*Biochemical Pharmacology*, Vol. 38, No. 6, pp. 895–899, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00


# HEPARIN FRAGMENTS REGULATE COLLAGEN PHENOTYPE AND FIBRONECTIN SYNTHESIS IN THE SKIN OF GENETICALLY DIABETIC MICE

C. ASSELOT, J. LABAT-ROBERT and P. KERN

Laboratoire de Biochimie du Tissu Conjonctif, UA CNRS 1174, faculté de Médecine, Université Paris XII, 8 rue du Général Sarrail, 94010 Creteil, France

(*Received* 25 *August* 1988; *accepted* 31 *August* 1988)

**Abstract**—The biosyntheses of interstitial collagens of type I and III and of fibronectin were studied in genetically diabetic KK mice, as compared to control C57 Black mice, as well as the effect of low-$M_r$ heparin fragments (CY 222) on these biosyntheses.

An increased production of type III collagen, as compared to type I collagen, could be demonstrated in explant cultures of KK mice skins. Fibronectin biosynthesis was also increased. *In vivo* treatment of KK mice with 1 mg/kg of CY 222 decreased the biosyntheses of type III collagen and of fibronectin to normal levels. These experiments suggest that low-$M_r$ heparin fragments can modulate the expression of extracellular matrix macromolecules.

The regulation of expression of genes coding for extracellular matrix (ECM) macromolecules is an essential part of cell differentiation, morphogenesis and embryonic development [1–3]. Several diseases, genetic and acquired, were shown to be accompanied by qualitative and quantitative modifications of the expression of these genes [4–6]. For example overproduction of collagen has been described in liver fibrosis or cirrhosis, [7] pulmonary fibrosis [8], arteriosclerosis [9]. Diabetes is accompanied by perturbations of the biosyntheses of collagens, proteoglycans and structural glycoproteins [10–18].

We reported previously on the relative increase of collagen type III in skin from genetically diabetic KK mice [19] and in human diabetic conjunctiva [20]. Furthermore, we have demonstrated an increase of tissue fibronectin biosynthesis both in human diabetic and in KK mouse skin [21, 22]. Previous reports suggested a regulatory effect of heparin on the biosyntheses of collagenous and non collagenous macromolecules of ECM [23–26].

Preliminary studies in our laboratory have shown that low-$M_r$ fragments which possess the binding site to antithrombin III, but lack hemorrhage inducing properties (referred to as CY 222. CHOAY Laboratories) are also capable to modulate the biosyntheses of collagen type III and fibronectin by smooth muscle cells in culture [27].

We wish to report here on the *in vivo* effects of CY 222 on the biosynthesis of skin extracellular matrix in genetically diabetic KK mice. We have focused our attention on interstitial collagens (type I and type III) and fibronectin.

## MATERIALS AND METHODS

Acrylamide, *N-N'*-methylene bisacrylamide, salts, solvents and buffer systems were purchased from Merck (Darmstadt, F.R.G.).

CY 222 (obtained from Choay laboratories) is a preparation of low-$M_r$ heparin fragments obtained by nitrous acid depolymerization of heparin, major species having a molecular weight between 2000 and 3000 daltons [28].

*Animals used.* Non-obese, genetically determined, spontaneously diabetic KK mice were bred in our laboratory. The mutation was maintained by brother–sister matings. C57 black mice were used as controls. All animals were kept in standard conditions and fed purina chow. Physiological parameters of the KK strain have been previously described [19, 29, 30]. Essentially, the diabetic mice exhibited a moderate increase of plasma insulin concentration without significant change in glycemia.

*Treatment with CY 222.* KK mice and C57 black mice treated groups (two-month-old males) were injected with CY 222: i.m. injection of 1 mg/kg; three injections per week during six weeks.

Non-treated mice (control group) were injected with isotonic saline solution. The experimental groups were classified as: (a) C57 black mice (control); (b) CY 222-treated C57 black mice; (c) non-treated, diabetic, KK mice; (d) CY 222-treated KK mice.

*Collagen biosynthesis in skin explant cultures.* Skin explant cultures were performed essentially as previously described [19, 20, 31]. Briefly, animals were shaved and killed. Dorsal skin was removed and dissected free of fat. Tissue was cut into small pieces and then incubated at 37° for 24 hr in Dulbecco's modified Eagles medium containing L (2.3.4.5 ($^3$H))proline (specific activity 105 Ci mM, 50 μCi ml; purchased from Amersham, France), β amino propionitrile (50 μg ml) penicillin (4000 U ml) and ascorbic acid (100 μg ml). Incubation was stopped by cooling at 4°. The medium was discarded and the tissue extensively washed with large excess of incubation buffer containing 1% proline [31].

*Limited pepsin digestion.* The tissue was homogenized and subjected to limited pepsin treatment at 4° for 2 × 24 hr (100 μg pepsin per mg collagen). Solubilized material was submitted to differential salt precipitation at acid pH (using dialysis against 0.5 M acetic acid containing 0.7 M NaCl and then 2 M NaCl) [31]. Hydroxyproline was determined in each fraction [32].

*SDS polyacrylamide gel electrophoresis.* Electrophoretic separation of collagen chains was performed according to Laemmli [33] with 7.5% acrylamide gel. In order to separate $\alpha_1$(I) and $\alpha_1$(III) chains, the method of interrupted electrophoresis was used [34]. Quantitative analysis of stained gels and radioactivity determinations were performed as previously described [31].

*Quantification of collagen types by CNBr peptides analysis.* Independently from the previous experiments, skin samples were directly treated with CNBr as described elsewhere [20, 31] CNBr peptides were resolved by SDS PAGE [35]. Type I and type III collagens were quantified using $\alpha_1$(I) CB8 and $\alpha_1$(III) CB8 as peptide markers. Calculations were made as described [20].

*Fibronectin biosynthesis in skin explant cultures.* Fresh minced skin of each animal (about 0.5 g) was incubated at 37° for 24 hr in 5 ml of Dulbecco's modified Eagle's medium containing methionine at 10% of its normal level and 100 μCi (1 Ci = 37 GBa) of [$^{35}$S]methionine (Amersham) and supplemented with 10% fetal bovine serum (FloBio, Paris) previously depleted of fibronectin by passage onto a gelatine Sepharose column. After incubation, the medium was collected and exhaustively dialyzed for 48 hr against several changes of 10 mM Tris–HCl buffer (pH 7.4) containing the protease inhibitors (phenylmethylsulphonyl fluoride, N-ethylmaleimide, EDTA, at 2 mM).

The tissues were washed three times with Dulbecco's phosphate buffered saline (without $Ca^{2+}$ and $Mg^{2+}$, pH 7.4; Seramed Biochrom K.G., Berlin) and homogenized in 5 ml of boiling 1% Nadodecyl sulfate. The aqueous phase was removed after centrifugation at 10,000 *g* for 15 min and dialyzed against $CH_3COOH$ 0.2 M containing the same protease inhibitors as above. The insoluble proteins were removed by centrifugation.

Fibronectin was immunoprecipitated from aliquots of the culture medium and tissue extracts.

## RESULTS

### *Collagen biosynthesis*

Total collagen present in whole skin was not significantly different in the mice strains with or without CY 222 treatment (total skin hydroxyproline values, expressed as mg/g dry wt, ranged from 97 (±8) for control mice and 100 (±11) for CY 222-treated control mice to 95 (±9) for KK mice and 89 (±8) for treated KK mice). After incubation with ($^3$H)proline during 24 hr, tissue samples were pepsin solubilized.

In all groups, nearly 90% of total skin collagen was solubilized. Collagenous material was salt precipitated at acid pH. The 0.7 M NaCl fraction representing more than 95% of starting material was analyzed by SDS PAGE. Figure 1 shows typical

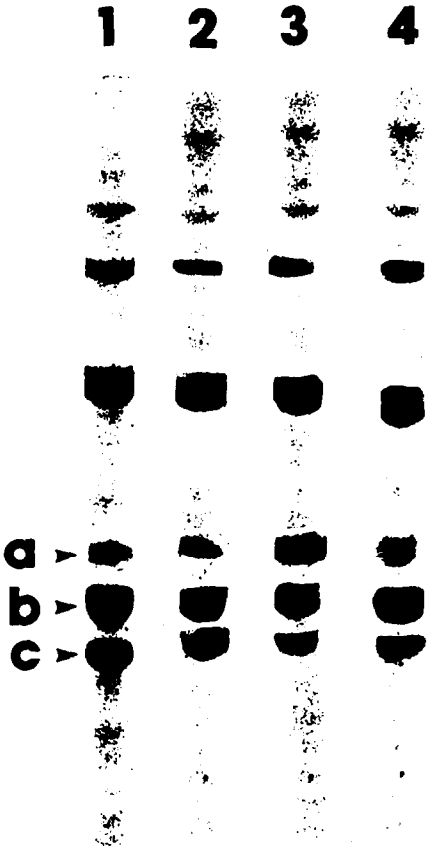


Fig. 1. SDS–PAGE analysis of pepsin-solubilized collagen molecules precipitated by 0.7 M NaCl. Precipitation and electrophoresis were performed as described in material and methods using interrupted electrophoresis. Gels stained with Coomassie blue were analyzed at 560 nm. Migrating positions of standard α chains were: a, $\alpha_1$(III); b, $\alpha_1$(I); c, $\alpha_2$(I). Lane 1, control mice; lane 2, CY 222-treated control mice; lane 3, diabetic KK mice; lane 4, CY 222-treated KK mice.

electrophoretic patterns of pepsin soluble collagen from all mice groups. Quantitative densitometric analysis of the data show significant variations and are summarized in Fig. 2. As we have previously shown [19], the percentage of type III collagen was significantly higher in KK mice skin as compared to control mice. After treatment of KK mice with CY 222 the proportion of type III collagen decreased significantly ($P < 0.01$).

Furthermore, each gel fraction containing $\alpha_1$(I) or $\alpha_1$(III) chains was hydrolyzed and the specific activity of hydroxy($^3$H)proline determined. The data obtained for type I collagen did not exhibit significant variations (Fig. 3A). However, we noted a slight increase of type I collagen biosynthesis in the KK mice treated group as compared to non-treated diabetic mice. The incorporation of radioactivity in type III collagen of KK mice skin was higher than in controls (Fig. 3B) as we have previously found [19]. This increased biosynthesis of type III collagen was corrected by treatment with CY 222 ($P < 0.01$).

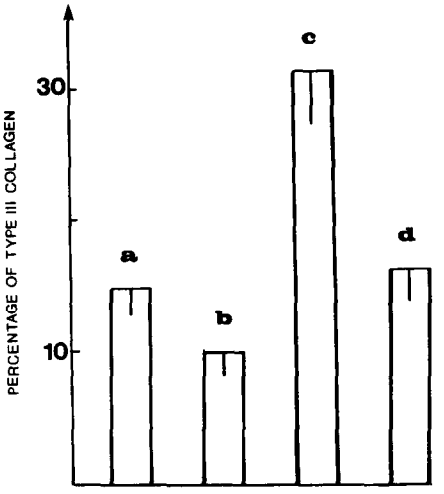


Fig. 2. Relative proportion of type III collagen. The percentage of type III collagen was determined using densitometric analysis of SDS PAGE (see Fig. 1). The results are expressed as $\frac{[\text{type III}] \times 100}{[\text{type I}] + [\text{type III}]}$. Each value is the mean (±SE) of six independent determinations. a, control mice; b, CY 222-treated control mice; c, KK mice; d, CY 222-treated KK mice. Significance of the difference between a and c, $P < 0.01$; difference between c and d, $P < 0.01$.

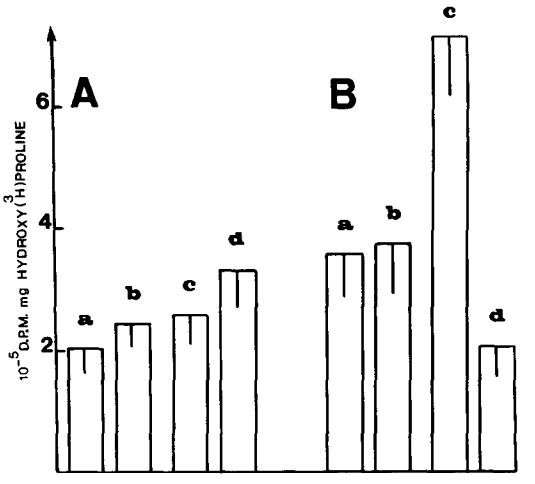


Fig. 3. Specific radioactivity of hydroxy ($^3$H)proline in type I and type III collagens. Skin explant culture and labeling procedures are described in methods. $\alpha_1$ chains from type I and type III collagens were separated as described in Fig. 1. Specific radioactivity of hydroxy ($^3$H)proline was determined in each isolated $\alpha$ chains as indicated in text. Each value is the mean (±SE) of six independent experiments. (A) Type I collagen; (B) type III collagen a, b, c and d as in Fig. 2. In B, significance of difference between a and c: $P < 0.01$; between c and d: $P < 0.01$.

Independently of the previous experiments, skin samples from all mice groups were digested with CNBr. The CNBr peptide mixtures were separated by SDS PAGE (data not shown). Densitometric analysis using $\alpha_1$(I)CB8 and $\alpha_1$(III)CB8 is indicated in Table 1 and shows a significant increase of type III collagen proportion in diabetic mice skin. This alteration is modified by treatment of KK mice with CY 222. The proportion of type III collagen decreased to normal levels.

*Fibronectin biosynthesis*

Fibronectin was expressed as the ratio of the radioactivity present in the immunoprecipitate to total incorporated radioactivity. The specificity of the immunoprecipitation procedure is shown in Fig. 4. The differences were not significant between treated and non-treated mice, diabetic or controls for the radioactivity of the fibronectin immunoprecipitated from the culture medium. The percentage ranged between 0.36 (±0.06) and 0.29 (±0.03) for control and CY 222 treated mice to 0.49 (±0.03) and 0.40 (±0.06) for diabetic and diabetic CY 222 treated mice.

In SDS extracts, fibronectin biosynthesis was increased (117%, $P < 0.01$) in diabetic mice (Fig. 5). When control mice were treated with heparin fragments there was no significant variation in fibronectin biosynthesis by comparison to non-treated control mice, only a slight (non-significant) decrease of fibronectin biosynthesis could be noted in the treated mice. There was, however, a significant decrease ($P < 0.01$) of fibronectin biosynthesis in SDS extracts of treated diabetic mice, as compared to the non-treated diabetic mice, as shown in Fig. 5.

## DISCUSSION

We have examined the *in vivo* effects of low-

Table 1. Determination of proportion of type III collagen in skin by CNBr peptide analysis

| Mice group | Percentage of type III collagen |
|---|---|
| A Control mice | 14 (±2) |
| B Control mice + CY 222 | 11 (±1) |
| C KK mice | 29 (±3)* |
| D KK mice + CY 222 | 16 (±2)** |

Analysis and calculation using the relative proportions of $\alpha_1$(I)CB8 and $\alpha_1$(III)CB8 as indicated in methods. The results are the mean (±SD) of six independent determinations.

* Difference as compared to control group A, $P < 0.01$.

** Difference as compared to KK group C, $P < 0.01$.

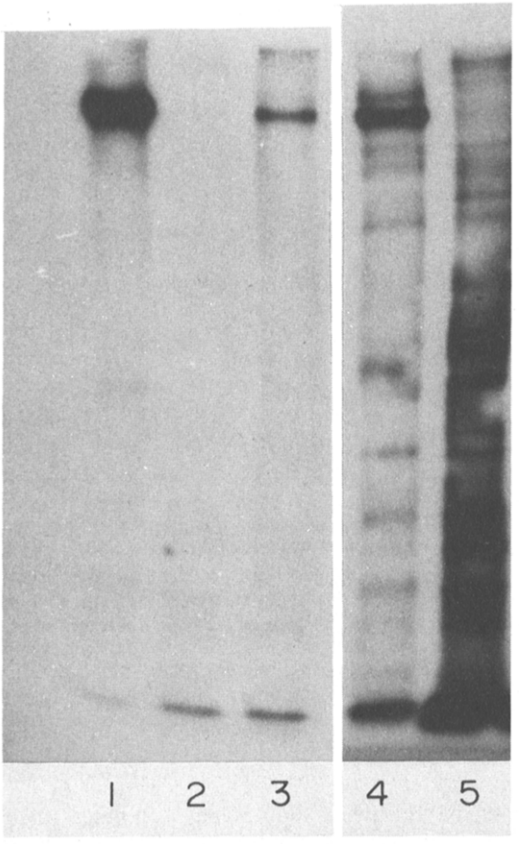


Fig. 4. Fluorography of SDS–PAGE electrophoresis showing the specificity of the fibronectin immunoprecipitate: 1, immunoprecipitate of the incubation medium of skin explants; 2, supernatant of the immunoprecipitated SDS skin extracts; 3, immunoprecipitate of the SDS extract of mouse skin explants; 4, total proteins in the incubation medium; 5, total proteins in the SDS extract. For the details of the extraction conditions of skin explants and for the SDS–PAGE electrophoresis, see Materials and Methods.

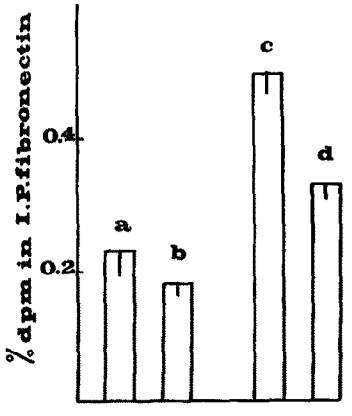


Fig. 5. Incorporation of $^{35}$S-methionine in mouse skin explants. Percentage of radioactivity incorporated in immunoprecipitable fibronectin. Skin explant culture and labelling procedures are described in Materials and Methods. Each value is the mean (±SE) of six determinations a, b, c and d as in Fig. 2. Difference between a and c are significant: $P < 0.01$; between c and d: $P < 0.01$.

molecular weight heparin fragments (referred as CY 222) on the biosyntheses of collagen and fibronectin in the diabetic KK mice skin. Our results indicate that repeated injections of CY 222 to these hereditary diabetic mice decreased the synthesis of fibronectin and reduced the proportion of type III collagen towards values found in control C57 black mice.

Diabetes mellitus is accompanied by various defects in ECM [10–22]. Several authors have described the disturbances of capillary basement membranes [10, 11, 13]. Furthermore alterations of structural glycoproteins were shown [17, 18] as well as modifications of proteoglycans [14, 36, 37] and qualitative and quantitative variations of collagen metabolism [14, 18–20].

We have previously demonstrated an increase of type III to type I collagen ratio in the skin of hereditary diabetic KK mice [19]. We have also found an increased fibronectin biosynthesis in the same model [21, 22]. Similar modifications were evidenced in human diabetic ECM [20–22], showing that diabetes mellitus can be considered as a connective tissue disease.

Recent studies have indicated that ECM components interact with each other and with cells [38–41]. For example, extracellular glycosaminoglycans affect the biosynthesis of collagen and proteoglycans [34–37]. Particularly the regulatory role of heparin-like glycosaminoglycans has been demonstrated concerning the biosynthesis of collagenous and non-collagenous proteins [25, 26].

Interestingly, addition of heparin to smooth muscle cells in culture decreased the amount of type III relative to type I procollagen [25]. Studies on a similar model in our laboratory have demonstrated a relative inhibition of fibronectin and type III collagen production by smooth muscle cells treated with CY 222 [27].

In the present report we have been able to perform an *in vivo* regulation of the biosyntheses of type III collagen and fibronectin in diabetic KK mice skin.

As far as collagen biosynthesis is concerned, CY 222 decreased the proportion of type III collagen in diabetic mice skin demonstrated with CNBr peptides analysis of native tissues and independently by SDS PAGE analysis of pepsin solubilized material. Furthermore, a direct effect of CY 222 decreasing type III collagen Hypro ($^{3}$H) specific activity was also documented. These results indicate a decrease of newly synthetized type III collagen in CY 222-treated tissues.

For fibronectin, we demonstrated an increased processing of newly synthesized fibronectin (monomeric form) to the pericellular (SDS-extractible) polymeric form in diabetic mouse skin fibroblast cell cultures [22]. The present results obtained in *ex vivo* experiments confirm these findings and show that *in vivo* CY 222 treatment of diabetic mice decreased the processing of newly synthetized fibronectin and its accumulation in the pericellular stroma.

*Acknowledgements*—Supported by C.N.R.S. (UA 1174), Conseil scientifique de l'Université Paris XII, the Choay Laboratories and their Director Dr Jean Choay. We gratefully acknowledge the able technical assistance of Mr J. C. Derouette.

## REFERENCES


1. Gay S and Miller EJ, *Collagen in the Physiology and Pathology of Connective Tissue*, pp. 34–106. G. Fischer, Stuttgart, 1978.
2. Hawkes S and Wang JL, *Extracellular Matrix*. Academic Press, New York, 1982.
3. Hay ED (Ed.), *Cell Biology of Extracellular Matrix*. Plenum Press, New York, 1981.
4. Lapière CM and Nusgens B, Collagen pathology at the molecular level, In: *Biochemistry of Collagen* (Eds. Ramachandran, GV and Reddi AH), pp. 337–447. Plenum Press, New York, 1976.
5. McKusick VA, *Heritable Disorders of Connective Tissue*, Mosby Co., St. Louis, MO, 1972.
6. Prockop DJ, Chu M, Dewer W, Myers JC, Pihlajaniemi T, Ramirez F and Sippola M, Mutations in osteogenesis imperfecta leading to the synthesis of abnormal type I procollagens. *Ann NY Acad Sci* **460**: 289–297, 1983.
7. Gerlach U, Pott G, Rauterberg S and Voss B, *Connective Tissue of the Normal and Fibrotic Human Liver*. Georg Thieme, Stuttgart, 1982.
8. Madri SA and Furthmayer H, Collagens polymorphism in the lung. *Hum Pathol* **11**: 353–366, 1980.
9. Barnes MJ, Collagen in atherosclerosis. *Collagen Rel Res* **5**: 65–97, 1985.
10. Kefalides NA, Basement membrane research in diabetes mellitus. *Collagen Rel Res* **1**: 295–299, 1981.
11. Spiro RG, Search for a biochemical basis of diabetic microangiopathy. *Diabetologia* **12**: 1–14, 1976.
12. Andreassen TT, Seyer-Hansen K, Oxlund H, Biochemical changes in connective tissues induced by experimental diabetes. *Acta Endocr* **98**: 432–436, 1981.
13. Rohrbach DH and Martin GR, Structure of basement membrane in normal and diabetic mice. *Ann NY Acad Sci* **401**: 203, 1982.
14. Pihlajaniemi T, Myllyla R, Kirivikko KI and Tryggvason K, Effects of streptozotocin diabetes, glucose and insulin on the metabolism of type IV collagen and proteoglycan in murine basement membrane forming EHS tumor tissue. *J Biol Chem* **257**: 14914–14920, 1982.
15. Hasslacher C, Reichenbacher R, Gechter F and Timpl R, Glomerular basement membrane synthesis and serum concentration of type IV collagen in streptozotocin diabetic rats. *Diabetologia* **26**: 150–154, 1984.
16. Schneider SL and Kohn RR, Effects of diabetes mellitus on the solubility of collagen from human skin, tracheal cartilage and dura mater. *Exp Gerontol* **17**: 185–194, 1982.
17. Karttunen T, Risteli J, Autio-Harmainen HB and Risteli L, Effect of age and diabetes on type IV collagen and laminin in human kidney cortex. *Kidney Int* **30**: 586–591, 1986.
18. Högemann B, Voss B, Alternwerth FJ, Schneider M, Rauterberg J and Gerlach U, Concentration of 7S collagen and Laminin P1 in sera of patients with diabetes mellitus. *Klin Wochensch* **64**: 382–385, 1986.
19. Kern P, Moczar M and Robert L, Biosynthesis of skin collagens in normal and diabetic mice. *Biochem J* **182**: 337–345, 1979.
20. Kern P, Sebert B and Robert L, Increased type III: type I collagen ratio in diabetic human conjunctival biopsies. *Clin Physiol Biochem* **4**: 113–119, 1985.
21. Labat-Robert J, Phan Thanh L, Leutenegger M and Robert L, Biosynthesis of dermal fibronectin in experimental and human diabetes. *Biochem Soc Trans* **12**: 673–674, 1984.
22. Phan-Thanh L, Robert L, Derouette JC and Labat-Robert J, Increased biosynthesis and processing of fibronectin in diabetic fibroblasts. *Proc Natl Acad Sci USA* **84**: 1911–1914, 1987.
23. Wever J, Schachtschabel DO, Sluke G and Wever G, Effect of short or long term treatment with exogenous glycosaminoglycans on growth and glycosaminoglycan synthesis of human fibroblasts (WI-38) in culture. *Mech Age Devel* **14**: 89–99, 1980.
24. Ivaska K, Effect of extracellular glycosaminoglycans on the synthesis of collagen and proteoglycans by granulation tissue cells, *Acta Physiol Scand* **494**: 1–53, 1981.
25. Majack RA and Bornstein PJ, Heparin and related glycosaminoglycans modulate the secretion of collagen and proteolgycans by granulation tissue cells, *Cell Biol* **99**: 1688–1695, 1984.
26. Majack RA and Bornstein PJ, Heparin regulates the collagen phenotype of vascular smooth muscle cells: induced synthesis of an Mr 60000 collagen. *Cell Biol* **100**: 613–619, 1985.
27. Kern P, Labat-Robert J, Derouette JC and Robert L, manuscript in preparation.
28. Kern P, Picard J, Caron M and Veissiere D, Decreased binding of insulin to liver plasma membrane receptors in hereditary diabetic mice. *Biochem Biophys Acta* **389**: 281–289, 1975.
29. Bârzu T, Van Rijn JLML, Petitou M, Molho P, Tobelem G, and Cean J, Endothelial binding sites for heparin. *Biochem J* **238**: 847–854, 1986.
30. Opperman W, Ehrenreich W, Patel D, Espinoza T and Camerini-Davalos RA, Related factors in the progression of microangiopathy in KK mice. In: *Early Diabetes Advances in Metabolic Disorders*, Vol. 2 (Eds. Camerini Davalos RA and Cole HS), pp. 271–280. Academic Press, New York, 1973.
31. Kern P, Robert L, Courtois Y and Laurent M, Selective decrease of type I collagen synthesis in Fraser mice skin. *Biochim Biophys Acta* **286**: 174–179, 1985.
32. Bergman I, Loxley R, Two improved and simplified methods for the spectrophotometric determination of hydroxyprolin. *Anal Chem* **35**: 1861–1863, 1963.
33. Laemmli UK, Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond)* **227**: 680–685, 1970.
34. Sykes B, Puddle B, Francis M and Smith R, The estimation of two collagens from human dermis by interrupted gel electrophoresis. *Biochem Biophys Res Commun* **72**: 1472–1480, 1976.
35. Light ND, Estimation of type I and III collagens in whole tissue by quantitation of CNBr peptides on SDS-polyacrylamide gels. *Biochim Biophys Acta* **702**: 30–36, 1982.
36. Weiss RE, Gorn AH and Nimni E, Abormalities in the biosynthesis of cartilage and bone proteoglycans in experimental diabetes. *Diabetes* **30**: 670–677, 1981.
37. Rohrbach DM, Hassel JR, Kleinman HK and Martin GR, Alterations in the basement membrane (heparan sulfate) proteoglycan in diabetic mice. *Diabetes 31*: 185–188, 1982.
38. Bissel MJ, Hall HG and Parry G, How does the extracellular matrix direct gene expression? *J Theor Biol* **99**: 31–68, 1982.
39. Bitterman PB, Rennard SI, Adelberg S and Crystal RG, Role of fibronectin as a growth factor for fibroblasts. *J Cell Biol* **97**: 1925–1932, 1983.
40. Aumailley M, Krieg T, Razaka G, Muller PK and Bricaud H, Influence of cell density on collagen biosynthesis in fibroblast cultures. *Biochem J* **206**: 505–510, 1982.
41. Benya PD and Shaffer JD, Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**: 215–224, 1982.