76. *Active Synthesis of Collagen by Albumin-Producing Liver Parenchymal Cell Clones in Culture*†)

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Recent studies have revealed that various cell types of non-mesenchymal origins carry a protocollagen prolyl hydroxylase activity which is known to be essential for collagen biosynthesis.[1)-3)]

In the normal liver, small amounts of collagen fibers are often observed in the space of Disse, while deposition of the fibers are detected in the perihepatocellular area as well as portal and proliferating periductular regions with progressing hepatic fibrosis.[4)] Recent report on the findings of a high enzymatic activity of protocollagen prolyl hydroxylase in the isolated rat liver parenchymal cells has suggested a possibility of collagen production by the cells,[5)] though presence of the enzyme does not warrant the collagen production.[3),5)]

In our previous papers, the production of collagen by several cloned and uncloned liver epithelial cell lines have been demonstrated by hydroxyproline analysis and/or histological observations.[6),7)] This paper is concerned with quantitation and type analysis of collagen produced by albumin-producing rat liver parenchymal cell lines in order to elucidate the molecular mechanism of collagen metabolism in normal and cirrhotic livers.

Materials and methods. Two liver epithelial clones (BB and BC) derived from a JAR-2 suckling rat were generously supplied by the Tissue Culture Laboratory, Yokohama City University School of Medicine and other cell lines of rat liver epithelial strain (M),[8)] mouse skin fibroblast (L-929),[9)] and rat lung fibroblast (RLG-1)[8)] were kindly donated by Department of Cancer Cell Research, Institute of Medical Science, Tokyo University. The cells were cultured in Ham F-12 medium containing 10% calf serum or Dulbecco's modified

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Table I. Collagen production by liver parenchymal cells in culture

Cell line	Albumin ng/ml	α-feto-protein ng/ml	Cell type	Characteristic	Collagen synthesized*6 %
BB-[24]*1,*2	16	—	Rat liver	Contact inhibited	2.04
BB-[26]*1,*2	19	—	epithelial	clone	1.62
BC-[36]*1,*2	50	—	Rat liver	Contact inhibited	1.67
BC-[36]*1,*3	38	—	epithelial	clone	1.08
BC-[38]*1,*2	24	—			1.93
M*3	—*5	3.3	Rat liver	Transformed	1.30
M*2		—	epithelial		0.95
L-929*3 (213-12)	—	—	Mouse skin fibroblastic	Transformed clone	0.70
RLG-1*3	—	—	Rat lung fibroblastic	Transformed clone	0.23
3T3 P-1*4			Mouse	Contact inhibited	1.90
3T3 P-3*4			embryonic	subclones of Balb	1.96
3T3 714*4				3T3 A-31	0.91

Late logarithmic phase cells were labeled as described[11),12)] in 3 ml of Ham F-12 medium or Dulbecco's MEM supplemented with 0.1 mM ascorbic acid and 0.5 mM β-aminopropionitrile. After a 15-min preincubation at 37°C, 100 μCi of [^{3}H]-proline was added to each dish, which was incubated at 37°C under 95% air/5% CO_2 for 3 hr. [^{3}H]-labeled proteins were prepared from cell and medium fractions as described[13)] and a part of each fraction was used for quantitation of collagen with protease-free collagenase.[10)] Aliquots of the media cultured at the same time with no radioactive material were used for radioimmunoassay of rat serum albumin and α-fetoprotein.[14)]

*1 Figure in bracket means passage level of the clone. *2 Cells were grown in Ham F-12 medium +10% calf serum. *3 Cells were grown in Dulbecco's MEM+10% fetal calf serum. *4 Data were cited from previous experiments.[11)] *5 Less than sensitivity limit (1 ng/ml). *6 Calculated assuming that collagen has an imino acid content 5.4 times higher than other proteins[13)] and is the percent radioactive proline incorporated into collagen compared to total protein in 3 hr.

Eagle medium (MEM)*) supplemented with HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (3 g/l) and with 10% fetal calf serum. Penicillin G (50 mg/l) and dihydrostreptomycin (50 mg/l) were also added to both media. Antibiotics were generously donated by Toyo Jozo Co., Ltd. For labeling protein, cells were cultured in Ham F-12 medium or Dulbecco's medium without serum but supplemented with 0.1 mM ascorbic acid, 0.5 mM β-aminopropionitrile, an inhibitor of lysine-derived cross-link formation, and 100 μCi of [2,3-^{3}H] proline (20 Ci/mmole, New England Nuclear, Boston, Massachu-

*) Abbreviation: MEM for modified Eagle's medium.

setts). Purified *Clostridium histolyticum* collagenase was obtained from Amano Pharmaceutical Co., Ltd., Nagoya. The enzyme, when used in the presence of N-ethylmaleimide,[10] did not release any appreciable amount of radioactivity from ^{3}H-tryptophan (an amino acid lacking in collagen)-labeled chick embryo proteins, showing the specificity of this enzyme preparation.

Results and discussion. *Production of collagen by liver epithelial cells.* Table I summarizes characteristics of the cells employed and relative amount of collagen synthesized by them. Cloned liver epithelial cells (BB and BC) were shown to produce collagen at a level of 1.1–2.0% of total proteins when cultured in the medium of either Ham F-12 or Dulbecco's-MEM, which was in the same level with those of contact inhibited 3T3 cells and two to nine times higher than those with fibroblastic cell lines of L-929 (0.70%) and RLG-1 (0.23%) cultured in parallel. When collagen production per DNA was compared, the liver cells were two to three times higher than the fibroblastic cells. BB and BC cells of the same passage also produced rat serum albumin in the early to middle log phases as well as the late log phase. These results show that the liver epithelial clones are capable of producing collagen and serum albumin at the same time. Collagen synthesis observed here may not be due to contaminating fibroblastic cells, but to parenchymal cells, since both BB and BC cells were cloned and only epithelial cells were observed in culture which produced similar levels in amount of collagen in different subculture passages. Transformed liver epithelial cells (M) which lost albumin-producing activity were also shown to possess a capability of producing appreciable amounts of collagen (Table I). The results described above indicate that liver parenchymal cells do possess a collagen producing capacity intrinsically.

When the degree of collagen deposit in cultured cell layers were compared morphologically by electron microscope, the degree with M cells were higher than with BC cells and only a faint collagen deposition was observed with BB cells,[7] though quantitation of collagen by the collagenase digestion method have shown that BB cells produced collagen as much as BC cells and slightly higher than that with M cells. It is now well accepted that collagen is produced and secreted by collagen-forming cells as procollagen which possesses extention peptides at the N- and C-termini and after processed by procollagen peptidases, procollagen turned to collagen giving rise to electron microscopically detectable collagen fibers. The discrepancy between the results obtained by the morphological and enzyme digestion methods could be explained by differences in the rate of secretion and/or processing of procollagen produced by these cells.

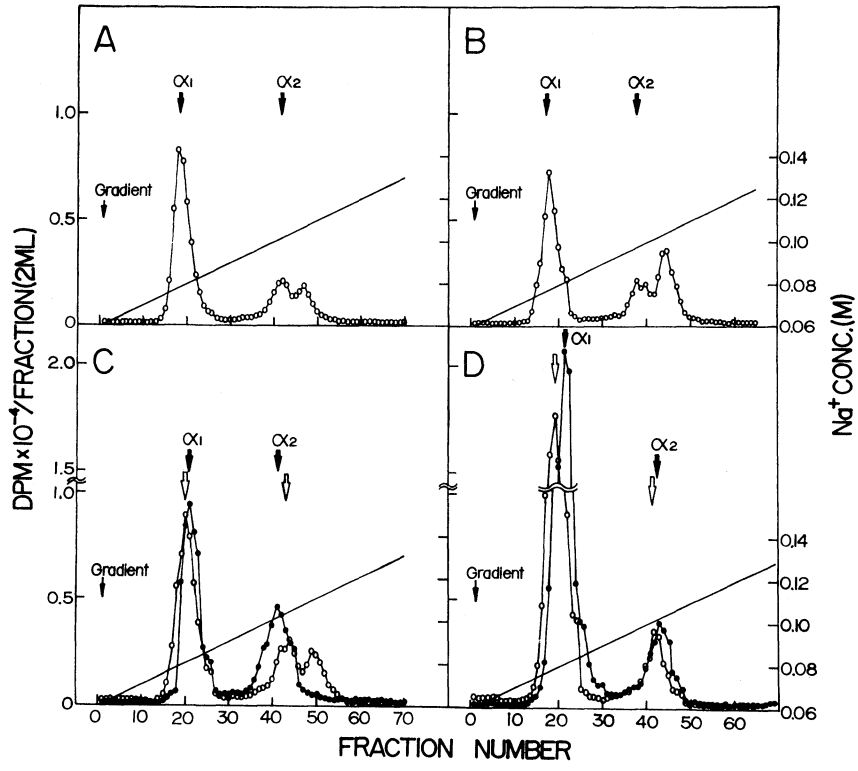

Fig. 1. Carboxymethyl-cellulose chromatograms of collagen produced by liver parenchymal cells. Radioactive collagen from the medium of cell cultures was prepared as described previously[11] and further purified by ammonium sulfate precipitation (176 mg/ml) then applied to a CM-cellulose column (1.0 ×7 cm) with 3 mg of carrier lathyritic rat skin collagen. The column was washed with starting buffer (0.06 M sodium acetate/4 M urea at pH 4.8), then developed with a NaCl concentration gradient from 0 to 100 mM with 100 ml each of starting and limit buffer at 43°C. (A) BB cells and (B) BC cells cultured in Ham F-12 medium. (C) BC cells and (D) M cells cultured in Dulbecco's MEM. Chromatography was done in the absence (○——○) or presence (●——●) of 1 mM dithiothreitol. Arrows indicate the elution positions of carrier rat skin collagen subunits. ——: Na^+ concentration.

Partial characterization of the type of collagen produced by liver epithelial cells. Fig. 1 shows carboxymethylcellulose chromatography of collagens in cultured media of BB, BC and M cells. The presence of three different collagen chain components were observed with BB and BC cells cultured in Ham F-12 (Fig. 1, A and B). The first two components were eluted at α_1- and α_2-chain fractions and the third one eluted after α_2-chain was found to correspond to a rat type III collagen,[15] which could be recovered around α_2-chain fraction by reduction, as observed with type III collagen, though further evidence

is required to confirm it. Essentially no change in collagen types was observed with BC cells in Dulbecco's MEM (Fig. 1C). The relative content of the third component in each cell culture system was estimated to be approximately 15%, 30% and 20% respectively (Fig. 1, A–C). On the contrary, less than 3% of the third component was detected with M cell culture (Fig. 1D). This could be explained by the effect of transformation or difference in the passage of subculture of the cells from BB and BC cell lines.

The ratios of α_1/α_2 in the above culture systems were 3.7, 3.4, 2.4 and 4.7, respectively, which are all higher than that, i.e., 2.3, expected for type I collagen labeled with radioactive proline.[11] These results suggest that the cell lines employed here may produce type X collagen composed of three identical α_1(X) chains.

Recent report on hepatic diseases has suggested that chronic hepatitis caused by hepatitis B virus may result in liver cirrhosis giving rise to a primary hepatic carcinoma.[16] Our recent studies have demonstrated that sarcoma virus and some carcinogen affect collagen production by Balb 3T3 cells, changing the phenotype of the material.[11] Epithelial-mesenchymal interactions were also shown to induce specific collagen gene(s) during tissue differentiation *in vitro*[17] and addition of dibutyryl cyclic AMP to increase collagen production.[12),18)]

About 70% of the liver cells are known to be parenchymal cells. Studies on collagen production by the parenchymal cells with special reference to type analysis and on factors affecting collagen production may lead to elucidate the molecular mechanisms of the relation among virus infection, liver cirrhosis and hepatic carcinoma.

Summary. Two lines of albumin-producing rat liver parenchymal cell clones (BB and BC) have been shown to synthesize collagen at a level of 1–2% of total proteins produced which was two to nine times higher than those by the fibroblastic cells (L-929 and RLG-1). Type analysis of the collagen by carboxymethylcellulose chromatography has shown that the former two cell lines synthesized type I collagen, $[\alpha_1(\mathrm{I})]_2\alpha_2$, type X, composed of α_1 chains and type Y similar to type III collagen, while a transformed liver cell line (M) did the former two collagen types.

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