

## Defective collagen metabolism in Saudi patients with hernia

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**SUMMARY.** Fibroblastic cell cultures were derived from the hernial sac and some of the surrounding muscles (rectus sheath, internal oblique, and/or cremasteric muscle) of 130 Saudi patients with different types of herniation, and from 21 control subjects. The rates of cell proliferation were studied for 39 days. Results suggested decreased rates of proliferation of cells derived from patients compared to controls. *In vitro* studies of the rates of incorporation of <sup>14</sup>C proline into the muscle biopsies revealed decreased rates of label incorporation in the samples derived from patients compared to controls. However, no differences were detected between rates of collagenase activities of the biopsies obtained from patients compared to those of controls. These findings suggest that collagen synthesis is probably defective in the studied group of Saudi patients with hernia.

*Additional key phrases: human fibroblast collagenase activity*

The surgical repair of inguinal herniation is one of the commonest surgical operations in the Kingdom of Saudi Arabia in general and at King Abdulaziz University Hospital (Jeddah, Saudi Arabia) in particular.<sup>1</sup> However, no studies have been conducted in the Kingdom to determine whether biochemical differences exist between normal individuals and hernia prone subjects. Ample evidence accumulated over the last two decades from investigations on hernia patients of North America and Europe relates hernia to some metabolic disorders of collagen biosynthesis and/or breakdown.<sup>2-10</sup> However, understanding of the exact nature of such collagen metabolic syndromes is far from complete. Two main assumptions seemed most likely to be linked to hernia, namely, decreased biosynthesis of collagen,<sup>2-4,6-8</sup> or, alternatively, a disturbed equilibrium between collagen synthesis and collagenolysis at the hernia site.<sup>5,9,10</sup>

The present study was therefore undertaken in order to elucidate this question in Saudi patients who have as yet not been subjected to any similar biochemical investigation.

### MATERIALS AND METHODS

#### Patients

A total of 151 cases (130 hernia patients and 21 controls) were included in the study. The mean

ages were 43.3 and 41.8 years for male and female hernia patients, respectively. All cases were consecutive patients presenting at King Abdulaziz University Hospital over a period of 1 year. The distribution of cases by types of herniation is shown in Table 1.

#### Controls

Twenty-one patients of matched age (mean 42 and 40 years for men and women, respectively), sex and physical parameters undergoing lower abdominal operations other than hernial repair, i.e., those free from hernial defects, were included in the investigation. They were mainly patients suffering from acute appendicitis admitted to King Fahd Hospital in Jeddah. Twenty-one biopsies of the internal oblique muscle were collected. Biopsies from regions closer to the inguinal region could not be always obtained. The collected control biopsies were, therefore, considered as close as feasible.

#### Processing of biopsies

##### *Hernia patients*

During the hernial repair surgical operation (the usual repair technique performed for inguinal hernia is the modified Bassini herniorrhaphy) the hernial sac was routinely biopsied by ordinary surgical scalpel and transferred immediately to a special sterile container filled with sterile Eagle's

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TABLE 1. *Hernia cases by type*

Type	Men		Women		Total	
	Number	(%)	Number	(%)	Number	Total (%)
Direct-right*	55	51%	4	18%	59	45%
Direct-left*	25	23%	2	9%	27	21%
Direct-bilateral*	10	9%	—	—	10	8%
Indirect	11	10%	—	—	11	8.5%
Paraumbilical	5	4%	10	45%	15	11.5%
Incisional	1	1%	5	23%	6	4.5%
Epigastric	1	1%	1	5%	2	1.5%
Total	108	—	22	—	130	—

\*Total direct 90 men (83% of male cases) and six women (27% of female cases), i.e. total of 96 subjects (74% of all cases).

Minimum Essential Medium (MEM). In some cases, when the surgical repair permitted, smaller samples of the internal oblique muscle and/or the cremasteric muscle were also biopsied and kept in MEM. In the less common types of herniation, the hernial sac and any adjacent skeletal muscle were biopsied.

#### Controls

Samples of the internal oblique muscles were biopsied, kept in MEM and processed similarly.

#### Cell culture studies<sup>4,8,11-13</sup>

Fibroblast cultures were derived from the biopsied tissues of the patients and controls. Primary explants were followed by secondary subcultures. The generation time data presented pertain to secondary subcultures when the fibroblasts attained reasonable proliferation rates.

#### Technique

##### Explantation

The tissue after biopsy was brought to the laboratory in MEM and dissected free of fat and adhering material. The fascia was minced and divided into approximately four portions. Each section was transferred to a 25-ml sterile screw-cap plastic culture flask (Corning Glass) containing 10 mL of MEM. The explants were incubated at 37°C in carbon dioxide incubator (Forma Scientific). The medium was changed at 3 to 4 day intervals.

Hernial culture cells attained a monolayer 8 to 10 weeks after explantation (compared to 5 weeks in the case of control).

##### Secondary Subcultures

The medium and debris were decanted and the cells in the monolayer were incubated with

trypsin/Versen working solution (5 mL) at 37°C for 30 min. The resulting cell suspension was centrifuged at 1500 rpm for 5 min. The cells were resuspended in fresh medium, and daughter cultures were derived in separate culture flasks. After a known number of cells ( $4$  to  $7 \times 10^5$ ) from each specimen could be harvested, they were suspended uniformly in 10 mL of MEM. The suspension was divided into 10 flasks (1 mL each), containing 3 mL of MEM. The medium was changed each 3 to 4 days. The total number of cells were counted by a haemocytometer at 5 day intervals. The duration of this phase of the experiment was 39 days, chosen because the number of cells increased approximately threefold over this period. All these procedures were conducted in a laminar flow hood (Miniflo, Italy) with a relative manometer pressure of 8 mm water and under strict sterile conditions.

The overall duration of each complete cell culture experiment was approximately 16 weeks from the time of sampling of the biopsy.

##### Calculation of cell generation time<sup>4,11</sup>

From the growth data, linear regression equations and correlation coefficients ( $r$ ) were computed. From the constants of the regression equations, the total number of cell divisions and the average generation time in 39 days of the experimental period were calculated from:

$$\text{Regression equation: } Y = aX + b$$

where  $Y$  = cell population on day  $X$ ;  $a$  = slope;  $b$  = intercept  
Total number of cell divisions

$$= \frac{\log(39 \times \text{slope} + \text{intercept}) - \log(\text{intercept})}{\log 2}$$

Average generation time = 39/total number of cell divisions. The total number of cell divisions is defined as the number to double the cell

population and the average generation time is defined as the average time required for the cell population to double.

#### **Tissue incorporation of $^{14}\text{C}$ -proline<sup>4</sup>**

##### *Incorporation technique*

Samples of the internal oblique muscles from control subjects and hernia patients were brought to the laboratory in MEM as described in the above method and transferred to a Petri dish containing Krebs-Henseleit bicarbonate buffer (KH buffer). Any adhering fat or blood was removed by cleaning and the tissue was rinsed in buffer once.

The tissue was then placed in fresh buffer solution and cut into four pieces of approximately equal size. Each was divided further into about six equal squares and immediately transferred into a 10 mL Erlenmeyer flask containing 1.9 mL incubating medium (two parts of KH buffer plus one part of a solution containing 4000 units of penicillin G, 3 mg of streptomycin sulphate, 8  $\mu\text{mol}$  of dextrose per mL of 0.9% NaCl).

L-proline- $^{14}\text{C}$ , 250 mCi/ml (Amersham, Buckinghamshire, UK), was diluted with incubating medium so that the activity was 1.25 mCi per mL. A 0.1 mL aliquot of this diluted solution was then added to the incubation mixture at zero time. The flasks were thoroughly flushed with a gas mixture (95%  $\text{O}_2$  plus 5%  $\text{CO}_2$ ). The contents were incubated at 37°C and the reaction was stopped at 30 min, and at 1 h, 2 h and 4 h by addition of 0.2 mL incubation medium containing 300  $\mu\text{g}$  of cyclohexamide (to prevent protein synthesis) and 470  $\mu\text{g}$  of 2,2'-dipyridyl (to stop hydroxylation).

The media were separated from tissue. The tissue was washed twice with 2 mL of cold deionized water and washings were pooled with the corresponding medium. The washed tissue was treated with acetone and dried at 60°C for 16 h *in vacuo*. The dry weight of each tissue was recorded before the determination of radioactivity.

##### *Measurement of Radioactivity*

Each tissue was hydrolyzed in 1.0 mL 6 N HCl at 130°C for 6 h under nitrogen. The hydrolysate was neutralized with 10 N NaOH and the neutral solution was adjusted to 2.0 mL with deionized water.

For measurement of total radioactivity each vial contained 0.10 mL of neutralized hydrolysate diluted to 1.0 mL with water, one drop of 4% stannous chloride and 10 mL of BBS-3 Fluoralloy-toluene scintillation counting medium

[Fluoralloy (Beckman), 6.8 g; Bio-Solv BBS-3 (Beckman), 200 mL; toluene (scintillation grade), 800 mL].

The samples were counted in an automatic LKB 1211 Rackbeta Liquid Scintillation Counter. Values were reported as disintegrations per minute (dpm) per milligram of dry tissue.

##### **Assay of collagenase activity<sup>14-16</sup>**

The method<sup>16</sup> depends on the preparation of soluble [ $^{14}\text{C}$ ] acetylated collagen as a substrate for the collagenase enzyme. The collagen cleavage products were separated from noncleaved collagen by precipitation with dioxane/methanol.

##### *Substrate preparation*

Purified Type I calf-skin collagen was acetylated with  $^{14}\text{C}$ -acetic anhydride. Over a 2-h period, 0.5 mCi of the labelled anhydride was added to 62.5 mL of a 2.0 g/L solution of collagen, with constant stirring at 10°C (pH 8). This solution was acidified with acetic acid to pH 4 then dialyzed for 96 h versus deionized water at 4°C. The dialyzed substrate was diluted with an equal volume of 1 mM acetic acid, and 50 mg of pepsin was added. The mixture was slowly stirred for 18 h at 4°C, then dialyzed again against deionized water for 48 h. The resulting substrate was lyophilized and stored at -20°C. The pepsinization step removes any collagen that may have been denatured during the labelling process. The produced substrate had a specific activity of 0.5 to 1.0  $\times 10^6$  dpm per milligram of protein.

##### *Preparation of fibroblast tissue matrix*

Homogenates of the tissue for use in the collagenase assay on labelled substrate were prepared by grinding the crude fibroblast culture matrix with Tris buffer (100 mmol/L, pH 7.5) containing 10 mmol/L calcium chloride, in an all-glass homogenizer. All tissue homogenates were prepared to yield a hydroxyproline concentration of 1 g/L.

##### *Separation of collagenase substrate*

Type I collagen and the collagenase substrate were separated by HPLC.<sup>15</sup>

##### *Collagenase assay*

The radiolabelled collagen solution (2.0 g/L in 1 mmol/L acetic acid) was added to an equal volume of 100 mmol/L Tris buffer (pH 7.5) containing 10 mmol/L calcium chloride and 0.4 mol/L NaCl. The cell culture sample (100  $\mu\text{L}$ ), prepared from the cultured fibroblasts of hernia

patients and controls, were added to the labelled substrate solution (100  $\mu$ L), with mixing. The reaction mixture was incubated for 90 min at 35°C, which is several degrees below the thermal denaturation point ( $T_m$ ) for purified native collagen. The reaction was then stopped by addition of 100  $\mu$ L of a solution containing 150  $\mu$ g of unlabelled collagen in 0.4 mol/L EDTA solution. The mixture was incubated for 30 min at 35°C, to allow for complete denaturation of all cleaved collagen molecules. To each sample or control tube was added 300  $\mu$ L of the mixture of precipitation solvents (1,4-dioxane/methanol, 4:1 by volume, cooled to 8°C) and the resulting mixture centrifuged at 6000 g for 15 min. An aliquot of each supernate (200  $\mu$ L) was added to 10.0 mL of Bray's solution and the radioactivity was measured.

The samples were counted in an automatic LKB 1211 Rackbeta Liquid Scintillation Counter as described above.

One unit (U) of enzyme activity represented the cleavage of 1  $\mu$ g of native collagen per minute at 35°C and pH 7.5.

To investigate the susceptibility of fibroblast extracellular matrix to degradation by collagenase, the competitive substrate method was used.<sup>16</sup> Each determination contained 100  $\mu$ g of <sup>14</sup>C-acetylated collagen with either 10 or 50  $\mu$ g of tissue homogenate (from control and hernia specimens). [Purified mammalian fibroblast collagenase (94 mU) was added to each tube to initiate the reaction.] The reaction mixture was incubated for 15, 30, 60 or 90 min, followed by quantification of cleaved substrate as described.

Rates of enzyme velocity were computed from this approach as per cent decrease in specific activity.

### STATISTICAL METHODS

Statistical analysis of data were carried out using Statgraphics personal computer program (Statistical Graphics Corporation, USA). Student's *t*-test was used to test the significance of the difference between the hernia and control samples. The program compares the means and variance from the two samples generating a calculated *t*-value and the corresponding significance level (*P* value).

Furthermore, Dunnett's *t*-test of significance between the control mean and several test means was performed.<sup>17,18</sup> This ensures the validity of frequent comparisons between many samples and one control.

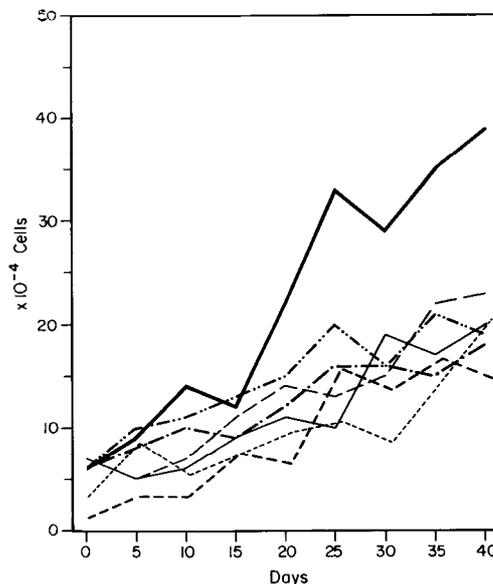


FIGURE 1. Rate of proliferation of cultured fibroblasts (randomly chosen specimens/patients versus control). — Control; --- S44; ···· S61; - · - · S73; - - - - S85; - · - · - S99; — S132.

### RESULTS AND DISCUSSION

The observed rates of proliferation of fibroblasts cultured from the 130 patients with different types of herniation as well as from 21 control biopsies were calculated as detailed in the section on Methods. Some representative sample plots are shown in Fig. 1. The samples were chosen at random to illustrate the rates obtained. For clarity of presentation only six lines plus control are shown in this graph. The calculated rates based on the regression equations for all samples were computed from the found cell population counts for each sample. These in turn yielded the total number of cell divisions and average generation time in days for each biopsy culture. The generation time for each sample was then computed relative to the overall mean value of the 21 controls.

The number of cell divisions for controls ranged from a low of 2.64 to a high of 3.29 with a mean value of 2.97 (SD=0.17). These corresponded to calculated average generation times of 14.77 and 11.85 days, respectively (mean 13.16, SD=0.79). For the hernia derived cell cultures the number of cell divisions ranged from 1.22 to 2.77 (mean 2.07, SD=0.30) corresponding to generation times of 31.85 and 14.09 days, respectively (mean 20.08, SD=3.10). When the generation times of hernia derived fibroblast

cultures were recalculated relative to control, it was immediately apparent that the rates of cell proliferation were decreased among these cells as compared to those of controls. The average generation times for cells derived from hernial patients were longer than normal cells by a factor which ranged from a low of only 7% to a high of over 100% (i.e. 2 to 2.5 times as long). Student's *t*-test was performed on each hernia sample as compared to control. The differences were significant at  $P < 0.01$ . In addition, Dunnett's *t*-test of significance between the control mean and several test means was done. All hernia samples were significantly different from control at  $P < 0.05$ . These cell generation times were considered as good indicators of intrinsic differences in the growth characteristics between normal and hernia derived fibroblasts.

Representative data on the incorporation of  $^{14}\text{C}$ -proline in the internal oblique muscle are shown in Table 2 for four samples chosen at random. In general the average rates of incorporation of the radioactive label of the hernia patient specimens were significantly lower than in control at all times of the incubation period. By extrapolation to zero time average values of  $377 \pm 25$  and  $211 \pm 18$  dpm of  $^{14}\text{C}$ -proline per mg dry tissue were obtained for the control and hernia specimens, respectively. Student's *t*-test as well as Dunnett's *t*-test indicated that the difference is statistically significant at  $P < 0.015$  and  $0.03$ , respectively. The order of incorporation thus followed a similar trend to that observed in the cell proliferation data.

Analysis by HPLC of the  $^{14}\text{C}$ -acetylated collagens revealed that acetylation did not alter the biophysical parameters of the protein. After acetylation, different forms of collagen migrated with the non-acetylated starting material. This confirmed the ability of collagenase enzyme to

TABLE 2. Rate of in vitro incorporation of  $^{14}\text{C}$ -proline in tissue biopsies from hernia patients and control (dpm/mg dry tissue)

Hour	Control	Selected hernia specimens					Mean $\pm$ SD
		S37	S54	S89	S101		
1	340 $\pm$ 30	200	170	150	125	161 $\pm$ 32	
2	395 $\pm$ 49	225	195	180	165	191 $\pm$ 26	
3	410 $\pm$ 41	290	260	230	195	244 $\pm$ 41	
4	395 $\pm$ 37	300	250	240	210	250 $\pm$ 37	
<i>n</i>	4	5	5	3	3	4	

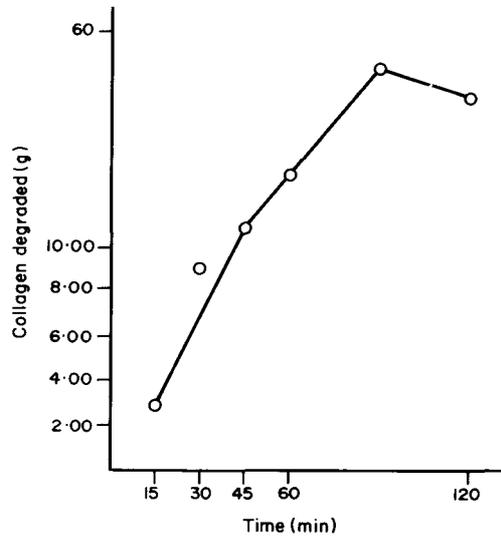


FIGURE 2. Cleavage of radiolabelled collagen by purified fibroblast collagenase as a function of time. Samples chosen randomly. (Each assay initially contained 100  $\mu\text{g}$   $^{14}\text{C}$ -acetylated collagen.)

utilize completely the acetylated form of collagen as substrate. On using the dioxane/methanol mixture to precipitate the noncleaved substrate, background radioactivity was reduced to a minimum. About 92% of the native substrate was precipitated by this mixture.

The amount of collagen degraded was found to be linear with time for approximately 90 min after which the reaction became substrate limiting (Fig. 2). A correlation diagram for enzyme

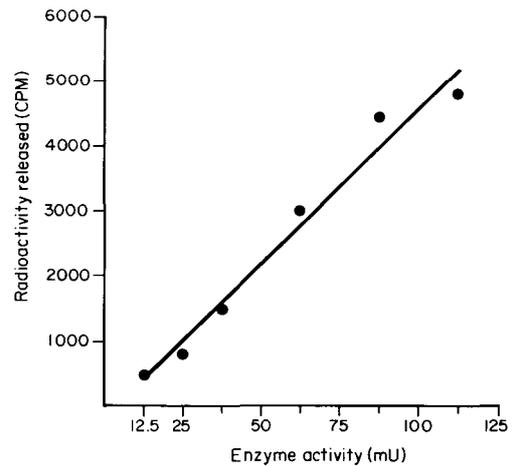


FIGURE 3. Correlation for enzyme activity versus radioactivity released (standard curve). ● Actual readings; — regression fit.

TABLE 3. Effect of fibroblast tissue matrix on the substrate specific activity

Source of matrix	Collagen added† (µg)	No. of samples	Decrease in specific activity (%)*		
			Mean	SD	SE
Control	71	21	44.3‡	5.4	1.18
	357	21	74.1§	4.6	0.99
Hernia	71	130	42.4‡	8.6	0.75
	357	130	76.0§	9.0	0.79

\*The mean decrease of reaction velocity from four time points (15, 30, 60 and 90 min).

†Calculated by multiplying micrograms of hydroxyproline by 7.14, which represents the correction for molecular mass of collagen.<sup>16</sup>

‡Difference not significant,  $P=0.32$ .

§Difference not significant,  $P=0.355$ .

activity versus radioactivity release is shown in Fig. 3, which indicates this assay is linear over a 10-fold range of enzyme activity.

The results of the study of fibroblast susceptibility to degradation by collagenase, for tissue matrices derived from hernia patients and controls (Table 3) revealed no significant difference ( $P>0.3$ ) in collagenase activity between the two groups. Hence it may be inferred that little or no difference in collagen breakdown exists between hernia patients and normal subjects.

Taken collectively, the data presented in this section indicated that fibroblast proliferation rates in cell and tissue culture derived from hernia patients are lower than control samples. Thus, one of the significant reasons why collagen may be reduced in the abdominal aponeuroses could be a general lower rate of synthesis. Tissue incorporation of <sup>14</sup>C-labelled proline, which was lower in biopsies obtained from the hernia sites, substantiated the assumption that decreased collagen integrity in hernia patients, if any, should be related to the rate of collagen production. Theoretically, however, these tissue incorporation experiments could be consistent with incorporation of the active proline either in collagen specifically or in any other protein being synthesized in the muscle. However, it is important to realize that irrespective of the type of protein in which the label is incorporated, the lower rate of incorporation of proline is a valid reflection of retarded cellular biosynthesis. This is in good agreement with the cell proliferation data.

Since collagen integrity is the outcome of a balance between synthesis and breakdown, the above data indicate that such balance is defective in the Saudi hernia patients studied relative to controls as a result of decreased synthesis. This agrees with many previous data on non Saudi patients.<sup>2-4,6-8</sup>

Collagenase activity data showed no detectable difference between controls and hernia patients in rates of collagen breakdown. Therefore, for these Saudi patients at least, the data presented are not consistent with the suggestion<sup>5,9,10</sup> that increased collagenase activity is the main reason for defective collagen imbalance as was suggested in some other reports.

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