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A PURIFIED STRUCTURAL GLYCOPROTEIN DETERMINES A STRONG INHIBITION OF PROTEIN SYNTHESIS IN FIBROBLAST CULTURES

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Summary : An homogeneous fraction of structural glycoproteins (SGP) purified from the connective matrix of rabbit skin, exerts a strong inhibitory influence on the protein syntheses that occur in cultured fibroblasts both from rabbit and human skin. The effect is dose dependent from 0.7 to 8.4 x 10^{-6} M. The inhibition of collagen synthesis parallels that of the bulk of proteins.

INTRODUCTION

Structural glycoproteins (SGP) are a group of very slightly soluble proteins of the connective matrix (1) that become extractable only after collagenase digestion of the tissue. Their structure is very similar in most of the species (2, 3, 4) and their biological functions are still unknown. In this paper, we demonstrate that a SGP fraction, purified from rabbit skin, has a drastic inhibitory influence on the protein syntheses that occur in cultured fibroblasts both from rabbit and human skin.

MATERIAL AND METHODS

Chemicals : Most of the chemicals were purchased from Prolabo (Paris). L-ascorbic acid and urea were obtained from Merck and β-aminopropionitrile from Sigma. Bacterial collagenase was purchased from Worthington (CLSPA grade) and purified in the laboratory according to Peterkofsky (5). Uniformly labelled (^{14}C)-L-proline (sp. act. 250 mCi/m mole) (code CB 18) was obtained from the Centre de l'Energie Atomique (CEA, Saclay, France). (^{3}H)-inulin (sp. act. 300 mCi/m mole) (code TRA 326) was purchased from the Radiochemical Center (Amersham, GB).

Cell cultures : The reagents for fibroblast cultures were purchased from Flow. The cells were derived in the laboratory from explants of adult rabbit dermis or from human newborn foreskin (obtained on parental consent), depending on the case (6). They were routinely grown in Eagle's minimal essential medium (MEM) supplemented with 10 % fetal calf serum (FCS). The doubling time was approximately 2 days in the case of rabbit fibroblast cultures and 3 days as regards human fibroblast cultures. They were routinely checked for the absence of contamination, mainly in which concerns mycoplasma. Any contaminated strain was discarded. When necessary, the cells were stored either frozen at - 140°C or in a resting state under MEM containing only 1 % FCS.

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In the case of incubation experiments, we used MEM supplemented with essential aminoacids (MEM solution) and vitamins (MEM solution) in order that their concentration was twice that in MEM alone. This medium will be referred to as "incubation medium" in what follows. All the experiments described in the following sections were conducted both on human and rabbit fibroblasts. The viability of the cells was checked by the trypan blue test(7).

Structural glycoproteins (SGP) : The preparation of a definite fraction of SGP from rabbit skin was described in a previous paper (4). Briefly, insoluble collagen is digested by purified bacterial collagenase, the remaining insoluble fraction is extracted by a buffered 8 M urea solution. This solution is reduced, alkylated and fractionated on a Sepharose 4 B column. The main peak contains a homogeneous protein (MW 16 000 d) giving a single band in agarose and acrylamide gel electrophoreses and having a constant composition in the preparations obtained from 8 different batches of animals.

Prior to the incubation experiments, a convenient amount of lyophilized SGP was weighed, dissolved in 0.1 ml of 8.0 M urea solution, diluted to a satisfactory concentration with the incubation medium and sterilized by filtration through a Millipore filter with a pore size of 0.22 μm. The final concentration of urea did never exceed 0.032 M. In every experiment, control cultures containing the same amount of urea were processed in parallel.

Incorporation of (^{14}C)-proline into proteins and collagen: The cells obtained by trypsinization were seeded in equal amounts (10^5 cells) into 24 plastic Petri dishes of 5 cm diameter and maintained in MEM containing 10 % FCS up until they are confluent. The medium was replaced for 24 h by fresh MEM containing only 0.5 % FCS. The incubation was initiated by adding to every dish 5 ml of incubation medium without FCS, 0. 28 mM L-ascorbic acid, 0.7 mM β-aminopropionitrile, 0.35 mM unlabelled proline and 0.2 μCi of (^{14}C)-L-pro.

The first 4 dishes served as controls and the following ones received amounts of SGP ranging from 0.7 to 8.4 x 10^{-6} M. Every concentration of SGP was tested in quadruplicate. The incubation period lasted 4, 15 or 24 h, depending on the type of experiment. The medium was harvested, dialysed against repeated changes of distilled water and an aliquot of the non-dialysable fraction was counted for total protein radioactivity. The proteins contained in 4 ml of the remaining solution were precipitated by addition of ammonium sulfate to the concentration 1.33 M. The precipitate was processed for the evaluation of the collagen radioactivity according to the method described by Peterkofsky (5) and the radioactivity of hydroxyproline liberated from this collagen measured according to Juva and Prockop (8).

The remaining cell layer was incubated for 10 min in a solution of 0.06 % trypsin + 0.05 % EDTA in Ham's saline and scraped off with a rubber policeman. The cell suspension was centrifuged at 2 000 g for 15 min and the DNA contained in the cell pellet extracted according to Tedesco (9). The amount of DNA was measured by the colorimetric technique of Burton (10) with a solution of calf thymus DNA (Sigma, D 1501) as a standard.

Intracellular proline pool : Additional experiments have been performed in the same conditions except that 0.2 μCi/ml of (^{3}H)-inulin was added to the medium together with 0.2 μCi/ml of (^{14}C)-L-pro. At the end of the incubation period (varying from 30 min. to 24 h according to the purpose of the experiment), the medium was removed, the cells destroyed by adding 5 ml of 10 % TCA to every dish and the non-protein radioactivity extracted in this solution by incubating the dishes for 1 h at + 4°C with gentle stirring from time to time. The TCA solution was centrifuged at 30 000 g for 30 min at + 4°C, the total radioactivities of ^{14}C and ^{3}H were counted. The ^{3}H radioactivity served as a measure

of the level of contamination of the cell pellet by the extracellular medium sticking to the cells. Four ml of supernatant were fixed on a Dowex 50 x 8 column (120 x 6 mm) equilibrated in 0.1 M HCl solution and the amino acids were eluted together by 20 ml of 2 M HCl solution. After concentrating to dryness, the residue was submitted to an ion exchange chromatography in a Beckman amino acid analyser model Multichrom B 4255 equipped with a M 72 resin (11) connected to a fraction collector, for the measure of both concentration and radioactivity of every aminoacid.

Enzymatic digestions : In order to demonstrate that the biological activity of SGP depends on its structural integrity, the preparation was subjected to heat denaturation in sealed tubes for prolonged periods and to digestions with pepsin (EC 3.4.23.1, Worthington) (enzyme/substrate ratio 1/25, W/W, for 40 hrs) and with a neutral protease extracted from B. Polymyxa (Dispase, E. C. 3.4.24.4, Boehringer) (enzyme/substrate ratio 1/25, W/W, for 72 hrs), under sterile conditions.

RESULTS

Fig. 1 shows the effect of increasing concentrations of SGP on the incorporation of ^{14}C-pro into the proteins secreted in the medium by human fibroblasts. The amount incorporated in the presence of 8.4×10^{-6} M SGP is only 10 % that obtained in the non-inhibited cells. The effect is dose-dependent.

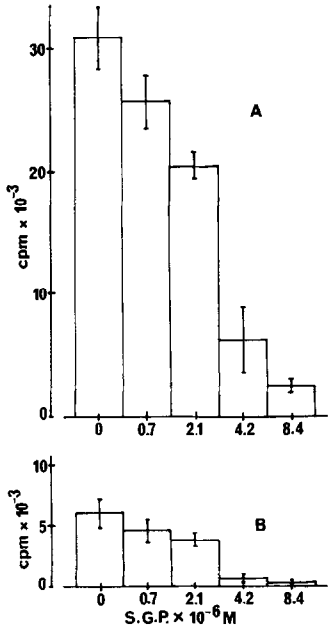

Fig. 1: Effect of increasing amounts of SGP on the incorporation of ^{14}C pro into total proteins (diagram A) and collagen (diagram B) secreted in the medium by confluent human fibroblasts. For details see text. Every bar is the mean $\pm$ 1 SD of the determinations made in 4 Petri dishes in the presence of SGP from the same batch. 6 different batches of SGP were essayed with the same result. No serum was added to the Petri dishes. Rabbit fibroblasts give the same results. The effect is significant with $p < 0.05$, even for a concentration of 0.7×10^{-6} M.

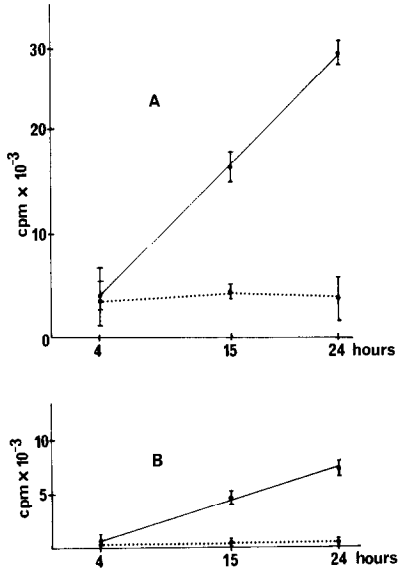

Fig 2: Kinetics of the incorporation of (^{14}C)-pro into proteins (diagram A) and collagen (diagram B) secreted in the medium by confluent human fibroblasts. Every data is the mean ± 1 SD of determinations obtained from 4 dishes. No serum was added to the Petri dishes. Full line:controls ; dotted line : SGP 4.2 x 10^{-6} M.

Fig. 2 shows that the incorporation of ^{14}C-pro into total proteins and collagen is linear with time and that a significant decrease in the incorporation in both total proteins and collagen occurs when SGP are added at the concentration 4.2 x 10^{-6} M. A similar effect is observed when the rabbit SGP are added to rabbit fibroblast cultures.

We checked whether a variation in the free proline pool in the cells could be responsible for the inhibition of incorporation of pro into the proteins. Table I demonstrates that there is no significant variation of this pool.

When the SGP are boiled 4 hrs in a sealed tube, they do not exert any more inhibiting effect. After pepsin digestion, the inhibiting influence of SGP was only slightly decreased. When SGP was digested by Dispase, no inhibiting activity remained in the treated sample.

DISCUSSION

These experiments demonstrate an inhibiting effect of the SGP extracted from rabbit skin on the protein syntheses occuring in human and rabbit fibroblastic strains. The species to species structural and antigenic similarities demonstrated for SGP (12, 13, 14) probably explain the absence of species specificity for this effect.

TABLE I

The intracellular pool of proline measured in human fibroblasts. Proline is isolated by ion exchange chromatography (see text). Every data is the mean ± 1 SD of the determinations made on 4 separate dishes. There are no significant differences between the controls and the samples incubated in the presence of SGP.

		incubation period		
		30 min	4 H	24 H
intracellular proline concentration (n mole/dish)	control	25.9 ± 2.5	33.0 ± 7.1	36.4 ± 9.1
	2.1 x 10^{-6} M SGP	29.7 ± 6.8	37.8 ± 6.5	40.5 ± 2.6
intracellular proline radioactivity (cpm/dish)	control	9360 ± 770	13080 ± 2290	15400 ± 3190
	2.1 x 10^{-6} M SGP	10036 ± 1980	13020 ± 2355	14145 ± 2700
specific activity of intracellular proline (cpm/n mole)	control	363 ± 34	399 ± 40	425 ± 50
	2.1 x 10^{-6} M SGP	339 ± 24	344 ± 11	349 ± 41

The active concentrations of SGP cover a range that seems to correspond with the physiological concentrations obtained from tissue extractions despite the difficulty of evaluating the SGP concentrations, for which specific methods are still lacking. It was verified by the trypan blue method that the cells exposed up to 2 x 10^{-6} M remain all alive. Beyond this concentration some cells have a tendancy to leave the wall. When the cells have been incubated for 24 h with 0.7 x 10^{-6} M SGP, if the medium is replaced by new medium containing 10 % FCS, the protein and collagen syntheses return to their normal level, demonstrating that the effect of SGP is fully reversible.

The SGP is exceptionally resistant to denaturation and protease action. It takes 4 hrs at 110°C in sealed tube for it to be denatured. Only dispase, a neutral protease of strong and unspecific activity, was found capable of suppressing its inhibiting activity. This demonstrates that this activity is actually depending on the integrity of a short sequence of the protein that is respected by pepsin digestion and only destroyed by dispase.

A recent paper by Aalto (15) described also an inhibiting effect of structural glycoproteins extracted from connective tissue. Despite interesting similitudes in action with our SGP fractions, differences are evident : Aalto's SGP are of larger molecular weight than ours and are extracted from inflammed tissues, not from normal ones.

Up to now, there was no physiological role of the SGP clearly established. Our experiments demonstrate that they may exert a regulatory role on the fibroblast metabolism. An indirect confirmation comes from the fact that tissues rich in SGP are less rich in collagenous fibers, and vice versa (16, 17).

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REFERENCES :

1. Robert, L. and Dische, Z. (1963) Biochem. Biophys. Res. Communic. 10, 209-214
2. Robert, L. and Comte, P. (1968) Life Sci. 7, 493-497
3. Wolf, I., Fuchmans, W., Weiser, M., Furthmayr, H. and Timpl, R. (1971) Eur. J. Biochem. 20, 426-431
4. Randoux, A., Cornillet-Stoupy, J., Desanti, M. and Borel, J.P. (1976) Biochim. Biophys. Acta 446, 77-86
5. Peterkofsky, B. and Diegelman, R. (1971) Biochemistry 10, 988-994
6. Simpson, W.F. and Stulberg, C.S. (1975) Tissue Culture Assoc. Manual 1, 211-213
7. Schen, T.F., Stucker, H.J. (1975) Biochem. J. 150, 455
8. Juva, K. and Prockop, D.J. (1966) Anal. Biochem. 15, 77-83
9. Tedesco, T.A., and Mellman, W.J. (1966) Experim. Cell. Res. 45, 230-258
10. Burton, K. (1956) Biochem. J. 62, 315-323
11. Szymanowicz, A.G., Malgras, A., Randoux, A. and Borel J.P. (1979) Biochim. Biophys. Acta 576, 253-262
12. Anderson, J.C. (1976) Intern. Rev. Conn. Tissue Res. 7, 251-322
13. Robert, L. and Robert, B (1970) Exposés annuels de Biochimie Médicale (Masson, Paris) pp 189-277 vol. 30
14. Robert, L. (1971) Presse Med. 79, 2277-2283
15. Aalto, M., Potila, M. and Kulonen, E. (1979) Biochim. Biophys. Acta 587, 606-617
16. Robert, L. (1971) Presse Med. 79, 2277-2283
17. Anderson, J.C., Labedz, R.I. and Kewley, M.A. (1977) Biochem. J. 168, 345-351