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Effects of retinol on proliferation, cell adherence and extracellular matrix synthesis in a liver myofibroblast or lipocyte cell line (GRX)

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Summary. We have studied the effect of retinol on an established murine cell line (GRX), representative of liver connective tissue cells. This cell line has myofibroblast characteristics; under retinol treatment it is induced into the lipocyte (Ito-cell) phenotype. Retinol decreased the proliferation rate in the entire cell population. It increased cell adherence to the substrate, which was correlated with the increased secretion of fibronectin. Collagen secretion was specifically decreased, whilst the total protein secretion remained stable. Heparan sulphate was decreased in the pericellular compartment, but other glycosaminoglycans were not affected by retinol treatment. Modulations of pericellular components induced by retinol may alter the relations among liver mesenchymal cells, and may be related to vitamin-A-induced modifications of the homoeostasis of hepatic connective tissue and hepatic fibrosis.

Keywords: retinol, lipocyte, Ito-cell, myofibroblast, collagen, glycosaminoglycan, fibronectin, hepatic fibrosis

In normal liver, the ratio between connective tissue cells and hepatocytes is low. However, many chronic injuries of hepatic tissue lead to the formation of extensive fibrosis, with large increases in both connective tissue cell populations and extracellular matrix. Activation of liver connective tissue cells (LCTC) is thus associated, and probably causally related, with the development of hepatic fibrosis.

The two major connective tissue cell populations of hepatic lobules are described as peri-sinusoidal cells with myofibroblastic characteristics and lipocytes (fat-storing cells, Ito-cells). They are frequently considered to be two phenotypes of a single hepatic connective tissue cell lineage (Bioulac-Sage & Balabaud 1985). Hepatic lipocytes were observed to be associated with collagen fibres in normal and fibrotic hepatic parenchyma (Kent *et al.* 1976). In an in-vivo study of lipocytes, Clement *et al.* (1984) have demonstrated synthesis of extracellular matrix molecules, involving fibronectin and collagen types I, III and IV. In-vitro studies have also indicated that lipocytes are one of

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the major potential sources of collagen and associated extracellular matrix molecules in liver tissue (Senoo *et al.* 1984; Friedman *et al.* 1985; Gressner & Haarman 1988; Gressner *et al.* 1989).

An increase in number of lipocytes is known to be associated with hepatic cirrhosis due to hypervitaminosis A (Jacques *et al.* 1979). Conversely, an increase in number of liver myofibroblasts, associated with a proportional decrease in number of lipocytes, was observed in chronic alcohol intoxication in both humans and experimental models (Minato *et al.* 1983; Mak *et al.* 1984). The transition of lipocytes into activated collagen-secreting myofibroblasts has therefore been proposed as one mechanism leading to increased fibrogenesis in alcohol-induced cirrhosis and fibrosis (Mak *et al.* 1984). The expression of lipocyte versus myofibroblast phenotype therefore may be one of the principal control points for the homoeostasis of normal and pathological secretion of extracellular matrix in liver.

We have established a continuous cell line, termed GRX, from fibro-granulomatous lesions induced in mouse liver by schistosomal infection (Borojevic *et al.* 1985a). It has been maintained since then by serial passages and has been recloned several times: it has now undergone more than 500 population doublings. Nevertheless, GRX cell line is dependent upon a high concentration of serum (maximal growth at 30% foetal bovine serum), it does not grow in soft agar, and is not tumorigenic in syngeneic mice, indicating that this is not a fully transformed cell line. It has myofibroblastic characteristics, secretes a complex extracellular matrix, and displays *in vitro* biological and biochemical characteristics of primary cell lines isolated from human or mouse fibrotic livers (Borojevic *et al.* 1985b; Monteiro & Borojevic 1987). Like the in-vivo induction of lipocyte hyperplasia by hypervitaminosis A, retinol treatment of GRX cells induces their conversion into the lipocyte phenotype (Margis & Borojevic 1989a; Borojevic *et al.* 1990). Differential study of lipid accumulation and analysis of induction of enzymes involved in lipid synthesis have shown that the conversion of GRX cells displaying the myofibroblast phenotype into lipocytes represents induction of the full expression of a different phenotype, and not simply a metabolic response of the cell line to a specific stimulus (Margis & Borojevic 1989a; Borojevic *et al.* 1990). Since this conversion has been related to the control of liver fibrosis, we have studied the in-vitro modification of proliferation, cell adherence and secretion of the three major components of extracellular matrix (collagen, glycosaminoglycans and fibronectin) in GRX cells, together with their retinol-induced conversion from myofibroblasts into lipocytes.

Materials and methods

Cell cultures

GRX-123 cells were obtained from the Rio de Janeiro Cell Bank (BIO-RIO, Rio de Janeiro, Brazil). They were routinely cultivated in Dulbecco's medium (low glucose, Sigma Chemical Co., St Louis, MO) supplemented with 10% foetal bovine serum (Cultilab, Campinas, SP, Brazil) and 3 g/l of HEPES (Sigma), at 37°C, under normal air atmosphere.

Induction of the fat-storing phenotype

Freshly trypsinized cells were plated (5×10^5 cells/25 cm^2 flask) and left to adhere for 2 h. They were washed with balanced salt solution (BSS) and incubated in the medium described above, supplemented with 10 μM all-*trans* retinol (Sigma), dissolved in absolute ethanol. Control cells were incubated in the standard medium containing 0.1% ethanol.

Cellular growth and proliferation rate

An aliquot of 3×10^5 cells were plated per 25 cm^2 tissue culture flasks. They were left to adhere for 2 h and subsequently incubated in

the control or the retinol-containing media. Every 2 days, three flasks of control and retinol-treated cells were trypsinized and cells were counted.

The proliferation rate of individual clones was estimated by plating cells at cloning density (250 cells per 25 cm^2 flask). Cells were left to adhere overnight and subsequently, the number of cells in 150 different clones was monitored daily, in control and retinol-treated cultures, under a Leitz inverted microscope equipped with phase contrast.

Estimation of cell adherence

The adherence of cells to the plastic substrate was estimated by their resistance to trypsinization. 10^5 cells were plated per well, in 24-well plates (Nunclon). They were left to adhere for 2 h, and incubated for 36 h in control or retinol-containing media. Cells were then washed twice with calcium and magnesium-free BSS (CMF-BSS), and incubated with 1 ml/well of trypsin solution (0.125 mg/ml trypsin and 0.025 mg/ml EDTA in CMF-BSS), with constant agitation on a gyratory shaker at 60 r.p.m. Trypsinization was interrupted after every 2 min in triplicate wells, by fixation with 1 ml of saturated aqueous solution of picric acid, containing 4% formalin. When all wells were trypsinized and fixed, the fixative with detached cells was discarded and the cells that remained adherent were quantified by a modified Coomassie Brilliant Blue R-250 method (CBBR-250) (Margis & Borojevic 1989b).

Collagen quantification

Cells were plated as described for assessing cell growth and proliferation, and left to attain early confluence (approximately 10^6 cells/flask). Cell cultures were washed and incubated with 4 ml of the standard or retinol-containing medium, supplemented with 1 μCi ^{14}C-proline (273 mCi/mM specific activity, New England Nuclear, Boston, Ma), 100 μg/ml ascorbic acid and 50 μg/ml beta-amino-propionitrile (Sigma).

Procollagen was precipitated from cell culture supernatants as described by Ramshaw *et al.* (1984). Briefly, 3 ml of supernatant was neutralized with NaOH supplemented with 50 μg of carrier collagen type I. Collagen was subsequently precipitated by incubating with 10% polyethylene glycol PEG-400 (Sigma), for 24 h at 4°C. The precipitate was solubilized with 0.2 ml glacial acetic acid. Radioactivity was quantified by liquid scintillation counting in a Beckman LS 1803 counter, using the automatic quenching compensation program with internal gamma-emitting radiation source, to express the results in d.p.m.

Total protein was precipitated from 1 ml of supernatant with trichloroacetic acid (TCA, 12.5% w/v, final concentration) for 24 h at 4°C. The precipitate was centrifuged (15 000 *g*, 10 min), washed in 10% TCA and centrifuged again. The pellet was solubilized in formic acid and radioactivity quantified as above.

Fibronectin analysis

Cells were plated as described for collagen synthesis quantification. They were pretreated for 24 h with retinol or with standard medium and incubated for 4 h with 50 μCi of ^{35}S-methionine (generously supplied by Professor M. A. Rabello, Universidade Federal do Rio de Janeiro) in 1 ml of standard medium. Cells were washed with BSS and disrupted by addition of 200 μl of standard urea lysis buffer. Samples were centrifuged and supernatants stored at -20°C for later analysis.

Fibronectin present in the cellular layer was analysed by: (a) standard 5% polyacrylamide gel electrophoresis (PAGE-SDS) in a discontinuous gel buffer (Laemmli 1970), and (b) two dimensional isoelectric focusing followed by PAGE-SDS, described by O'Farrel (1974), using ampholines in the pH 5–7 and 3.5–10 ranges (4:1).

Fibronectin was differentiated from other proteins by immunoblotting (Symington

1984), using goat polyclonal anti-fibronectin and peroxidase-coupled anti-goat-IgG antibodies (both from Sigma). Radiolabelled fibronectin was quantified after exposure of dried gels to X-Omat radiography film (Kodak, São Paulo, Brazil) and by densitometric analysis of autoradiograms.

Glycosaminoglycans analysis

Cell cultures in early confluence were incubated in fresh Eagle's medium without sulphate (Eurobio, Paris, France) containing 10% foetal bovine serum, with or without 10 μM all-*trans* retinol. Each 25 cm^2 culture flask was supplemented with 40 μCi $H_2{}^{35}SO_4$ (Instituto de Pesquisas Energéticas e Nucleares, São Paulo, Brazil), for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. At the completion of this labelling period cells attained full confluence. Media were decanted and cells were washed twice with phosphate-buffered saline (PBS, pH 7.4). Sulphated glycosaminoglycans were isolated from the cell culture as previously described (Silva *et al.* 1990). Briefly, cells were detached by mild trypsinization with 2 ml CMF-BSS containing 0.05% trypsin and 0.05 EDTA, for 10 min at room temperature. Cells were separated from the supernatant by centrifugation: this supernatant contained *pericellular* glycosaminoglycans. The pellet was subsequently digested with 3 ml of the same trypsin solution, for 24 h at 37°C, releasing the *intracellular* glycosaminoglycans. Both fractions were centrifuged and the glycosaminoglycans in the supernatant were precipitated with 95% ethanol and maintained at −10°C for 24 h. The precipitates were collected by centrifugation, washed with 80% ethanol, vacuum dried and dissolved in 0.2 ml distilled water.

^{35}S-sulphated glycosaminoglycans were identified by their electrophoretic mobilities in agarose gels, in parallel with known standards, and by the pattern of their enzymatic degradation with chondroitinases AC and ABC (Silva *et al.* 1989).

Three independent experiments were done in quadruplicate and results were analysed by Student's *t*-test: *P*-values equal or inferior to 0.01 were considered significant.

Results

GRX cells have a fibroblastoid morphology. Like most cell lines of smooth muscle lineage, they showed a relatively low level of contact inhibition, and typically grew at hyperconfluence in the form of 'hills and valleys'. In the presence of retinol, GRX cells became polygonal. They adhered to the substrate more strongly than they adhered to each other, and consequently formed a regular monolayer, with all cells spread directly on the substrate. The present study shows that retinol-treated cells acquire an increased adhesiveness to the substrate, as judged by their increased resistance to trypsin (Fig. 1).

The effect of retinol on GRX cell proliferation is shown in Fig. 2. Both proliferation rate and cell density at the plateau were decreased in retinol-treated cultures. The decreased cell density at confluence can be correlated with the increased adhesiveness

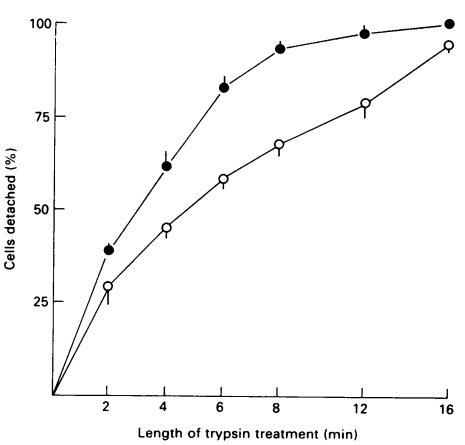

Fig. 1. Resistance of GRX cells to trypsinization, after 36 h incubation in ●, standard and in ○, retinol-supplemented medium, expressed as percentage of cells that detached from the substrate after 0–16 min of trypsin treatment. Mean values and standard deviations of three experiments are given.

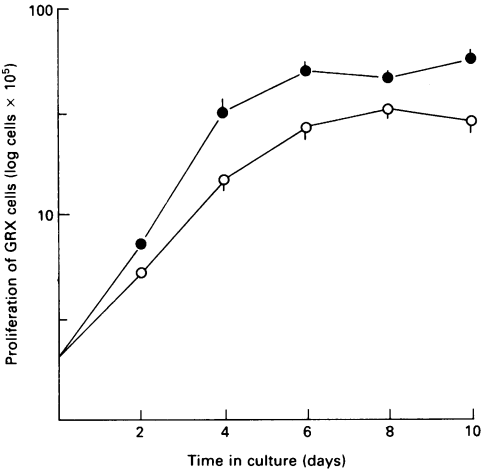

Fig. 2. Proliferation of GRX cells in ●, standard and in ○, retinol-supplemented medium, during 10 days of culture. Results are expressed as log cells × 10^5, and represent mean values and standard deviations of four experiments.

and consequent spreading on the substrate. The proliferation rate was reduced from the start of the culture period, in both low and high density cultures. This could reflect either a decreased proliferation rate of the whole cell population, or the induction of a non-proliferative subpopulation of terminally differentiated cells. This question was addressed in the study of proliferation at cloning density. This showed the proliferation decrease to affect the entire cell population, since no differentiation into two subpopulations was detected (Fig. 3). After 4 days of culture, a net decrease of one division was observed in retinol-treated cells. This inhibition of cell proliferation was dose dependent and increased steadily from 1 to 20 μM retinol (results not shown). These concentrations of retinol were not toxic to GRX cell as determined by the trypan-blue dye exclusion test.

Collagen secretion by GRX cells *in vitro* was influenced by the cell density. Since retinol treatment modified cell proliferation and consequently cell density in parallel cultures, we had to establish whether the observed differences in collagen secretion were due directly to the presence of retinol or to the decrease of cell density. This was done by monitoring the relationship between cell density and the collagen secreted into the supernatant by GRX cells under standard conditions, in the presence of beta-aminopropionitrile (Fig. 4). The relative inhibition of collagen synthesis in the presence of retinol is shown in Fig. 5. This inhibition was specifically of collagen synthesis since total protein synthesis did not change throughout the 8 days of culture. The ratio between collagen and total protein decreased from 50% at 2 days to less than 20% after 12 days of retinol treatment.

Modifications of protein synthesis induced in GRX cells by retinol were studied by both one and two-dimensional SDS-PAGE electrophoresis of proteins from cells and their supernatants, after incubation with ^{35}S-methionine. The synthesis of several unidentified proteins was modified. Fibronectin was identified by immunoblotting. Densitometric analysis of autoradiograms of electrophoretic separations of synthesized proteins, and the subsequent integration of surfaces corresponding to the fibronectin band, has shown that the incorporation of methionine into fibronectin was increased in retinol-treated cells (Fig. 6). Under standard cell culture conditions, the fibronectin band appeared as a symmetrical peak, while in retinol-treated ones, a subfraction of fibronectin with a slightly higher molecular mass could be identified (Fig. 6). Similarly, in two-dimensional electrophoresis, the first isoelectric-focusing run detected a slight shift of the fibronectin spot to the basic pH (not shown).

Total glycosaminoglycan synthesis, as estimated by ^{35}S incorporation, was significantly decreased by retinol treatment. The decrease ($P<0.01$) involved essentially the pericellular fraction of glycosaminoglycans since incorporation into the intracellular fraction remained approximately constant throughout 48 h. GRX 123 cells in effect synthesized heparan sulphate and chondroitin 4-sulphate. While the ^{35}S incorporation

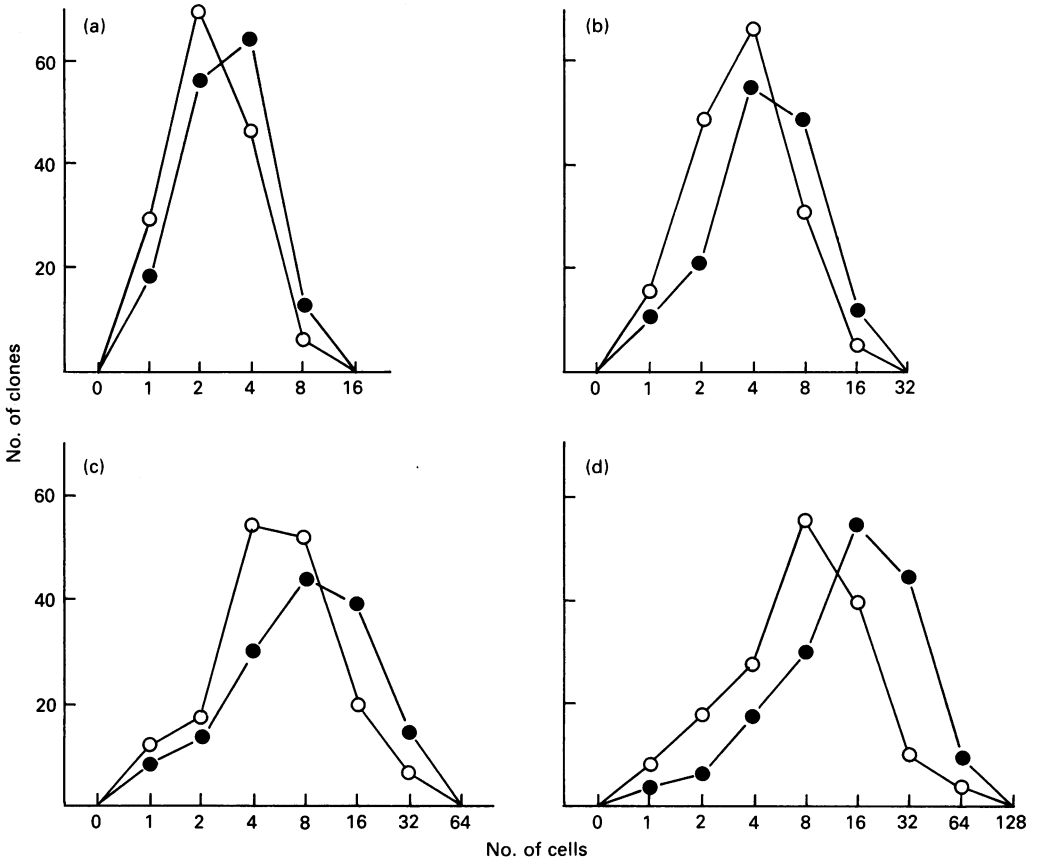

Fig. 3. Proliferation of GRX cells at cloning density, in ●, standard and ○, retinol-supplemented medium during a, 1; b, 2; c, 3; d, 4 days of culture. The number of clones containing from 1 to 128 cells is shown for each day.

into chondroitin remained constant throughout the experiment, its incorporation into heparan was decreased (Fig. 7).

Discussion

Liver is a major site for uptake, storage and mobilization of retinol. Although hepatocytes have been shown to incorporate and metabolize retinol from chylomicrons, more than 90% of stored retinoids in the liver are found in lipocytes (Knook *et al.* 1989).

Studies of primary in-vitro cultures of rat lipocytes have shown that retinol modifies their proliferation rate and their metabolism (Davis *et al.* 1987; Davis & Vucic 1988). However, in primary cultures of hepatic myofibroblasts, only a minor effect of retinol was observed and a full expression of the lipocyte phenotype was not described (Geerts *et al.* 1989). The cloned murine cell line GRX, used in the present study, was established from fibrotic lesions in liver: it expresses morphological and biochemical features which are characteristic of hepatic myofibroblasts (Borojevic *et al.* 1985a). These cells are sensitive to retinol, which induces a full expression of the lipocyte phenotype, and has a pleotropic effect on cell metabolism (Margis

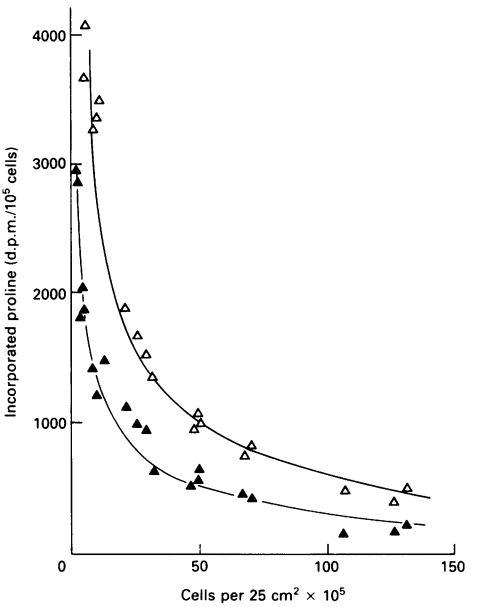

Fig. 4. Incorporation of radiolabelled proline into △, total proteins and ▲, collagen secreted by GRX cells into the supernatant during 24-h culture in the standard medium supplemented with beta-aminopropionitrile. The incorporated proline is expressed as d.p.m. per 10^5 cells, and the cell density as the number of cells per 25 cm^2 tissue culture flask. Note that the ratio between proline incorporation into total proteins and collagen is independent of cell density.

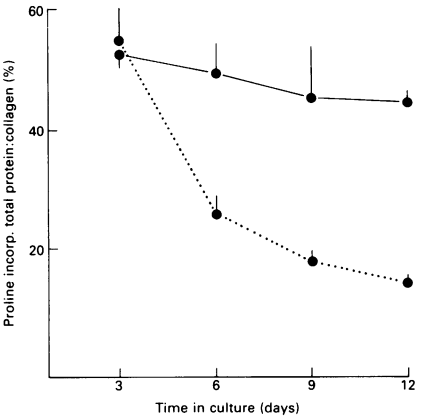

Fig. 5. Ratio (expressed as percentage) between proline incorporation into total proteins and collagen secreted into the supernatant by GRX cells, maintained in the ——, standard medium or . . .., the retinol-containing medium, supplemented with beta-aminopropionitrile, during 12 days of culture.

& Borojevic 1989a; Borojevic *et al.* 1990). This experimental model has now been used to correlate the shift from the myofibroblast to the lipocyte phenotype in the liver connective tissue cells with controls of their proliferation and secretion of extracellular matrix.

Retinol treatment of GRX cells decreased the cell density at confluence and their overall proliferation rate. The former effect may be related directly to increased adhesiveness: the increased spreading of cells on the substrate induces a more efficient contact inhibition, lowering the final cell density of the culture. The same phenomena cannot be involved in proliferation decrease, since this was observed also in low density cultures.

We have shown that the conversion of hepatic myofibroblasts into lipocytes is a full re-programming of their phenotypic expression (Borojevic *et al.* 1990). The present study of proliferation inhibition induced by retinol at cloning density has shown that it involved the entire cell population equally. The induction of the lipocytic phenotype in hepatic myofibroblasts is thus not a terminal differentiation; if it were, a proliferative undifferentiated subpopulation plus a population of differentiated non-proliferating cells would be expected, as occurs when pre-adipocytes differentiate into adipocytes (Scott *et al.* 1982). Instead, there is a potentially reversible shift between the two phenotypes, consistent with the notion of a single population of connective tissue cells in hepatic lobules. The relative proportion of the two phenotypes is probably coordinated by external conditions, such as vitamin-A status and the presence of potentially fibrogenic molecules in hepatic tissue. The vitamin-A-induced 'hyperplasia' of fat-storing cells in liver is consequently due not to their increased proliferation but to the increased conversion of liver connective tissue cells into the lipocyte phenotype.

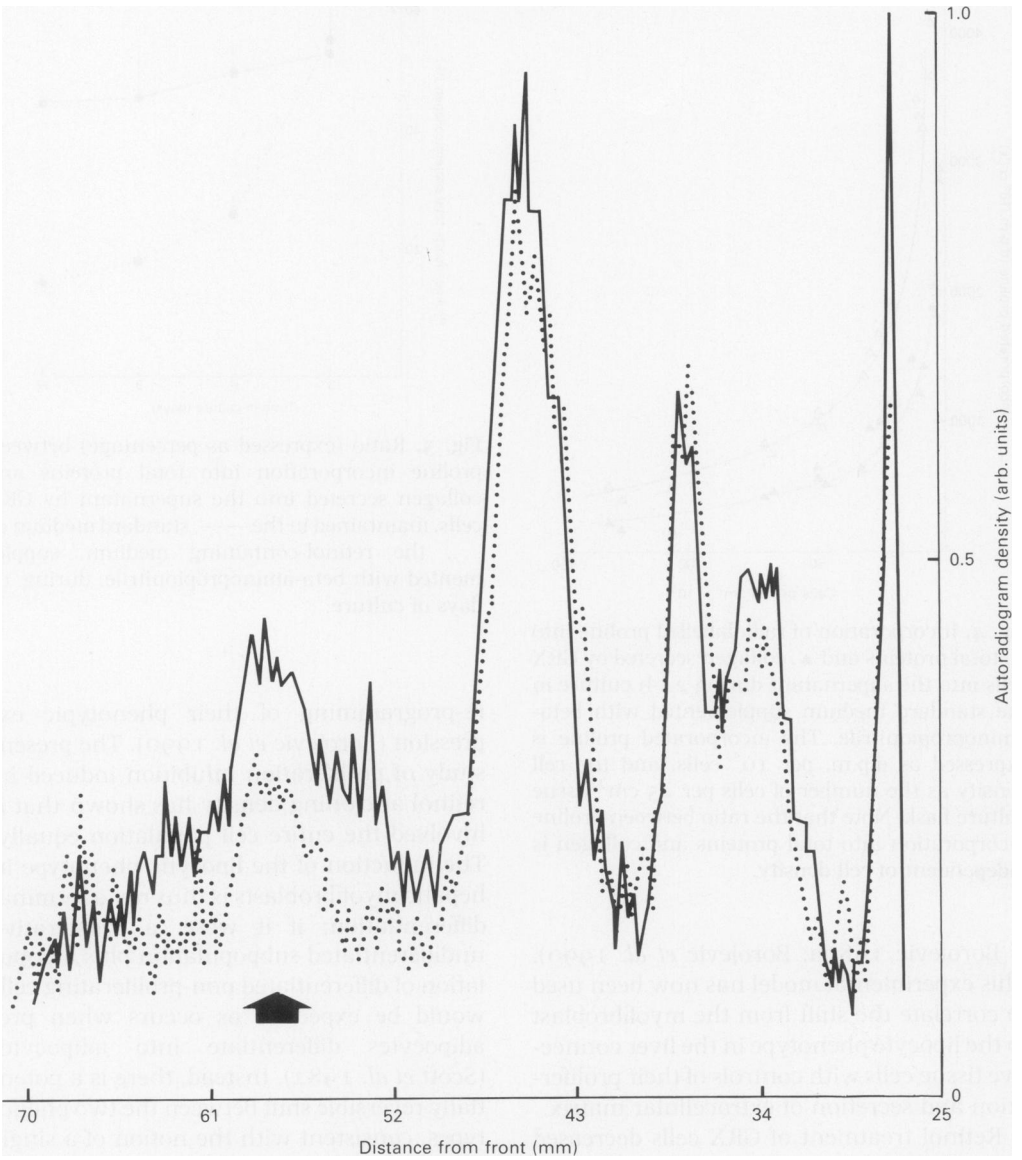

Fig. 6. Densitometry of the autoradiogram of SDS-PAGE analysis of proteins secreted into the culture medium by GRX cells, maintained in . . ., the standard and ——, retinol-supplemented medium. Results are expressed in arbitrary densitometric units and the distance (in mm) from the front of the autoradiogram. The peak identified as fibronectin by immunoblotting is indicated by an arrow.

The adhesion of cells to the substrate is mediated by cell surface associated molecules. The increase of fibronectin secretion, concomitant with increased adhesiveness of retinol-treated cells, suggests a causal relationship between the two phenomena. A similar observation was made with fibroblast cell lines treated with retinoids (Jetten *et al.* 1979). A simultaneous slight variation of the physicochemical properties of fibronectin

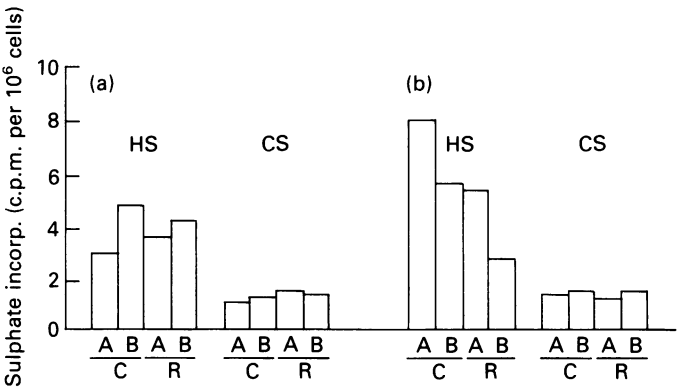

Fig. 7. Radiolabelled sulphate incorporation into heparan sulphate (HS) and chondroitin sulphate (CS), during A, 24-h and B, 48-h culture of GRX cells in C, the standard and R, retinol supplemented medium. Results observed in a, the intracellular and b, pericellular fractions are shown separately. Results are expressed in c.p.m. per 10^6 cells/10^3.

suggests a modification of secreted fibronectin. Similar modifications have been described in pathological situations. They may change cell motility and proliferation, as well as the chemotactic properties of fibronectin (Unnisa & Hunter 1981; Imada *et al.* 1988).

By contrast, heparan sulphate was decreased in the pericellular compartment of retinol-treated GRX cells. This was apparently not due to its decreased synthesis since its presence in the intracellular compartment remained unaltered. This decrease is contrary to the effect of retinoids on fibroblast cell lines in which heparan sulphate secretion remained unaltered while dermatan sulphate secretion decreased (Jetten *et al.* 1979). A similar sensitivity to retinoids of dermatan sulphate, but not heparan sulphate, secretion was observed in hepatic lipocytes exposed to acetaldehyde (Gressner & Althaus 1988). The effect of retinol on heparan sulphate in GRX cells thus appears to be a specific phenomenon. It may be relevant in view of the many functions that are attributed to cell surface associated heparan sulphates. Of particular importance for this model are heparan sulphate regulation of lipid metabolism (Cisar *et al.* 1989) and its interaction with TGF-β (McCaffrey *et al.* 1989), known to be an important regulator of hepatic connective tissue cell proliferation and matrix secretion (Davis & Vucic 1989).

Modifications of fibronectin and pericellular heparan sulphate induced by retinol indicate alterations of the relationship among hepatic connective tissue cells, extracellular matrix, and associated endothelial and parenchymal cells. These observations may provide a new approach to the study of the pathogenesis of hepatic fibrosis associated with chronic hypervitaminosis-A in humans and experimental animals.

The specific decrease of collagen secretion induced by retinol in GRX cells is similar to effects observed in other cell types (Nelson & Balian 1984; Dickson & Walls 1985). This is consistent with reports on primary cell cultures of rat Ito-cells (Davis & Vucic 1989). The absence of modifications in collagen synthesis in 24–48 h, reported by Geerts *et al.* (1989), is consistent with our results since in our model a considerable decrease was also observed only after 6 days, when a full expression of lipocytic phenotype was induced. The in-vitro retinol-induced decrease of collagen secretion is also consistent with in-vivo observations of the prevention of hepatic fibrosis by moderate retinol administration (Senoo & Wake 1985). The severe

liver fibrosis and cirrhosis observed in elevated chronic hypervitaminosis-A remain to be explained. They are apparently not directly related to retinol modulation of collagen synthesis in liver connective tissue cells but should involve more complex cell and tissue controls of extracellular matrix deposits.

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