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Modulation of the morphology and glycosaminoglycan biosynthesis of human monocytes, induced by culture substrates

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Monocytes were isolated from human blood and cultured *in vitro* on plastic culture dishes or on fibronectin-coated dishes. After 5 days *in vitro*, the cells on plastic dishes displayed marked morphological changes compared with day 1, with an epithelioid appearance resembling that of foreign-body cells. This transition was inhibited in cells cultured on fibronectin-coated dishes. ^{35}S-labelled polysaccharides were isolated from the culture media after 24 h incubation periods with inorganic [^{35}S]sulphate. The cells cultured for 5 days on a plastic substrate synthesized, and secreted into the medium, an oversulphated galactosaminoglycan previously shown to contain 4,6-di-*O*-sulphated *N*-acetylgalactosamine units [Kolset, Kjellén, Seljelid & Lindahl (1983) *Biochem. J.* **210**, 661–667]. In contrast, ^{35}S-labelled polysaccharide produced by cells cultured on plastic for 1 day only, or on fibronectin for either 1 or 5 days, contained only minor amounts of such disulphated sugar units. These findings indicate that the formation of oversulphated chondroitin sulphate is coupled to the conversion of monocytes into epithelioid cells. Furthermore, they suggest that the overall process is induced by contact with artificial substrates, and that it may be regarded as the equivalent of a foreign-body reaction *in vivo*.

The ability to synthesize and secrete glycosaminoproteoglycans is a phenotypic expression shared by many types of cells. Alterations of the differentiative or functional state of cells are often reflected in qualitatively or quantitatively changed patterns of polysaccharide synthesis. One example of such effects is the loss of chondrogenic expression after treatment of chondrocytes with the uridine analogue, 5-bromodeoxyuridine, which causes inability to synthesize cartilage-specific proteoglycan (Palmoski & Goetinck, 1972; Levitt & Dorfman, 1974). Similar effects on polysaccharide synthesis have been observed, along with distinct morphological changes, after fibronectin treatment (West *et al.*, 1979) or viral transformation (Pacifici *et al.*, 1977) of chondroblasts. Also, specific changes in polysaccharide structure have been noted, such as a decreased sulphation of heparan sulphate resulting from viral transformation (Underhill & Keller, 1975; Winterbourne & Mora, 1978).

Human monocytes maintained *in vitro* on plastic or glass substrates display changes in morphology and in functional properties that have been interpreted as an expression of differentiation into macrophage-like cells (for references see Kolset *et al.*, 1983). This transition, which is completed after 4–5 days in culture, is accompanied by a switch in polysaccharide synthesis, from the production of chondroitin 4-sulphate to the formation of an oversulphated galactosaminoglycan containing 20% or more of hexuronosyl-*N*-acetylgalactosaminyl-4,6-di-*O*-sulphate disaccharide units (Kolset *et al.*, 1983). The results obtained by Kolset *et al.* (1983) did not allow any conclusion as to whether this change in sulphation is an obligatory prerequisite and/or sequel to the differentiation process, or rather an unrelated effect of the culture conditions. In the present paper we show that the changes in morphology and in sulphation pattern are both prevented when the cells are grown on fibronectin-coated culture dishes.

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Experimental

Materials

Chondroitin sulphate from bovine nasal septa was a gift from Dr. Å. Wasteson, University of

Uppsala, Uppsala, Sweden. Heparin (Stage 14) from pig intestinal mucosa was purchased from Inolex Pharmaceutical Division, Park Forest South, IL, U.S.A., and purified further as described (Lindahl *et al.*, 1965). Mono- and di-*O*-sulphated hexuronosyl-2,5-anhydro[1-^{3}H]mannitol disaccharides were prepared from heparin and separated as described by Thunberg *et al.* (1982). Inorganic [^{35}S]sulphate (carrier-free) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Bacterial chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4) was from Seikagaku Fine Chemicals, Tokyo, Japan. Papain and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Penicillin and streptomycin were from Gibco Bio-Cult, Paisley, Renfrewshire, Scotland, U.K., Percoll was from Pharmacia Fine Chemicals, Uppsala, Sweden, and Whatman DEAE-cellulose (DE-52) from Whatman Biochemicals, Maidstone, Kent, U.K. Fibronectin, purified as described by Vuento & Vaheri (1979), was a gift from Dr. S. Johansson, University of Uppsala, Uppsala, Sweden.

Methods

Hexuronic acid was determined by the carbazole method (Bitter & Muir, 1962), and radioactivity was determined in a Packard model 2405 liquid-scintillation spectrometer, with Packard emulsifier-scintillator 229. Additional methods used are described in the legends to Figures.

Preparation of fibronectin-coated substrate. Glass cover-slips were introduced into 16mm Costar wells (Costar, Broadway, Cambridge, MA, U.S.A.) and were overlayered with 20μg of fibronectin/well in 0.5ml of phosphate-buffered saline (0.14M-NaCl, 2mM-KCl, 8mM-Na_2HPO_4, 1.5mM-KH_2PO_4, pH7.4). The wells were left at room temperature overnight to dry. Salt was removed by five washes with the same buffer before the cells were seeded.

Cell-culture experiments. The procedure for isolating human monocytes has been described in detail (Pertoft *et al.*, 1980). In short, defibrinated blood was layered on top of a 54% (v/v) Percoll solution in 0.15M-NaCl and centrifuged at 1000*g* for 20min at room temperature in a swing-out rotor. Cells at the interphase were collected, washed and seeded in F-10 medium, containing 20% autologous serum and 100 units each of penicillin and streptomycin/ml, either directly on the Costar plastic or on to fibronectin-coated cover-slips (2×10^6 cells/well). Non-adherent cells were washed away after 2h of incubation at 37°C in an atmosphere of CO_2/air (1:19). Incubation was continued in F-10 medium with 20% autologous serum and antibiotics as described above. During prolonged cultivation, medium was changed every second day.

For biosynthetic labelling of glycosaminoglycans, the cultures were washed with sulphate-depleted medium ($MgCl_2$ substituted for $MgSO_4$) and re-incubated in this medium (1ml/well) containing 20% autologous serum and inorganic [^{35}S]-sulphate (50μCi/ml). Labelling was continued for 24h, either directly after establishing the cultures (day 1) or from day 4 *in vitro*. After completion of exposure to the [^{35}S]sulphate, labelled polysaccharide was isolated from the spent culture media by digestion with papain followed by gel chromatography, as described by Kolset *et al.* (1983).

Enzymic degradation of glycosaminoglycans. Samples of labelled polysaccharide (approx. 10000c.p.m. of ^{35}S) were incubated with 0.01 unit of chondroitinase ABC in 0.1ml of 0.05M-Tris/HCl, pH8.0, containing 0.05M-sodium acetate, 0.05M-NaCl and 0.1mg of bovine serum albumin/ml (Yamagata *et al.*, 1968). After 60min of incubation at 37°C, samples (100μl) were directly applied to Whatman 3 MM paper and subjected to high-voltage paper electrophoreis.

Scanning electron microscopy. After completion of the respective labelling periods, monocytes were prepared for microscopic examination. Cells adhering to the Costar-well plastic were recovered by cutting out the bottom of the well after the cells had been fixed for 30min at room temperature in 0.1M-cacodylate buffer, pH7.3, containing 2.5% (v/v) glutaraldehyde and 0.1M-sucrose. Cells on fibronectin-coated cover-slips were fixed as described above, and the cover-slips were recovered from the wells. The cells were then dehydrated in ethanol and critical-point-dried (Hitachi CPI, Tokyo, Japan) in CO_2. The specimens were coated with gold (Polaron, SEM Coating Unit E 5000 V) and examined with a high-resolution Hitachi scanning electron microscope (HHS/2 R) at 20kV and a tilt angle of 15°. Pictures were taken on Kodak Plus-X Pan film.

Results

Morphology of cells

The effect of cultivation of human monocytes on plastic or on fibronectin-coated glass surfaces was investigated by scanning electron microscopy. Cells maintained on a plastic surface for 5 days showed dramatic morphological changes (Figs. 1*b* and 1*d*), in accordance with other reports (Ødegaard *et al.*, 1974; Johnson *et al.*, 1977) and our own previous findings (Kolset *et al.*, 1983). The cells increased in size to about 3–5 times their diameter at day 1, and most of them acquired an epithelioid appearance, with a centrally located

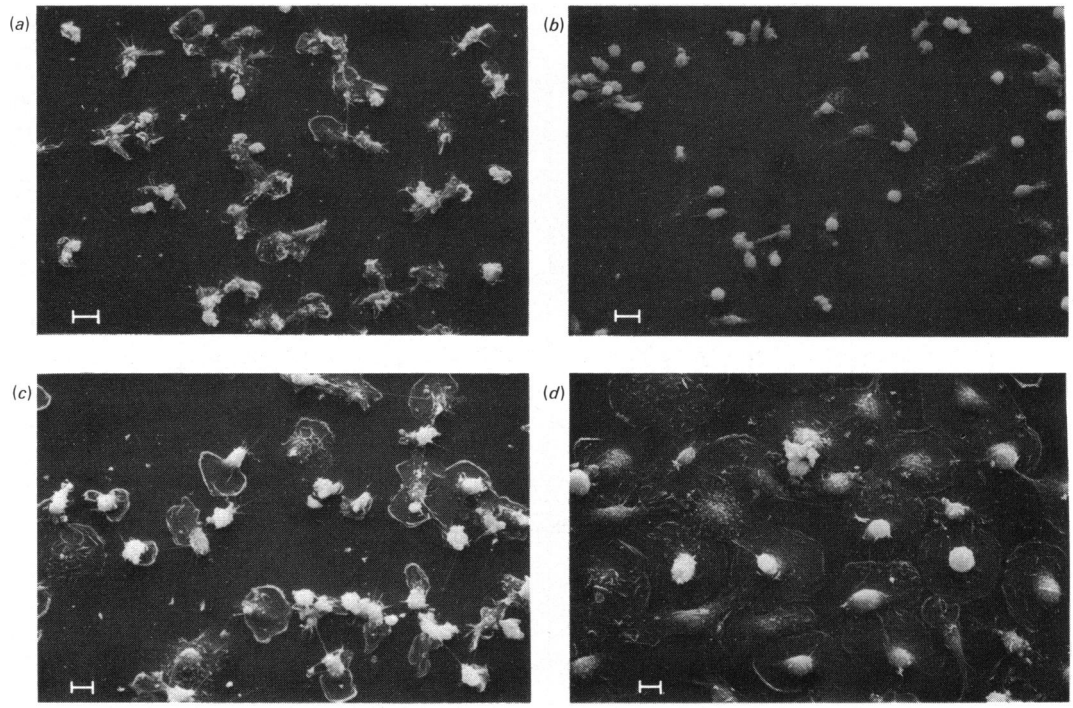

Fig. 1. *Scanning electron micrographs of human monocytes cultured on different substrates*
Cells cultured on fibronectin-covered (*a*, *c*) or on plastic (*b*, *d*) substrate for 1 (*a*, *b*) or 5 (*c*, *d*) days. The bars represent 10μm.

nucleus. Cells seeded on glass surfaces (not coated with fibronectin) detached more frequently and seemed to thrive less well, but developed otherwise in a fashion similar to the cells on plastic surfaces (results not shown).

Monocytes cultured on a fibronectin-coated surface for 1 day (Fig. 1*a*) showed a more spread-out and irregular shape, with more ruffling of the membranes, than did the corresponding cells on a plastic substrate (Fig. 1*b*). After 5 days on a fibronectin coat the cells had spread out somewhat further (Fig. 1*c*), but otherwise essentially retained their appearance from day 1, with irregular shapes and excentric nuclei. A striking difference was thus noted between the cells cultured on plastic (Fig. 1*d*) and on fibronectin (Fig. 1*c*) for 5 days. The latter cells were much smaller and furthermore lacked the smooth, regular, epithelioid character of the plastic-supported cells. However, on prolonged incubation (7–10 days; results not shown) an increasing proportion of the monocytes cultivated on fibronectin-coated surfaces gradually acquired the same morphological properties as displayed by the cells after 5 days on a plastic substrate.

Structure of glycosaminoglycans

^{35}S-labelled glycosaminoglycans were isolated as single polysaccharide chains from the culture media, as described previously (Kolset *et al.*, 1983). Analysis by ion-exchange chromatography of the polysaccharide released from cells cultured on plastic dishes showed conspicuous retardation of the day-5 (Fig. 2*d*) as compared with the day-1 (Fig. 2*b*) material. Furthermore, paper electrophoresis of products obtained on digesting the day-5 material with chondroitinase ABC showed a prominent peak (approx. 35% of total ^{35}S) of di-*O*-sulphated disaccharide (Fig. 3*d*); the corresponding component derived from day-1 polysaccharide appeared in much lesser quantity (Fig. 3*b*). These results conform to our previous finding, that prolonged cultivation of monocytes on plastic dishes induces the formation of an oversulphated galactosaminoglycan that contains a high proportion of 4,6-di-*O*-sulphated *N*-acetylgalactosamine residues (Kolset *et al.*, 1983). In contrast, analysis of polysaccharide produced and secreted by monocytes maintained on a fibronectin-coated substrate failed to reveal any significant increase in sulphation of day-5 as compared with day-1 material. The two polysaccharide preparations thus were indistinguishable on ion-exchange chromatography (Figs. 2*a* and 2*c*) and yielded similarly low proportions of disulphated disaccharides after enzy-

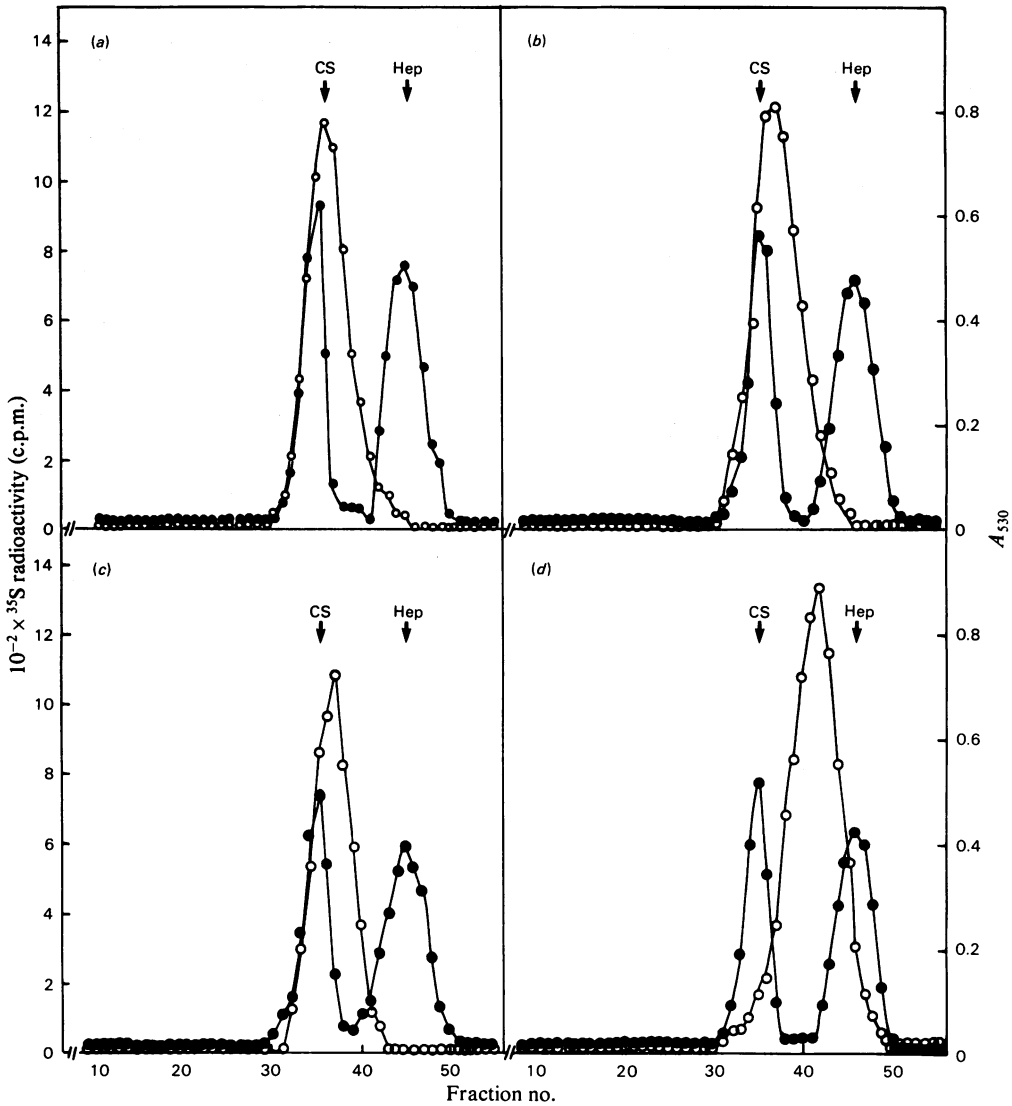

Fig. 2. *Ion-exchange chromatography of ^{35}S-labelled glycosaminoglycans from human monocytes*
Samples containing 10000 ^{35}S c.p.m. were applied to a column (1 cm × 7.5 cm) of Whatman DE-52 DEAE-cellulose along with internal standards of chondroitin 4-sulphate (CS, 1 mg) and heparin (Hep, 2 mg). The column was eluted at a rate of 9 ml/h with a gradient (starting at fraction 10) of 0.05–1.5 M-LiCl in 0.05 M-acetate buffer, pH 4.0. Fractions (approx. 3 ml) were collected and analysed for radioactivity (○) and for hexuronic acid (A_{530}; ●). The various samples of ^{35}S-labelled polysaccharide were obtained from monocytes cultured on fibronectin (*a*, *c*) or plastic (*b*, *d*) substrates for 1 (*a*, *b*) or 5 (*c*, *d*) days.

mic degradation (Figs. 3*a* and 3*c*). It is therefore concluded that the morphological change and the alteration in polysaccharide structure expressed by monocytes on plastic dishes are both impeded on fibronectin substrates.

Discussion

Monocytes isolated from human blood have been widely used in studies on the differentiation of circulating monocytes into tissue macrophages. During cultivation on glass or plastic dishes *in vitro*, these cells dramatically increase their size (Figs. 1*b* and 1*d*) and simultaneously acquire novel functional properties. The differentiated cells thus produce fibronectin (Alitalo *et al.*, 1980), expose Fc and complement-factor-C_3 receptors on their surface (Newman *et al.*, 1980; Kaplan & Gaudernack,

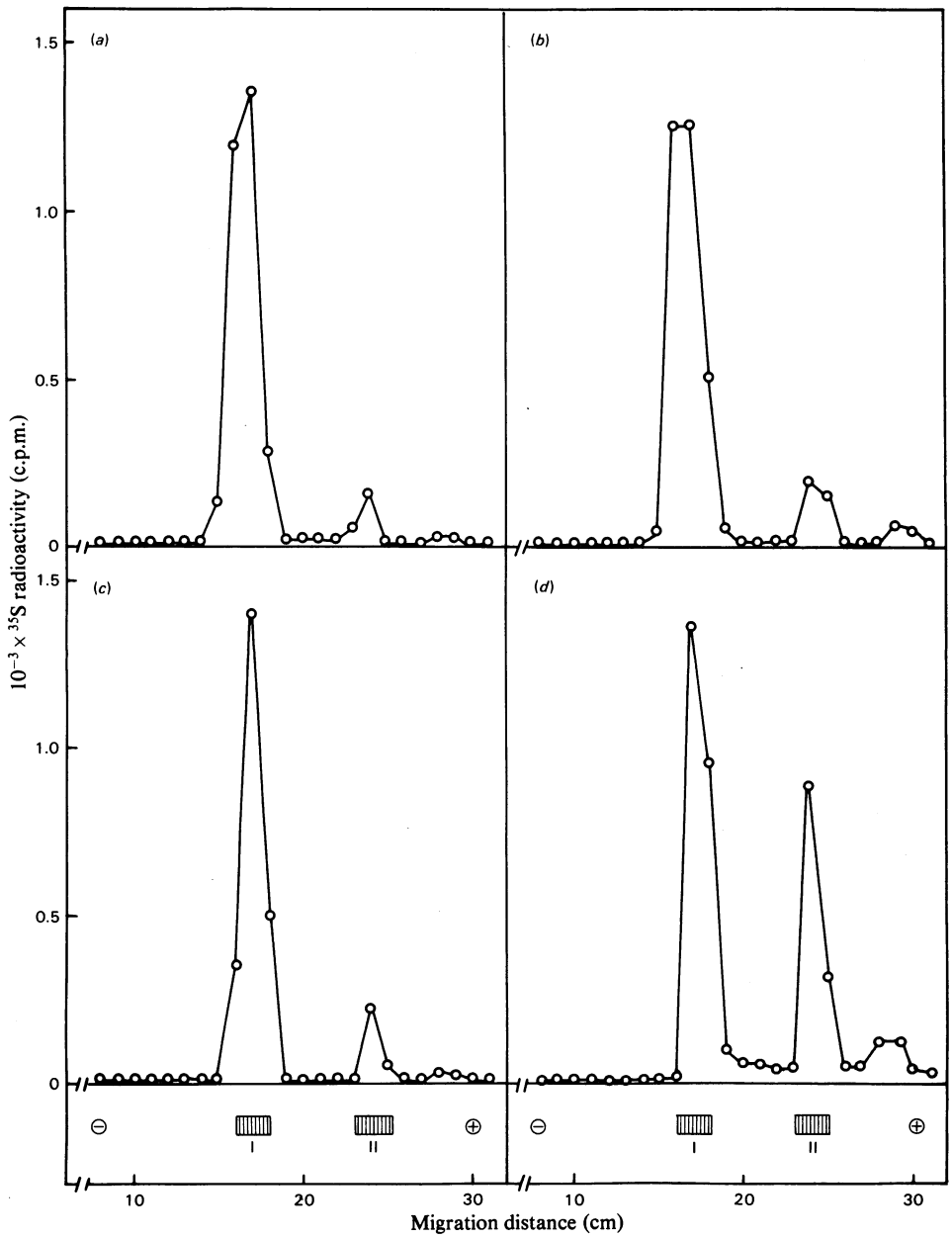

Fig. 3. *Paper electrophoresis of disaccharides obtained on digestion of ^{35}S-labelled glycosaminoglycans with chondroitinase ABC*

Samples of labelled polysaccharide were digested with chondriotinase ABC as described in the Experimental section. The products were applied to strips of Whatman 3MM paper and separated by high-voltage electrophoresis in 1.6M-formic acid, pH 1.7, at 40V/cm for 80min. The samples represent polysaccharide produced by monocytes cultured on fibronectin (*a*, *c*) or plastic (*b*, *d*) substrates for 1 (*a*, *b*) or 5 (*c*, *d*) days. The standards are (I) monosulphated and (II) disulphated hexuronosyl-2,5-anhydro[1-^{3}H]mannitol disaccharides. Less than 10% of the labelled digestion products remained at the starting points, indicating >90% galactosaminoglycan.

1982), and reorganize their cytoskeletal and contractile elements (Lehto *et al.*, 1982). In a previous paper (Kolset *et al.*, 1983) we described yet another property acquired in the course of this transition, i.e. the ability to produce and secrete an oversulphated galactosaminoglycan. This poly-

saccharide, which contains *N*-acetyl-D-galactosamine 4,6-di-*O*-sulphate residues, and is sometimes referred to as chondroitin sulphate E, is not commonly found in mammalian tissues, but has been identified in various cultured mammalian cells (see Kolset *et al.*, 1983, for references). The functional role of the disulphated disaccharide unit is unknown. However, our present observation that the differentiation and the chondroitin sulphate biosynthesis of the monocyte can be modulated in parallel strongly suggests that these processes are somehow interrelated.

The modulation alluded to was achieved by substituting a fibronectin-coated surface for plastic or glass surfaces as a culture substrate for the monocytes [see also van Ginkel *et al.* (1977); Kaplan & Gaudernack (1982) and Kaplan (1983) regarding other substrate-induced effects on monocytes]. The mechanism of this modulation is intriguing. Fibronectin, a glycoprotein found in plasma, connective tissues and basement membranes, is produced by a variety of cells, and has been functionally implicated in highly diverse biological phenomena, including embryonic development, oncogenic transformation, cell adhesion (for references, see Hynes & Yamada, 1982), and phagocytosis by macrophages (Gudewicz *et al.*, 1980). Moreover, fibronectin has been found to modulate differentiation in various cell systems. It thus inhibits chondrogenesis (Pennypacker *et al.*, 1979) and fusion of myoblasts (Podleski *et al.*, 1979), but promotes adrenergic differentiation in neural crest cells (Sieber-Blum *et al.*, 1981). Little is known of the mechanism(s) of action of fibronectin in these various systems. Interestingly, data have been presented (Bevilacqua *et al.*, 1981) suggesting the occurrence of receptors for fibronectin on the surface of human monocytes. However, the modulatory effect of fibronectin described in the present paper seems not to involve a receptor-mediated mechanism, since soluble fibronectin (added, as cold-insoluble globulin, with the autologous serum to all cultures) could not prevent the differentiation of monocytes on glass or plastic substrates. Such an effect was observed only with the immobilized protein serving as culture substrate.

Attempts to understand the nature of the interaction between monocytes and their fibronectin substrate must take note of the fact that mononuclear phagocytes are notoriously attracted to 'foreign' surfaces *in vitro* as well as *in vivo*. It is thus logical to assume that prolonged cultivation of monocytes on a glass or a plastic substrate would lead to the development of so-called foreign-body cells similar to those encountered under certain pathological conditions *in vivo*, whereas a more natural substrate, such as fibronectin, would promote the formation of cells more akin to the normal macrophage or histiocyte of connective tissues. This suggestion is in agreement with findings pertaining to monocytes cultured on collagen (Kaplan & Gaudernack, 1982; Kaplan, 1983) or on fibrin (S. O. Kolset, unpublished work) gels. Interestingly, the cells in the latter case (as in the present study; see the Results section) gradually assumed an epithelioid appearance on prolonged cultivation (7–10 days), even in the presence of the 'natural' substrate. Microscopic inspection revealed that this transition occurred only where the fibrin gel had been eroded such that the cells had direct contact with the foreign surface beneath the coat. This finding reinforces our notion that the reactions displayed by the cells on plastic or glass surfaces, including the formation of oversulphated galactosaminoglycan, may be regarded as an equivalent *in vitro* of a foreign-body reaction *in vivo*. The modulating effects of the 'natural' substrates (fibronectin, fibrin or collagen) would thus partly be explained in terms of a steric hindrance that prevents the cells from reaching contact with a stimulatory agent.

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