it was further shown (26) that a compound which appeared to be an acidic metabolite of vitamin A and vitamin acid was a component of ATP-sulfurylase (ATP: sulfate adenyltransferase, EC2.7.7.4), an enzyme involved in the first step of activation of sulfate. In the present study, it was shown that vitamin A promoted mucopolysaccharide synthesis in the granulation tissue of skin wound in rats. The effect of vitamin A and aspirin on acid mucopolysaccharide synthesis probably is another mechanism of action of these compounds on wound healing.

SUMMARY

The reversal of healing retardation action of aspirin by vitamin A and vitamin E has been demonstrated in rats. In the absence of aspirin, vitamin A promoted healing. It also increased acid mucopolysaccharide synthesis in the granulation tissue.

Inflammation and acid mucopolysaccharide synthesis are two essential features in the early stage of wound healing. Aspirin inhibited both features and vitamin A promoted both features. Two possible mechanisms of action of these two compounds are suggested.

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Tensile strength—healing wound

Separation of Acetylsalicylic Acid and Salicylic Acid by Sephadex Gel Filtration

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Acetylsalicylic acid and salicylic acid can be quantitatively separated by Sephadex gel filtration. A comparative study has been made by using different types of Sephadex gels.

THE AMOUNT of acetylsalicylic acid (ASA) in the biological tissues is generally determined as salicylic acid (SA) after it is hydrolyzed in the presence of the preexisted amount of SA (1). This method has large error when SA present is in great excess of ASA, a situation which often occurs in practice. A few attempts have been made to determine ASA and SA separately. Rowland and Riegelman (2) described a gas-liquid chromatography method for the determination of SA and ASA (as its silyl derivative) in plasma. Earlier, Cotty and Ederma (3) introduced a direct method for the measurement of ASA in human blood. They removed SA by reaction with ceric ammonium nitrate from salicylates mixture. These methods require two extraction procedures to remove salicylates from plasma and also require the preparation of ASA or SA derivatives. Potter and Guy (4) described a simple method for the separation of plasma protein bound SA and free SA by subjecting plasma directly to Sephadex gel filtration. This method, with modifications, can be used for the

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