Inhibition of collagen fiber formation by dextran sulfate in hepatic cells

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ABSTRACT

When the hepatic cell strain (M) was cultured, the cells were covered with a collagen fiber network. On the other hand, when M cells were cultured in the presence of dextran sulfate at 10 $\mu\text{g/ml}$, no collagen fiber network was formed. The inhibitory effect of dextran sulfate was concentration-dependent; no significant effect was observed below 1 $\mu\text{g/ml}$, inhibition was evident at 5 $\mu\text{g/ml}$, and no collagen fibers were formed above 10 $\mu\text{g/ml}$. Western blotting showed that collagen in M cells differed from rat type I collagen.

Liver cirrhosis is a fatal disease in humans and occasionally leads to liver cancer. At present, there is no effective treatment for this disease. In the northern part of Japan, the ethanol extract of berries of Japanese amelopsis has been used for liver diseases as a folk medicine. In *in vitro* experiments, it was shown that this ethanol extract changed thick collagen fibers into numerous thin fibers in hepatic cells (M) (10). It is possible that carbohydrates contained in the ethanol extract would affect cells. Our recent studies showed that lignin derivatives (acetyl and sulfonyl) (19) and dextran sulfate (20) activated macrophages derived from rat bone marrow and induced multinucleated giant cells. These studies led me to examine whether or not the polyanions inhibit the collagen fiber formation.

To my knowledge, only the M cells produce a collagen fiber network (14, 15), although the production of collagen or procollagen was detected in fibroblasts (3, 5, 12, 23) or chondrocytes (6) in culture. M cells were kindly supplied by Dr Toshiko Takaoka of Memorial Institute of Tissue Culture. This cell line was established by Katsuta and Takaoka (9) from normal rat liver cells, and then were transformed by the treatment with a high concentration of 4-dimethyl-aminoazobenzene.

In M cells in culture, thin fiber-like structures appeared, and increased with time. The structures were identified as collagen fibers by silver impregnation (14, 15).

When M cells were cultured for 10 days, they were covered with a collagen fiber network as

shown in Fig. 1A. Collagen fibers were stained red with Sirius red F3BA, and other proteins were stained green with fast green FCF (13). When M cells were cultured in the presence of dextran sulfate at 10 $\mu\text{g/ml}$, no collagen fiber was formed (Fig. 1B).

The inhibitory effect of dextran sulfate on collagen fiber formation was concentration-dependent: no significant effect was observed below 1 $\mu\text{g/ml}$, inhibition was evident at 5 $\mu\text{g/ml}$, and no collagen fibers were formed above 10 $\mu\text{g/ml}$. A similar dose-response effect of dextran sulfate was observed in the multinucleation of macrophages (20). At 10 $\mu\text{g/ml}$, dextran sulfate did not affect the growth of M cells.

In order to determine whether dextran sulfate inhibits collagen synthesis, cell lysates were examined by Western blotting. Rat tail tendon collagen was used as a standard for rat type I collagen. The proteins on the nitrocellulose sheets were exposed to antibodies against rat type I or III collagen. The collagen in M cells was identified as type I by carboxymethylcellulose column chromatography (8). In addition, types I, III and IV collagens were identified in the bovine liver parenchyma by electron microscopy using peroxidase-labeled antibody (16). The collagen in M cells as well as in rat tail tendon reacted immunologically with the antibody against rat type I collagen, but did not with that against rat type III collagen. Standard rat type I collagen showed $\alpha 1(\text{I})$, $\alpha 2(\text{I})$ and β bands as shown in Fig. 2. However, the cell lysate obtained from M cells

Fig. 1 Micrographs of M cells stained with Sirius red F3BA and fast green FCF. M cells were cultured on the cover-slips in the tissue culture glass tubes for 10 days in the absence (A) or presence (B) of dextran sulfate ($10 \mu\text{g/ml}$), and washed with phosphate-buffered saline without Mg^{2+} and Ca^{2+} (PBS), pH 7.4. The washed cells were kept in 10% formaldehyde in PBS overnight at room temperature, and then exposed to a slow water stream for 4–6 h to remove formaldehyde from the cells at room temperature. The cells were then stained for 30 min at room temperature using the 'Collagen Stain Kit' (Collagen Gijutsu Kenkyukai, Tokyo, Japan). Stained cells were washed with water and dried in air. Dextran sulfate (molecular weight, approximately 8,000) was obtained from Sigma Chemical Company (St Louis, MO, U.S.A.). $\times 200$

showed two strong bands. One band appeared at the position between the $\alpha 1(\text{I})$ and β bands, and another at the position of the $\alpha 1(\text{I})$. The former might be the $\text{pro}\alpha 1(\text{I})$. Judging from these results, the majority of the collagen molecules in M cells seems to consist of $[\alpha 1(\text{I})]_3$. Thus, it is likely that the structure of collagen in M cells differs from that of rat type I collagen. Hata *et al.* (8) showed that the ratio of $\alpha 1/\alpha 2$ in M cells was much higher than the value expected for type I collagen, and they suggested the existence of abnormal collagen composed of three identical $\alpha 1(\text{I})$.

There were many broad bands which moved faster than the main bands. These broad bands may represent immature collagens or collagens metabo-

lized in cells before the cell harvest. Collagenase inhibitor, phenylmethylsulfonyl fluoride (PMSF), antipain and pepstatin A were added to the cell lysate to prevent proteolysis. Further studies are necessary to identify the nature of many collagen-related bands.

When M cells were treated with dextran sulfate, the quantity of the two major bands significantly decreased. However, many minor bands were still detected. Bateman and Golub (1) reported that dextran sulfate contributed to the processing of procollagen to collagen in skin fibroblasts in culture. Since the collagen components ($\text{pro}\alpha 1$, $\alpha 1$, $\text{pro}\alpha 2$ and $\alpha 2$) were not found in M cells cultured in the presence of dextran sulfate, the failure to

Fig. 2 Western blotting and gel electrophoresis of cell lysates. M cells were cultured in the presence or absence of dextran sulfate (10 μ g/ml), for 10–14 days. The cells washed with PBS were harvested, centrifuged at 2,000 rpm for 10 min, and the cell pellets were frozen at -80°C . After thawing, cells were homogenized in 0.1–0.2 ml of 50 mM Tris-HCl, pH 7.4, containing 20 mM EDTA, 0.2 mM PMSF, 0.5 mg/ml antipain, 0.28 mg/ml pepstatin A, and 1 mg/ml collagenase inhibitor with Phycotron, NITI-ON I Rikakikai Seisakusho (Tokyo, Japan) at 25,000 rpm for 20 sec two times. The homogenates were centrifuged at 10,000 rpm for 10 min, and the supernatant was used for SDS-gel electrophoresis. Twenty microliter aliquots of the cell lysate were mixed with an equal volume of 140 mM Tris-HCl, pH 6.8, containing 22.4% glycerol, 10% 2-mercaptoethanol, 6% sodium dodecyl sulfate, and 0.02% bromophenol blue (21). The samples were heated at 100°C for 5 min and electrophoresed in 10% polyacrylamide-gel and 0.1% sodium dodecyl sulfate by the method of Laemmli (11). After electrophoresis, Western blotting was performed by a modification of the method of Burnette (2). Electrophoretic transfer was carried out in 25 mM Tris-glycine, pH 8.3, containing 20% methanol (v/v) with a Blotting Set (Funakoshi, Tokyo, Japan). After transfer, the nitrocellulose sheets were immediately immersed in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl (TBS) to remove sodium dodecyl sulfate, and were kept in TBS

containing 3% bovine serum albumin at 4°C overnight. The sheets were then incubated with diluted anti-collagen serum (1:500) in TBS containing 3% bovine serum albumin for 1 h at room temperature, and washed three times with TBS containing 0.05% Tween 20. To visualize the bands, the sheets were incubated with a second antibody to rabbit IgG (5 μ g/ml) conjugated with peroxidase in TBS containing 3% bovine serum albumin for 1 h at room temperature. The sheets, washed three times with TBS containing 0.05% Tween 20 to remove the free second antibody, were incubated with 3,3'-diaminobenzidine (0.2 mg/ml) in TBS containing H_2O_2 . Anti-rat type I collagen was purchased from Advance (Tokyo, Japan), and anti-rat type III collagen was from Chemicon International (Temecula, CA, U.S.A.). The peroxidase conjugated goat anti-rabbit Ig Fc fragment purified by affinity chromatography was purchased from Organo Technica (Cappel) (Durham, NC, U.S.A.). Standard rat type I collagen was isolated from rat tail tendon which was minced and stirred in 0.5 M acetic acid at 4°C overnight. After centrifugation at 100,000 g for 1 h, the supernatant was neutralized by the addition of 5 N NaOH, and NaCl was added to make a 4.4 M NaCl solution. The precipitate was collected by centrifugation at 10,000 rpm for 30 min, and used as standard rat type I collagen without further purification. Western blotting with antibody to rat type I collagen. Lane 1, rat type I collagen; lane 2, M cell lysate; lane 3, lysate of M cells cultured in the presence of dextran sulfate (10 μ g/ml)

form collagen fibers may not be due to the inhibitory effect of dextran sulfate on procollagen processing or collagen secretion. It was reported that dextran sulfate interacted with the viral glycoprotein gp120 that is involved in HIV binding to cells (17). Scott (18) showed electronmicroscopically that the proteoglycan molecules interacted with the collagen molecule in rat tail tendon. It is possible that dextran sulfate inhibits the interaction of collagen with certain glycoproteins or proteoglycans that are important extracellular components for collagen fiber formation. In fact, collagen fibers seem to be surrounded with other materials (Fig. 1A).

Lignin derivatives (acetylated and sulfonated) induced multinucleation of macrophages (19), and showed anti-viral activity *in vitro* (22, 24), as did dextran sulfate (25). However, lignin derivatives did not affect the formation of collagen fibers in M cells (data not shown). Dextran (average molecular weight, approximately 11,000) also did not inhibit collagen fiber formation (data not shown). Judging from these results, multinucleation of macrophages and anti-viral phenomena appear to be induced by polyanions such as lignin derivatives and

dextran sulfate. On the other hand, both dextran structure and its negative charges seem to be required in the inhibition of collagen fiber formation.

It was reported that interferon- γ inhibited the synthesis of collagen mRNA *in vitro* (6, 23) and *in vivo* (7), and that α, α' -dipyridyl inhibited prolyl and lysyl hydroxylases and resulted in accumulation of unhydroxylated procollagen (4). In addition, the monovalent ionophore monensin inhibited the secretion of both procollagen and fibronectin from human fibroblasts in culture (12). Since collagen synthesis is an important metabolic pathway in normal metabolism *in vivo*, these agents are not applicable for chemotherapy. Dextran sulfate could be a chemotherapeutic agent, if this compound affects only abnormal collagen fiber formation. Even if dextran sulfate could not be directly used for chemotherapy of liver cirrhosis, the fact that dextran sulfate inhibits collagen fiber formation may contribute to the development of drugs for this lethal disease.

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REFERENCES

1. BATEMAN J. F. and GOLUB S. B. (1990) Assessment of procollagen processing defects by fibroblasts cultured in the presence of dextran sulfate. *Biochem. J.* **267**, 573-577
2. BURNETTE W. N. (1981) "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**, 195-203
3. CONRAD G. W., DESSAU W. and VON DER MARK K. (1980) Synthesis of type III collagen by fibroblasts from the embryonic chick cornea. *J. Cell Biol.* **84**, 501-512
4. FIEDLER-NAGY C., BRUCKNER P., HAYASHI T. and PROCKOP D. J. (1981) Isolation of unhydroxylated type I procollagen folding of the protein *in vitro*. *Arch. Biochem. Biophys.* **212**, 668-677
5. GAY S., MARTIN G. R., MÜLLER P. K., TIMPL R. and KÜHN K. (1976) Simultaneous synthesis of types I and III collagen by fibroblasts in culture. *Proc. Natl. Acad. Sci. USA* **73**, 4037-4040
6. GOLDRING M. B., SANDELL L. J., STEPHENSON M. L. and KRANE S. M. (1986) Immune interferon suppresses levels of procollagen mRNA and type II collagen synthesis in cultured human articular and costal chondrocytes. *J. Biol. Chem.* **261**, 9049-9056
7. GRANSTEIN R. D., MURPHY G. F., MARGOLIS R. J., BYRNE M. H. and AMENTO E. P. (1987) Gamma-interferon inhibits collagen synthesis *in vivo* in the mouse. *J. Clin. Invest.* **79**, 1254-1258
8. HATA R., NINOMIYA Y., NAGAI Y., SAKAKIBARA K. and TSUKADA Y. (1978) Active synthesis of collagen by albumin-producing liver parenchymal cell clones in culture. *Proc. Japan Acad.* **54** (B), 391-396
9. KATSUTA H. and TAKAOKA T. (1968) Cytobiological transformation of normal rat liver cells by treatment with 4-dimethylaminoazobenzene after Nagisa Culture. In *Cancer Cells in Culture* (ed. KATSUTA H.) University of Tokyo Press, Tokyo, University Park Press, Baltimore and State College, Pennsylvania, pp. 321-334
10. KATSUTA H. and TAKAOKA T. (1980) An attempt in tissue culture at preventing and treating the collagen fiber formation of liver cells. *Jap. J. Exp. Med.* **50**, 275-282
11. LAEMMLI U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
12. LEDGER P. W., UCHIDA N. and TANZER M. L. (1980) Immunocytochemical localization of procollagen and fibronectin in human fibroblasts: Effects of the monovalent ionophore, monensin. *J. Cell Biol.* **87**, 663-671
13. LÓPEZ-DE LEÓN A. and ROJKIND M. (1985) A simple micro-method for collagen and total protein determination in formalin-fixed paraffin-embedded sections. *J. Histochem. Cytochem.* **33**, 737-743
14. SAKAKIBARA K., SAITO M., UMEDA M., ENAKA K. and TSUKADA Y. (1976) Native collagen formation by liver parenchymal cells in culture. *Nature* **262**, 316-318
15. SAKAKIBARA K., TAKAOKA T., KATSUTA H., UMEDA M. and TSUKADA Y. (1978) Collagen fiber formation as a common property of epithelial liver cell lines in culture. *Exp. Cell Res.* **111**, 63-71
16. SANO J., SATO S., ISHIZAKI M., YAJIMA G., KONOMI H., FUJIWARA S. and NAGAI Y. (1981) Types I, III and IV (basement membrane) collagens in the bovine liver parenchyma: Electron microscopic localization by the peroxidase-labeled antibody method. *Biomedical Res.* **2**, 546-551
17. SCHOLS D., PAUWELS R., DESMYTER J. and DE CLERCQ E. (1990) Dextran sulfate and other polyanionic anti-HIV compounds specifically interact with the viral gp 120 glycoprotein expressed by T-cells persistently infected with HIV-1. *Virology* **175**, 556-561
18. SCOTT J. E. (1980) Collagen-proteoglycan interactions. Localization of proteoglycans in tendon by electron microscopy. *Biochem. J.* **187**, 887-891
19. SORIMACHI K., AKIMOTO K., YAMAZAKI S., NIWA A., TODA S. and YASUMURA Y. (1990) Multinucleation of macrophages with water-solubilized lignin derivatives *in vitro*. *Cell Struct. Funct.* **15**, 317-322
20. SORIMACHI K., AKIMOTO K., YAMAZAKI S., TODA S., NIWA A. and YASUMURA Y. (1991) Multinucleation of macrophages with dextran sulfate *in vitro*. *Agr. Biol. Chem.* **55**, 241-242
21. SORIMACHI K., IEIRI T., KOBAYASHI M., HORIUCHI R. and YASUMURA Y. (1989) Inhibitory effect of dibutyl cyclic adenosine monophosphate on the induction of alkaline phosphatase in human fetal liver cell line. *Biochim. Biophys. Acta* **1010**, 72-77
22. SORIMACHI K., NIWA A., YAMAZAKI S., TODA S. and YASUMURA Y. (1990) Anti-viral activity of water-solubilized lignin derivatives *in vitro*. *Agr. Biol. Chem.* **54**, 1337-1339

23. STEPHENSON M. L., KRANE S. M., AMENTO E. P., McCROSKERY P. A. and BRYNE M. (1985) Immune interferon inhibits collagen synthesis by rheumatoid synovial cells associated with decreased levels of the procollagen mRNAs. *FEBS Lett.* **180**, 43-50
24. SUZUKI H., OKUBO A., YAMAZAKI S., SUZUKI K., MITSUYA H. and TODA S. (1989) Inhibition of the infectivity and cytopathic effect of human immunodeficiency virus by water-soluble lignin in an extract of the culture medium of *Lentinus edodes* mycelia (LEM). *Biochem. Biophys. Res. Commun.* **160**, 367-373
25. UENO R. and KUNO S. (1987) Dextran sulphate, a potent anti-HIV agent in vitro having synergism with zidovudine. *Lancet*, **I**, 1379