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EFFECTS OF SEROTONIN, INDOMETHACIN AND OTHER ANTIRHEUMATIC DRUGS ON THE SYNTHESIS OF COLLAGEN AND OTHER PROTEINS IN GRANULATION TISSUE SLICES

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Abstract—Slices of sponge-induced granulation tissue, both at proliferating and mature (collagen-synthetizing) phases, were incubated in the presence of labelled proline. The addition of serotonin in the medium stimulated the synthesis of collagen, especially at the concentration of 10^{5}–10^{6} M. Indomethacin inhibited the synthesis of all proteins at high concentrations ($\lesssim 10^{-4}$ M) but stimulated at lower concentrations ($\leqq 10^{5}$ M). Phenylbutazon (but not myocrisin and certain other antirheumatic drugs) had similar effect as indomethacin. Bradykinin, histamine and vasopressin also stimulated the synthesis of proteins, including collagen.

THIS WORK was prompted by a desire to find out whether various substances, known to be effective as antirheumatic agents, would affect *in vitro* the synthesis of various proteins in the experimental granulation tissue,[1] which has been studied in this laboratory from the general metabolic point of view and suggested as an useful model for a solid fibroblastic tissue. Boucek and Alvarez[2,3] found that serotonin, related structurally to the most potent antiphlogistic drug indomethacin, has a cytospecific action on the fibroblasts; the lag phase is shortened, attachment of the cells on glass increased, their number increased and their survival improved. Through serotonin our interest touched such agents as histamine, known to be active in inflammatory processes, especially because strips of granulation tissue can be stimulated to contract by similar compounds.[4] Finally, our preliminary work[5] suggested that the synthesis of collagen in granulation tissue slices is sensitive to a pretreatment with neuraminidase, and reports in the literature indicate that the receptors of serotonin contain neuraminic acid.[6,7]

EXPERIMENTAL

Slices of granulation tissue. The experimental granulation tissue was induced subcutaneously in adult rats of Wistar strain with viscose-cellulose sponge as described in detail by Viljanto and Kulonen.[8] The granulomas were harvested after 6–25 days and sliced with Stadie–Riggs microtome. The conditions of treatment of the slices before the incubation have been discussed previously.[1]

Incubations. The medium used was Krebs–Ringer phosphate, pH 7·4, and the gas phase air. Glucose (22·4 mM) and carrier proline (2·87 mM) were added. The amount of collagen synthesis in granuloma slices depends on the extracellular concentration

of proline, if the latter is below about 1·0 mM.[9] The standard sample, about 300–400 mg of pooled granuloma slices in 3–5 ml of medium, was shaken in Erlenmeyer flasks at 37° in the shaking incubator (A. Gallenkamp & Co., Ltd., London E.C.2, England). After 30-min preincubation, 20–100 μc of ^{3}H-proline (TRA 82, Radiochemical Centre, Amersham, Bucks., England) was added and the incubation continued for 3 hr. The incubation was terminated by the immersion of the flasks into crushed ice.

Analyses of the slices. The slices were homogenized with the Ultra-Turrax® homogenizer (Janke & Kunkel KG, IKA Werk, Staufen i. Breisgau, Germany) in 80% ethanol, washed with ethanol until free proline was removed, and finally with diethyl ether. The homogenate was dried at 37° overnight, then weighed and rehomogenized in water and the mixture gelatinized at 130° in the autoclave for 2 hr. The collagenous solution was separated from the non-gelatinizable residue by filtration. Both fractions were hydrolysed at 130° for 3 hr and the radioactivities in hydroxyproline and proline fractions assessed according to Juva and Prockop.[10] The total radioactivity of the hydrolysates was determined by dissolving 200 μl aliquots into a solvent system consisting of 6 ml of ethylene glycol monomethyl ether (Methyl oxitol, Shell) and 10 ml of scintillation liquid containing 15 g of 2,5-diphenyl oxazole and 50 mg of 4-methyl-5-phenyloxazolyl-benzene (Packard Instrument Co.) in 1000 ml of distilled toluene. In the measurement of radioactive hydroxyproline, the solvent system did not contain methyl oxitol. The radioactivities were assayed with Packard Tri-Carb® liquid scintillation spectrometer Model 3214 (Packard Instrument Co., Inc., Downers Growe, Ill., U.S.A.). The assay method has been discussed thoroughly by Juva.[11]

The synthesis of collagen depends markedly on the developmental phase of the granuloma[1] and the incorporated activities vary between the experiments. Therefore the evaluation of the results was carried out on the per cent basis, especially because the activity of the added precursor was changed during the course of the work. Great care is necessary in pooling the slices to balance the eventual differences between the rats and the implantation sites.

When 100 μc of ^{3}H-proline had been added, the activity incorporated in collagen-hydroxyproline was (without corrections for destruction and counting efficiency) 19,100 $\pm$ 2200 (S.E.M., $n = 5$) cpm/100 mg dry tissue from 18–25 day granuloma and the activity in non-gelatinizable protein 264,000 $\pm$ 16,000 cpm/100 mg. All the experiments were carried out in duplicate. The mean difference within duplicate pairs was 15·0 $\pm$ 2·7 per cent (S.E.M., $n = 12$).

Experimental additions to the medium. Indomethacin (5-methoxy-2-methyl-1-(4-chlorbenzoyl)-indolyl-(3)-acetic acid was obtained from Messrs. Dumex A/S (Copenhagen, Denmark), serotonin from F. Hoffmann-La Roche & Co., Ltd. (Basle, Switzerland), myocrisin (Na-aurothiomalate) from Messrs. Pharma Rhodia (3460 Birkerød, Denmark), aminophenazon and acetosalicylic acid (Pharmacopoea Nordica) from Farbenfabriken Bayer AG (Leverkusen, Germany), chloroquine and hydroxychloroquine phosphates from Orion Oy (Helsinki, Finland), colchicine and histamine hydrochloride from E. Merck AG (Darmstadt, Germany), actinomycin D from Merck (West Point, Pa., U.S.A.), puromycin dihydrochloride from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.), bradykinin triacetate, L-epinephrine, oxytocin and (lysine)-vasopressin from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.) and phenylbutazon from Lääke Oy (Turku, Finland).

TABLE 1. EFFECTS OF SEROTONIN AND INDOMETHACIN ON THE INCORPORATION OF PROLINE INTO PROTEINS OF GRANULATION TISSUE SLICES

		Serotonin				Indomethacin			
		Control slices		+Indomethacin (10^{-4} M) added		Control slices		+Serotonin (10^{-4} M) added	
Age of granuloma	Concn. M	HYPRO	NC	HYPRO	NC	HYPRO	NC	HYPRO	NC
18–25 days	10^{-3}	114(2)	107(2)	85	112	10(2)	10(2)	14	13
	10^{-4}	138(2)	137(2)	93	88	47(2)	51(2)	69	52
	10^{-5}	145(3)	109(3)	91	109	111(2)	108(2)	89	47
	10^{-6}	155(2)	101(2)	101	112	121(2)	124(2)	80	68
	10^{-7}	147(2)	130(2)	91	115	198	90	78	97
	None	100	100	100	100	100	100	100	100
6–10 days	10^{-3}	56	60			10	18		
	10^{-4}	92	78			87	76		
	10^{-5}	135	100			134(2)	121(2)		
	10^{-6}	118(2)	89(2)			101	73		
	None	100	100			100	100		

The values are expressed in percentage of the respective control values. The number of experiments (if more than one), each in duplicate, are given in the parentheses. HYPRO bound hydroxyproline, NC non-gelatinizable residue, both evaluated originally in cpm/dry weight.

RESULTS

Table 1 shows that the addition of serotonin to the medium, especially in the concentrations of 10^{-5}–10^{6} M, stimulates the incorporation of proline to collagen-hydroxyproline, and indeed more than to non-collagenous proteins. When all the concentrations of serotonin are considered, the average incorporation of proline to collagen-hydroxyproline of the 18–25 day granulomas was 140·5 ± 11·2 per cent (S.E.M., $n = 11$) and to non-gelatinizable protein 116·1 ± 6·1 per cent, when compared to the controls. The effect of serotonin was statistically significant: in 18–25 day granulomas $t = 3{\cdot}62$ ($n = 10$), $P < 0{\cdot}005$; at the concentrations of 10^{-5}–10^{-7} M in all the granulomas $t = 4{\cdot}28$ ($n = 9$), $P < 0{\cdot}005$. In a young granuloma the higher concentrations seem to be toxic. No such effect of serotonin is observed in the presence of 10^{-4} M indomethacin.

Indomethacin itself inhibits the incorporation at higher concentrations, as expected. However, at low concentrations it seems to stimulate the synthesis of proteins, thus resembling serotonin. When the results at the concentrations of 10^{-5}–10^{-7} M indomethacin on all the granulomas are combined, the incorporation of proline to collagen-hydroxyproline is 129·1 ± 14·0 per cent compared to the respective controls. This finding also is statistically significant ($t = 2{\cdot}08$, $n = 7$, $P < 0{\cdot}05$). In the presence of 10^{-4} M serotonin the effects of indomethacin are smaller.

It is pertinent to look into the effects of biologically active amines and peptides (Table 2). Bradykinin, vasopressin and histamine exert a stimulating influence on the collagen synthesis in adult granulation tissue although not in a growing one.

TABLE 2. EFFECTS OF CERTAIN STIMULI ON THE INCORPORATION OF PROLINE INTO PROTEINS OF GRANULATION TISSUE SLICES

Age of granuloma	Substances, concn. (M)	Hydroxyproline		Non-collagenous protein cpm/dry wt
		Per 100 mg tissue, dry wt	cpm/ μmole	
14–19 days	Bradykinin(2) 10^{-5}	145(130–160)	142(130–154)	131(122–140)
	Vasopressin 10^{-5}	140	139	137
	Oxytocin 10^{-5}	87	70	76
	Histamine(3) 10^{-4}	133(103–154)	132(94–158)	109(87–130)
	Adrenalin 10^{-4}	110	118	131
6–7 days	Bradykinin 10^{-5}	99	44	110
	Histamine(3) 10^{-4}	99(69–143)	74(61–95)	90(81–98)
	Adrenalin 10^{-4}	107	90	79

The means and ranges are expressed in percentage of the respective control values. The number of experiments (if more than one), each in duplicate, are given in the parentheses.

To find out whether these effects on the protein synthesis were a general property of the antirheumatic drugs, certain additional compounds were tested (Table 3). Phenylbutazon seems to resemble indomethacin but myocrisin is not active. At the concentration of 10^{-4} M the effects of other antiphogistic drugs were not clear-cut. Actinomycin D, puromycin and colchicin all depressed the synthesis of proteins in this experimental system, as expected.

TABLE 3. EFFECT OF CERTAIN ANTIRHEUMATIC DRUGS ON THE INCORPORATION OF PROLINE INTO PROTEINS IN GRANULATION TISSUE SLICES

Addition	Concn. M	Hydroxyproline	Non-gelatinizable protein
Phenylbutazon	10^{-3}	14	13
	10^{-4}	28	40
	10^{-5}	85	109
	10^{-6}	109	127
Myocrisin	10^{-3}	100	105
	10^{-4}	126	104
	10^{-5}	101	86
	10^{-6}	92	134
Hydroxychloroquine phosphate	10^{-4}	72	84
Chloroquine phosphate	10^{-4}	86	85
Acetosalicylic acid	10^{-4}	83	100
Aminophenazon	10^{-4}	109	109
Colchicine	10^{-3}	54	59
Puromycin	10^{-4}	39	18
Actinomycin D	50 μg/ml	79	41

The age of the granulomas was 18–25 days after the implantation. The values are expressed in percentage of the respective control values and are averages of duplicate experiments.

DISCUSSION

The present results are pertinent to the findings of Boucek and Alvarez[2,3] but the influence of serotonin on collagen synthesis seems to be even more pronounced than on cellular proteins in general. These effects of serotonin prompt associations to the significance of nervous tissues in the regeneration,[12] to the stimulation of fibroblastic repair at the inflammation[13] and to the general mechanism of cellular stimulation. It is of great interest that the contraction of mature granulation tissue can be stimulated by the same substances which are known to induce a contraction of smooth muscle: e.g., serotonin, vasopressin, bradykinin.[4] Both the smooth muscle cells and fibroblasts respond to similar stimuli, perhaps by similar receptors, either by contraction or by synthetic functions.

There seem to be sites in the cell wall of the fibroblasts, which are susceptible to ouabain, to extracellular electrolyte composition, to neuraminidase or to serotonin[5,14] and which influence the synthesis of collagen. Cyclic AMP stimulated the amino acid transport and protein synthesis in embryonic bone cells by 20–50 per cent[15] and serotonin as well as ouabain are reported to increase the amount of cyclic AMP.[16]

Skidmore and Whitehouse[17] suggested that one of the actions of acidic anti-inflammatory drugs (cf. Ref. 18) was to reduce the synthesis of serotonin. However, the present consensus in the literature is against that hypothesis.[19] In small concentrations both indomethacin and serotonin are similarly stimulating the synthesis of collagen but at high concentrations indomethacin is much more suppressive. Both the hydroxyl and amine functions seem necessary for the action of serotonin on the fibroblasts.[2] The requirements for indomethacin as an antirheumatic agent include 5-methoxy and 3-acetic acid substitutions in the indole nucleus.[20] Indoles in general

seem to affect the formation of connective tissue[21] but the effect is reported to depend on the solubility to lipids.

Both the synthesis and breakdown of collagen may be affected by indomethacin but at different affinities so that there is an accumulation of collagen at low concentrations but a block in its formation at higher concentrations. The therapeutic concentration of indomethacin in the body fluids[22,23] is reported to be about 10^{-5}–10^{-6} M. Small amounts of indomethacin, administered locally, actually increase the tensile strength of a healing wound.[24] It is conceivable that an increase of collagen in the intercellular space would suppress the activities of the inflammatory cells and thus alleviate the rheumatoid symptoms.

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