Mechanism of Action of Retinyl Compounds on Wound Healing II: Effect of Active Retinyl Derivatives on Granuloma Formation

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Abstract □ The naturally occurring retinyl derivatives that promote wound healing as measured by their effects on tensile strength of healing wounds also stimulate granuloma formation induced by cotton pellets. The hexosamine and hydroxyproline contents in the granuloma affected by retinyl derivatives are also increased. The mechanisms of action of retinyl derivation on wound healing are discussed.

Keyphrases □ Retinyl compounds—effect on granuloma formation □ Granuloma formation—retinyl derivatives, effects □ Wound healing—mechanism of action, retinyl derivatives

Previous reports have shown that a few retinyl derivatives promote wound healing, and these compounds also reverse the wound-healing retardation action of anti-inflammatory agents (1–3). It is well known that inflammation and mucopolysaccharide synthesis are the two important features which are essential for subsequent healing.

One commonly used method for studying the anti-inflammatory action of drugs is measuring antigranulation effect with the cotton pellet method of Meier *et al.* (4). This method involves subcutaneous implanting of cotton pellets and measuring the size of the granuloma induced after a few days. Anti-inflammatory agents reduce the size or weight of granuloma as compared with that of the control. In the present study, it is found that those retinyl derivatives that promote healing increase the size or weight of the granuloma. These active retinyl derivatives are "inflammatory agents."

The promotion of mucopolysaccharide synthesis action of vitamin A in granulation tissue of an open wound has been demonstrated earlier (1). In the present study, it is found that both hexosamine and hydroxyproline contents in the granulomas of retinoic acid-treated animals are considerably higher than those of the controls. The mechanisms of action of retinyl derivatives on wound healing are discussed.

EXPERIMENTAL

Materials and Special Chemicals—Materials and chemicals used in this study included the following: crystalline hydroxy-L-proline;[1] retinoic acid,[1] all *trans*, Sigma grade, Type XX; synthetic crystalline, β-carotene,[1] Sigma grade, Type I; crystalline lycopene,[1] Blakeslea trispora origin; prednisone;[2] β-ionone[3] (n^{20}_D 1.584); α-ionone[3] (n^{20}_D 1.5030); glucosamine HCl,[4] reagent grade; 2,4-pentanedione[4] (acetylactone), reagent grade; *p*-dimethylaminobenzaldehyde,[4] reagent grade; dental cotton rolls,[5] size 1; polyvinyl sponge;[6] and retinyl palmitate[7] (400,000 units/g.). β-Ionone, as purchased, was a pure preparation, while α-ionone contained 31% of β-ionone. The isomer was satisfactorily separated by using a preparative gas chromatograph as described previously (3). The *p*-dimethylaminobenzaldehyde was twice recrystallized according to the procedure of Adams and Coleman (5).

METHODS

Implantation of Cotton and Polyvinyl Disks—The effect of retinyl compounds on granuloma formation in rats was evaluated with cotton pellet and polyvinyl sponge methods (4, 6). Cotton pellets are disks sliced from dental cotton rolls with a sharp razor blade. Disks weighing 20 ± 0.5 mg. were selected and placed in Petri dishes for sterilization in a steam autoclave for 40 min. The sterilized cotton disks were handled with sterilized instruments and an aseptic technique. Polyvinyl sponges were also prepared in the form of disks. The block of polyvinyl sponge was sliced with a heavy-duty butcher's cold-meat slicer into 3-mm. thick slices. A cork borer, 11 mm. in diameter, was used to cut the slices into uniform disks. The disks were then thoroughly washed with distilled water to remove the preservative and moistening agents. The washed disks were dried at 65° for 48 hr. Disks weighing 22 ± 0.5 mg. were selected and placed in Petri dishes for sterilization in a steam autoclave for 40 min.

Growth of granulation tissue into cotton pellets was induced by subcutaneous implantation at two symmetrical dorsolateral sites on Sprague-Dawley male rats weighing 120 ± 5 g. under ether anesthesia.

The cotton pellet implanted on the right side contained the retinyl compound, and the cotton pellet implanted on the left side served as the control. The compound was introduced to the pellet as its ether solution. The ether was completely evaporated before implantation. On the 7th day after implantation the animals were killed with ether and the body weights were taken. The granulomas were carefully removed and were weighed rapidly on a torsion balance.[8] After drying in an oven at 65° for 48 hr., the dried slices were weighed again.

In other experiments, two cotton disks without drug were implanted dorsolaterally and retinoic acid sodium salt was given orally or intraperitoneally on the day before the operation and on the 2nd day after the operation. On the 7th day after implantation, the granulomas formed were carefully removed, and their wet weights and dry weights were measured as described previously.

For polyvinyl sponge experiments, large Sprague-Dawley male rats weighing 230 ± 5 g. were used. Four sponge disks were implanted subcutaneously at symmetrical dorsolateral sites. Two disks containing drug and two control disks were implanted diagonally 2 cm. from each other. Prednisone was given orally on the day before the operation and also on the 2nd day after the operation. The dosage was 2.5 mg. per rat per day. On the 7th day after implantation, the granulomas were carefully removed and treated as already described.

Chemical Analysis—Individual dry disks were weighed and placed in a Pyrex 13 × 100-mm. culture tube with 1 ml. of 6 *N* HCl. The tube was sealed *in vacuo*, and the content was hydrolyzed at 140° in a constant-temperature heating block for 3 hr.[9] After cooling to room temperature, the seal of the tube was broken and the content was carefully neutralized with 6 *N* NaOH. The content was then filtered with the aid of suction. The original tube and the

[1] Sigma Chemical Co., St. Louis, Mo.
[2] The Upjohn Co., Kalamazoo, Mich.
[3] Aldrich Chemical Co., Inc., Milwaukee, Wis.
[4] Eastman Kodak Co., Rochester, N. Y.
[5] Johnson and Johnson, New Brunswick, N. J.
[6] Unipoint Laboratories, High Point, N. C.
[7] Eastman Kodak Co., N. J.
[8] Roller-Smith precision balance, Bethlehem, Pa.
[9] Hallikainen Instruments, Richmond, Calif.

Table I—Effect of Retinol and Retinoic Acid on Cotton Pellet-Induced Granuloma

	Group I		Group II	
	Left Side	Right Side (Control)	Left Side	Right Side (Control)
No. of animals	8		12	
Drug impregnated:				
Retinyl palmitate, 6000 I.U.	+	−	−	−
Retinoic acid, 2 mg.	−	−	+	−
Pellet wt., mg.	20.0 ± 0.5	20.0 ± 0.5	20.0 ± 0.5	20.0 ± 0.5
Granuloma wet wt., mg.	300.4 ± 7.2	220.3 ± 4.5	332.2 ± 4.9	218.7 ± 2.5
Left/Right	1.4		1.5	
Granuloma dry wt., mg.	54.3 ± 1.6	36.7 ± 2.0	50.9 ± 1.7	35.1 ± 1.9
Left/Right	1.5		1.5	
Change of body wt., g.	+49.5 ± 2.1		+56.1 ± 3.7	

Table II—Effect of Sodium Retinoate[a] on Granuloma Formation

	Group I	Group II	Group III
No. of animals	18	6	6
Drug given	—	RANa, oral	RANa, i.p.
Cotton pellet wt., mg.	20.0 ± 0.5	20.0 ± 0.5	20.0 ± 0.5
Granuloma wet wt., mg.	211.5 ± 4.8	264.9 ± 7.5	274.6 ± 9.9
Granuloma dry wt., mg.	28.6 ± 1.1	41.1 ± 1.6	42.3 ± 1.7

[a] Four milligrams of Na-retinoate was given to each rat on the day of operation and on the 2nd day after operation.

filter were rinsed several times with small portions of distilled water. The filtrate was brought to 10.0 ml. Aliquots were taken for hydroxyproline and glucosamine analyses. Samples containing 5–15 mg. of hydroxyproline were analyzed according to the method described by Neuman and Logan (7). Aliquots of the filtrate containing 0.010–0.030 mg. of glucosamine were analyzed according to the method of Elson and Morgan as modified by Boas (8).

RESULTS

The effects of retinoic acid and vitamin A on cotton pellet-induced granuloma are shown in Table I. Preliminary tests have been done to determine the proper amount of each compound to be used for appreciable, measurable effects. Either 6000 I.U. of retinyl palmitate or 2 mg. of retinoic acid is required to give about 50% increase in granuloma formation as compared with the control.

Both the wet weight and the dry weight of the granuloma induced by cotton pellets are about the same when either drug is applied. The granulomas of the controls are also similar in weight. The ratios of the granuloma induced by cotton pellets impregnated with either drug to the controls are all about 1.5. These differences are highly significant as determined by the Student t test ($p < 0.001$). These results clearly demonstrate that retinyl derivatives increase the size of granuloma induced by the cotton pellet. These results also indicate that retinyl palmitate and retinoic acid are of the same potency. Retinyl palmitate and retinoic acid also had the same activity when tensile strengths of healing wounds were compared (9). In both cases the rats gained body weight at a normal rate, which indicates that the amount of drug used was not showing any toxic effect.

The effects of the sodium salt of retinoic acid, given orally or intraperitoneally, on cotton pellet-induced granuloma are shown in Table II. Group I involves 18 rats receiving no drug. The average wet weight and dry weight of granuloma are about 212 and 29 mg., respectively. These values are about the same as those of the controls shown in Table I. This fact indicates that the implantation of a cotton pellet impregnated with 2 mg. of retinoic acid or 6000 I.U. of retinyl palmitate did not influence the size of their controls. In Group II, six rats were given sodium retinoate orally. The average wet weight and dry weight are significantly larger than those of Group I animals. In Group III, all six rats received sodium retinoate intraperitoneally; their granulomas are about the same as those of Group II animals but are significantly larger than those of the controls.

The effect of prednisone on granulomas induced by retinoic acid-impregnated polyvinyl sponge is shown in Table III. Group I rats received no oral administration of prednisone. The average wet and dry weights of all of the granulomas induced by polyvinyl sponges impregnated with 2 mg. of retinoic acid were 390.9 and 54.8 mg., respectively. The average wet and dry weights of granuloma induced by polyvinyl sponges were 289.5 and 32.9 mg., respectively. The ratio of wet and dry weights of the granulomas induced by retinoic acid-impregnated and control polyvinyl sponges was about 1.5. Group II rats were treated exactly as those of Group I, except prednisone was given to the rats on the day before the opera-

Table III—Effect of Prednisone on Retinoic Acid-Impregnated Polyvinyl Sponge-Induced Granuloma

	Group I (Control)		Group II (Prednisone)	
	With R. A. A	Control B	With R. A. C	Control D
No. of animals	8		6	
Retinoic acid-impregnated, 2 mg.	+	−	+	−
Sponge wt., mg.	22.0 ± 0.5		22.0 ± 0.5	
Granuloma wet wt., mg.	390.9 ± 16.9	289.4 ± 17.5	340.0 ± 15.4	240.5 ± 15.2
A/B	1.4		1.4	
Granuloma dry wt., mg.	54.8 ± 3.4	32.9 ± 3.0	46.3 ± 4.8	27.8 ± 2.3
C/D	1.6		1.6	
Granuloma wet wt., ratio				
A/C		1.15		
B/D		1.20		
Granuloma dry wt., ratio				
A/C		1.18		
B/D		1.18		

Table IV—Effect of a Few Retinol-Related Compounds on Cotton Pellet-Induced Granuloma

Group	No. of Animals	Drug Applied	Body Wt. Change, Av. g.	Granuloma Wt., mg. Wet: Expt.	Wet: Control	Wet: Expt./Control	Wet: p	Dry: Expt.	Dry: Control	Dry: Expt./Control	Dry: p
I	14	β-Carotene, 2 mg.	+53	339.0 ± 12.1	220.5 ± 9.3	1.5	<0.001	38.3 ± 2.0	26.6 ± 1.7	1.4	<0.001
II	6	Lycopene, 2 mg.	+50	244.8 ± 8.7	239.6 ± 6.4	1.0		23.6 ± 1.9	23.0 ± 1.5	1.0	
III	6	β-Ionone, 25 mg.	+47	305.3 ± 10.6	212.7 ± 7.6	1.4	<0.001	37.6 ± 2.6	24.3 ± 2.1	1.5	<0.001
IV	6	α-Ionone, 25 mg.	+49	252.0 ± 6.6	228.8 ± 3.8	1.1		27.5 ± 2.2	28.1 ± 1.7	1.0	

tion and also on the 2nd day after the operation. The average wet and dry weights of the granulomas induced by polyvinyl sponges impregnated with 2 mg. of retinoic acid were 340.0 and 46.3 mg., respectively. These weights are about 20% less than those of the animals receiving no prednisone (Group I). The wet and dry weights of the granulomas induced by polyvinyl sponges without retinoic acid were 240.5 and 27.8 mg., respectively. These weights are also about 20% less than those of the Group I animals. This experiment demonstrated again that retinoic acid enhanced granuloma formation induced by polyvinyl sponges and its activity was inhibited by prednisone. This also confirms the previous findings with another independent method: that prednisone inhibits and retinoic acid promotes healing (2, 3).

The effects of a few retinol-related, naturally occurring compounds in cotton pellet-induced granuloma are shown in Table IV. β-Carotene (Group I) enhances granuloma formation induced by the cotton pellet. It is almost as active as retinoic acid. Lycopene (Group II) has the same structure as β-carotene, except that it has an open trimethyl cyclohexene ring. It is not active. β-Ionone is active while α-ionone is not (Groups III and IV). The activities of these compounds on granuloma formation are essentially the same as their activities on the tensile strength of healing wounds (3).

The total hexosamine contents in the granulomas induced by cotton pellets in the presence and absence of 2 mg. of retinoic acid were 124.0 ± 3.5 and 94.0 ± 2.3 mcg., respectively. The dry weights of the respective granulomas were 55.4 ± 2.1 and 35.5 ± 0.8 mg. The hydroxyproline contents of the experimental and control granulomas were 259.2 ± 7.3 and 200.0 ± 8.5 mcg., respectively.

DISCUSSION

Inflammation and mucopolysaccharide synthesis are the two known important features in wound healing. Anti-inflammatory agents retard healing by their anti-inflammatory and inhibitory actions on mucopolysaccharide synthesis (2, 10–12). Retinol and a few retinyl derivatives can reverse the wound-healing retardation action of a number of anti-inflammatory agents (3, 10). This fact illustrates an important pharmacological principle of using an agent (retinol) to modify the untoward effect of a useful drug (anti-inflammatory agent). Retinol, or either one of the active retinyl derivatives alone, promotes healing (2, 3).

Meier *et al.* (4) introduced a method that makes quantitative studies of the action of cortisone on connective tissue possible. These workers used the cotton pellet as a foreign body to induce granuloma formation. Application of cortisone resulted in a diminution of granuloma size, which can be expressed quantitatively by determining its fresh and dry weights. Cortisone was effective by local as well as by general application, similar concentrations producing the same degree of inhibition of granuloma formed. Since then, this method has been extensively used for assaying steroid and nonsteroid anti-inflammatory agents. Retinol and retinyl derivatives that promote healing cause an increased granuloma mass induced by cotton pellets.

It is interesting to point out that this is the first demonstration of an agent given orally, intraperitoneally, or locally enhancing granuloma formation. This phenomenon definitely shows again the effect of retinol or its active retinyl derivatives and anti-inflammatory agents. It seems justified to call these retinyl compounds "inflammatory agents."

Grindlay and Waugh (6) used essentially the same implantation method to study tissue regeneration, except that they used polyvinyl sponges. The protein, lipid, and mucopolysaccharide contents of the granuloma have been studied.

Jackson *et al.* (13) pointed out that repair of connective tissue is the most basic feature in wound healing, and they used the formation of granuloma induced by polyvinyl sponges to study healing. Sandberg and Zederfeldt (14) found that the rate of gain in tensile strength and hydroxyproline in granuloma was directly related in both rats and rabbits. The results of the present experiment show that only those retinyl derivatives that are active in increasing tensile strength also increase the size and weight of granuloma induced by either the cotton pellet or polyvinyl sponge. Granuloma formation is a useful quantitative method to study wound healing.

The promotion of mucopolysaccharide synthesis action of retinol in granuloma tissue of an open wound has been reported (1). In the present study, both total hexosamine and hydroxyproline contents were increased in the granuloma induced by the cotton pellet impregnated with retinoic acid.

The results obtained in this study suggest that retinol and active retinyl derivatives promote healing by inducing inflammation and increase mucopolysaccharide synthesis mechanisms of action.

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