Collagen Synthesis by Human Fibroblast Strains.* (29551)

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When mouse fibroblasts are serially cultivated *in vitro,* they have a high probability of evolving into established lines having infinite growth potential(1,2). Several of such lines have been found to retain differentiated function, for upon reaching saturation density they begin to synthesize abundant collagen (3,4).

Human diploid fibroblasts appear to have a very low probability of developing into established lines(5). They grow well, retaining the diploid karyotype(5,6) but only for a finite period, about 50 cell generations under our conditions, and then enter a phase of declining growth rate(6), ending in cessation of growth. If, before their growth potential is exhausted, they are allowed to remain at saturation density for some time, they, like the mouse lines, produce appreciable amounts of collagen. These amounts may be increased by addition of ascorbic acid. The fraction of the total protein synthesized which is collagen may be estimated by exposing the cells to proline C^{14}, and comparing the radioactivity of the proline and hydroxyproline isolated from the proteins made by the culture. The ratio obtained may be regarded as an index of differentiation of the fibroblast.

Methods. Culture methods. All cultures were grown on the surfaces of plastic Petri dishes, in the Dulbecco-Vogt modification of Eagle's medium(7) containing 10% calf serum. In some experiments on collagen synthesis, the serum was exhaustively dialyzed against dilute salt solutions, and finally against medium before being used. The media of all cultures were changed 3 times weekly.

Cell strains. The origin and history of the strain "A" adult skin fibroblasts, and fetal lung fibroblasts used in these experiments have been described at length(6). The present experiments were performed on cultures initiated from stocks of glycerol-frozen cells put into storage after completing varying numbers of cell generations in culture. The keloid fibroblasts were obtained from small biopsy specimens‡ and cultivated in the same manner. They were found to grow relatively poorly, and could not be carried past 10 cell generations. In all cases the number of cell generations (doublings) shown in Table I does not include the number occurring in the primary culture, as this is not known.

Hydroxyproline determinations on cell layers. After removing the liquid medium, the remaining cell layer was washed twice with phosphate buffered saline (pH 7.2), scraped into tubes and hydrolyzed in 6 N HCl at 120° for 12 hours. Hydroxyproline was determined by the method of Prockop and Udenfriend(8). In some experiments, the results were corrected for the number of cells in the culture by trypsinizing and counting duplicate cultures.

Measurement of collagen and non-collagen ("Cell") protein synthesis by incorporation of C^{14} proline. 2.5 microcuries of randomly labelled C^{14} L-proline (158 millicuries per millimole) were added in fresh medium to cultures synthesizing collagen, and allowed to incubate for 12 hours. The liquid medium was separated from the cell layer and cold trichloracetic acid added to each to a concentration of 5%. The suspensions were dialyzed exhaustively, first against unlabeled proline and hydroxyproline, and then against several changes of dilute phosphate buffered salt solution. The nondialyzable material was hydrolyzed in 6 N HCl at 120° for 12 hours. Carrier proline and hydroxyproline were added to aliquots of the hydrolysates and reisolated by thin layer chromatography according to the method of Myhill and Jackson(9). The solvent front was allowed to travel approximately 36 cm to achieve good separation. The cellulose bearing the desired amino acids was removed with a teflon police-

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TABLE I. Accumulation of Collagen in the Cell Layers of Human Fibroblast Cultures.

Origin of cell strain	Previous history of cell strain: Transfers	Previous history of cell strain: Cell generations	Medium supplement	Days in culture without transfer	Hydroxyproline content of cell layer: mμMoles per petri dish	Hydroxyproline content of cell layer: mμMoles per 10^7 cells
Fetal lung	7	17	Dialyzed serum	10	40	
			Idem + Na ascorbate, 250 γ/ml	10	76	
" "	6	13	Whole serum	15	70	
			Idem + Na ascorbate, 250 γ/ml	15	260	
" "	8	16	Dialyzed serum	24		55
			Idem + Na ascorbate, 250 γ/ml	24		316
Adult skin Strain "A"	20	54	Whole serum	24		55
Keloid (adult)	4	4	Whole serum	24	4	
			Idem + Na ascorbate, 250 γ/ml	24	38	
" (child)	8	8	Whole serum	26	4.5	
			Idem + Na ascorbate	26	63	

man and eluted with absolute methanol at 65°C for 15 minutes. Aliquots of the eluate were counted by liquid scintillation and analyzed quantitatively for hydroxyproline(8) and proline(10). The total radioactivity incorporated by each culture into protein in the form of each of the 2 amino acids was calculated (Table II).

Results. Human fibroblast strains of embryonic lung, adult skin and keloids were inoculated at moderately high density (usually about 1:4-1:8 dilutions of dense parent cultures) and allowed to remain without further transfer for 10-26 days. The medium was changed 3 times weekly. In some cases, sodium ascorbate was added to the medium at each change. Finally, the cell layers were analyzed for hydroxyproline. The results are shown in Table I.

It can be seen that the fetal lung and adult skin cultures produced appreciable amounts of collagen, as indicated by the hydroxyproline content of the cell layers. This was true even when the serum supplement had been exhaustively dialyzed. However, addition of ascorbic acid to the medium always resulted in a greater accumulation of collagen in the cell layer. In the case of fetal lung fibroblasts, the value of 316 millimicromoles of hydroxyproline represents a collagen content of over 10% of the total protein in the cell layer. In the case of keloid fibroblasts, very low values were obtained for cultures unstimulated by ascorbic acid and addition of ascorbate resulted in relatively much greater stimulation than in the other strains.

Collagen secretion is considered to be of the merocrine type, soluble protein being discharged from vesicles at the cell surface, with aggregation of the protein into characteristic fibrils occurring extracellularly(11-13). Hydroxyproline determinations on the cell layer may therefore not be a complete measure of collagen synthesis if a significant quantity of collagen remains soluble in the liquid medium. To test this possibility and to analyze the ascorbate stimulation in greater detail, the following experiment was performed: A 17-day culture† of fetal lung fibroblasts was exposed to proline-C^{14} for 12 hours, and the radioactive amino acids incorporated into the protein of the medium and of the cells were isolated as described under *Methods*. Total radioactivity incorporated as proline and hydroxyproline was calculated. The results are shown in Table II.

† Initiated after the population had grown through 6 transfers and over 16 cell generations.

The data on hydroxyproline incorporation in the absence of ascorbate indicate that about ⅔ of the collagen synthesized is confined to the cell layer, the rest being lost to the medium as a soluble molecule. The proline counts incorporated represent mainly non-collagen protein, of which nearly 90% remains confined to the cell layer and represents the diverse cell proteins synthesized. The small fraction lost to the medium may be due to lysed cells (though the cells appeared to be healthy), to secretion of non-collagen proteins, and to the proline residues in the soluble collagen.

In the presence of ascorbate, there is a considerable increase in collagen synthesis, without appreciable effect on the synthesis of other proteins. However, precipitation of collagen in the cell layer is not more efficient, as an even greater fraction of the total collagen produced escapes into the medium.

From the data in Table II, it is calculated that a little over 3% of the protein synthesized during the 12-hour period by the ascorbate treated cells, and about 1% of that synthesized by the non-ascorbate treated cells were collagen. Presumably the "cell" protein turns over more rapidly than the collagen, so that total collagen accumulating in cultures may increase with time to values higher than 10% of the total protein.

Discussion. Collagen synthesis by dissociated cells has been studied by Jackson and Smith(14) in short term primary cultures of osteoblasts. Such cultures evidently do not remain in good condition beyond a week. From the present experiments it is clear that repeated dissociation of tissue fibroblasts and long term cultivation do not necessarily lead to loss of cellular ability to express this differentiated property. Even after 20 transfers, by which time each cell in the primary culture has given rise to 2^{54} progeny, human fibroblasts remain capable of synthesizing collagen. It therefore appears that they retain this property through most of their culture life.

As is well known (see review by B. Gould (15)), ascorbic acid plays an important role in collagen synthesis in animals. The cell strains used in the present experiments preserved their sensitivity to ascorbic acid as collagen synthesis and accumulation in the cell layer always increased in its presence.

From the experiment on proline incorporation by fetal lung fibroblasts (Table II), it

TABLE II. Synthesis of Collagen and Non- Collagen Protein by Fetal Lung Fibroblasts.

			Counts incorporated: Cells + medium combined	(a) HyPRO / $PRO_{n.c.}$ × 100	(b) Collagen / $Protein_{n.c.}$ × 100
Cells grown and exposed to Pro-C^{14} in absence of added ascorbate	Hydroxy-proline	Cell layer	9,760		
		Medium	4,120		
			13,880	2.8	.93
	Proline	Cell layer	442,000		
		Medium	66,800		
			508,800		
Cells grown and exposed to Pro-C^{14} in presence of added Na ascorbate (250 μg/ml)	Hydroxy-proline	Cell layer	21,600		
		Medium	20,000		
			41,600	9.2	3.1
	Proline	Cell layer	425,000		
		Medium	79,400		
			504,400		

Calculation of values in last 2 columns:

1. It is assumed that all counts incorporated into non-dialyzable hydroxyproline (HyPRO) represent collagen synthesis.
2. Proline (PRO) incorporation is mostly into non-collagen (n.c.) protein, but also into collagen. Since collagen contains 1.2 proline residues for each hydroxyproline residue, 1.2 × HyPRO counts must be subtracted from total proline counts incorporated to give non-collagen protein-incorporated proline ($PRO_{n.c.}$, Column a).
3. Since the HyPRO residue content of collagen is 12.2%, while the average PRO residue content of the cell proteins is 4.1%(17), HyPRO/$PRO_{n.c.}$ is multiplied by 4.1/12.2 to give the ratio of collagen synthesis to non-collagen protein synthesis (Column b).

is clear that a considerable fraction of the collagen produced fails to precipitate in the cell layer, and passes out into the medium. A similar phenomenon appears to occur *in vivo,* permitting soluble collagen to circulate in the blood(16). Ascorbic acid had no effect extracellularly on the precipitation of collagen fibers in culture, as the fraction of total collagen escaping from the cell layer to the medium was not reduced.

Keloid fibroblasts did not appear to have as great a capacity for growth or collagen synthesis under these culture conditions as fetal lung or adult skin fibroblasts. This is surprising, in view of their behavior *in vivo.* They did, however, respond to ascorbic acid by a relatively marked increase in collagen formation.

Summary. Human diploid fibroblast strains derived from fetal lung, adult skin and keloids, produce collagen *in vitro* when allowed to grow to confluence and remain without transfer. This property may be retained throughout most of their culture life. Keloid fibroblasts produced the smallest quantities of collagen and had the lowest growth potential. All strains produced more collagen when ascorbic acid was added to the medium. Up to 3% of the total protein being made in the presence of ascorbic acid consisted of collagen. An appreciable fraction of the collagen failed to precipitate in the cell layer and escaped to the medium both in the presence and in the absence of added ascorbate.

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Dimethyl Sulfoxide As a Protective Agent During Freezing and Thawing of Human Spermatozoa.* (29552)

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The survival of most types of cells during freezing and thawing depends upon pretreatment with a protective substance. Although the mechanism of freeze-thaw protection largely is unknown, the colligative properties of the widely used simply polyhydric alcohol, glycerol, seem ideally suited for this purpose. Pretreatment with glycerol has made possible preservation by freezing of a wide variety of cells and tissues(1). Lovelock and Bishop introduced the water-miscible organic solvent, dimethyl sulfoxide (DMSO), as a freeze-thaw protective agent(2). They found that DMSO compared favorably with glycerol in protecting bovine and human erythro-

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