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MALOTILATE REDUCES COLLAGEN SYNTHESIS AND CELL MIGRATION ACTIVITY OF FIBROBLASTS *IN VITRO*

A. POESCHL, D. REHN,* J.-M. DUMONT,* P. K. MUELLER and G. HENNINGS*
Max-Planck-Institut fuer Biochemie, Am Klopferspitz, D-8033 Martinsried, and *Zyma Muenchen/Nyon, Federal Republic of Germany

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Abstract—Diisopropyl-1,3-dithiol-2-ylidenemalonate (malotilate) was studied for and compared with cyanidanol, hydrocortisone and colchicin on its impact on fibroblast cultures. Under *in vitro* conditions, malotilate specifically reduces collagen synthesis of fibroblasts. In addition, malotilate is an efficient inhibitor of fibroblast migration as tested by a chemotaxis assay in a modified Boyden chamber. Our data may support the notion that malotilate is of potential interest for interfering with fibrotic processes.

In general, fibrotic processes develop in three phases: the first event is the lesion of a tissue which evokes in the second phase an inflammatory process and leads—in the third phase—to tissue repair [1]. The phase of tissue repair is characterized by increased synthesis and deposition of extracellular matrix components, e.g. collagens, proteoglycans and fibronectin [2–5]. If synthesis persists and deposition of matrix components continues, the crucial steps of a persistent fibrotic condition are triggered. A sequence of events analogous to that may underly fibrosis of lung and liver including that caused by chronic alcohol exposure. Histochemically, in early stages increased amounts of fibronectin are seen, whereas it is typical that in the late stage scanty fibroblasts are found [6].

Furthermore, histological examination of the cirrhotic stage suggests a tremendous overproduction and deposition of connective tissue components in particular of collagen. It is not clear at present which mechanisms are responsible for the increased deposition of extracellular matrix components. It is conceivable, however, that the fibroblasts seen in fibrotic processes had developed from other cell types, but it also appears possible that these fibroblasts were attracted by a gradient of a chemoattractant and had migrated out of the surrounding tissue [7].

In any case one may be intrigued by a therapeutic concept by which one would normalize excessive deposition of matrix components and also prevent chemoattraction of fibroblasts. Therefore, in our current investigation we studied the impact of malotilate on both collagen synthesis and chemotactic migration of fibroblasts under *in vitro* conditions. Our study was meant to validate observations from *in vivo* experiments which had demonstrated that the administration of malotilate has an antinecrotic [8] and antifibrotic [9, 10] effect on *in vivo* carbon tetrachloride-induced liver fibrosis in rats. Furthermore, we compared the efficiency of malotilate with that of other drugs which are known to show a beneficial effect on fibrotic processes.

MATERIALS AND METHODS

Chemicals. Diisopropyl-1,3-dithiol-2-ylidenemalonate (malotilate), produced by Nihon Nohyaku Ltd (Japan), was a gift of Zyma GmbH. Colchicin, (+)-cyanidanol-3 and hydrocortisone were provided to us by Zyma GmbH.

Cell culture. Skin biopsies from healthy human embryos were used to establish fibroblast cultures [11]. Cells were grown in plastic tissue culture flasks (Falcon, Becton, Dickinson, Heidelberg, F.R.G.) using Dulbecco's modified Eagles medium supplemented with sodium ascorbate (50 μg/ml), glutamine (300 μg/ml), streptomycin (50 μg/ml), penicillin (400 U/ml) and 10% fetal calf serum. The cultures were maintained in a moist atmosphere of 95% air, 5% CO_2 at 37°. Fibroblasts were passaged by trypsinization (0.1% Trypsin, 0.02% EDTA). The cells were used no longer than until the fifteenth passage. Viability of the cells after serial trypsinization was about 90% as judged by the Trypan blue exclusion test.

Metabolic labeling. Confluent monolayers of fibroblasts were trypsinized and replated on culture dishes at the initial cell density. After an attachment time of 2–3 hr, the cell layer was preincubated according to ref. 12. After 24 hr of preincubation the cells were incubated in parallel with 5 μCi/ml L-[2,3]-^{3}H-proline, β-aminopropionitril 100 μg/ml [12], ascorbate and the respective drugs in distinct doses. In one set of experiments, medium and cells were collected together after a 24-hr incubation, while in an other set of experiments medium was separated from cells. Cells were lysed by freeze-thawing (three times) and extracted with 1 M NaCl, 0.05 M Tris pH 7.4 at 4°. After centrifugation, soluble extracts were dialysed against 0.05 M acetic acid to remove dialysable radioactivity. Afterwards, the probes were lyophilised.

Pepsin treatment. Lyophilised probes were dissolved in 0.05 M acetic acid/HCl pH 2.0 and incubated with pepsin (3× crystallysed, Fa. Boehringer,

Mannheim, F.R.G.) in a final concentration of 0.1 mg/ml for 6 hr at 18°. The remaining non-digestable collagenous components were lyophilised in small aliquots and resuspended in the appropriate buffers for polyacrylamide slab gel electrophoresis [13].

SDS–polyacrylamide gelectrophoresis. Pepsin-treated lyophilized collagenous proteins were dissolved in SDS-sample buffer and separated on SDS-polyacrylamide gels (7% acrylamide, 0.2% bis-acrylamide) according to [13]. The gels were fixed in 2,5-diphenyloxazol, dried and exposed to an X-ray film (Kodak X-omat) at −70°.

Determination of the relative amount of collagen. To determine the proportion of newly synthesized collagen relative to the overall protein synthesis in extracts of cells and medium, the dialysed extracts were submitted to amino acid analysis. Dialysed probes were lyophilised in aliquots, hydrolysed for 24 hr at 110° with 6 N HCl plus mercaptoethanol (1:2000) under an N_2-atmosphere. The lyophilised samples were resuspended in 0.1 M Na_2-citrate buffer pH 2.2 in order to determine radioactive OH-proline and proline using an automated amino acid analyser (Beckman Multichrom) [14]. The amount of collagen relative to the overall protein synthesis was calculated according to the formula

$$\% \text{ Collagen} = \frac{2(\text{cpm Hypro})}{5(\text{cpm Pro-cpm Hypro}) + 2(\text{cpm Hypro})} \times 100$$

This formula is based on the fact that, firstly, hydroxyproline is a characteristic iminoacid of collagen molecules and, secondly, that hydroxyproline and proline occur in about equal amounts in type I collagen. Furthermore, the formula takes into account that Proline occurs about 5 times more often in collagen than in non-collagenous proteins.

Indirect immunofluorescence staining. The staining with antibodies was carried out according to refs. 15 and 16. Briefly, cultures of human embryonic skin fibroblasts were washed, fixed and stained with polyclonal (from rabbit) or monoclonal (from mouse) antibodies. The cells were incubated 30 min at room temperature in a moist chamber. We used fluorescein isothiocyanate-coupled goat anti-rabbit IgG or rabbit anti-mouse IgG and IgM in a dilution of 1:40 to 1:60 as a second antibody. The incubation was carried out for 30 min at room temperature in a dark moisture chamber. The stained cultures were studied by u.v.-microscopy with magnifications from 240- to 380-fold.

Chemotaxis assay. Freshly trypsinised cells were seeded on culture flasks and grown within two days to a cell density of 4–8 × 10^4 cells/cm^2. These confluent cell cultures were trypsinized, washed to remove serum and diluted to a uniform cell density of 1.5–2.0 × 10^6 cells/cm^2. These suspensions were used for chemotaxis assays in Boyden chamber as described [17, 18]: the Boyden chamber consists of two compartments (upper and lower compartment) which are separated from each other by a gelatin coated polycarbonate filter with pores of 8 μm in diameter. The lower compartment was filled with the chemoattractant while the upper chamber contains the cell suspension. As chemoattractant we used conditioned medium from fibroblast cultures (KM). Conditioned medium is defined as being particle free and serum deficient and is produced by a confluent culture of fibroblasts within 24 hr. It consists of all soluble components released into the medium. During incubation the fibroblasts actively migrate in the direction of the chemoattractant through the pores of the filter. After 4 hr of migration, the non-migrated cells were mechanically removed and the migrated cells on the lower side of the filters were fixed, stained and counted by light microscopy (magnification 400-fold). Each sample was assayed in triplicate, and cells in 10 high power fields were counted. Two experimental conditions were chosen:

Long term influence of agents: the freshly trypsinized and still proliferating cell cultures were treated with the drugs for 1–3 days on Petri dishes prior to the chemotaxis assay. These pretreated cell pools were submitted to chemotaxis assay. In this case we tested the behaviour of cells as a consequence of pretreatment with the drugs over a certain time-span (Fig. 1a).

Short term influence of agents: An uniformous pool of untreated cells was exposed to the different drugs only during the time of the chemotaxis experiment itself. In one set of experiments, the substances were

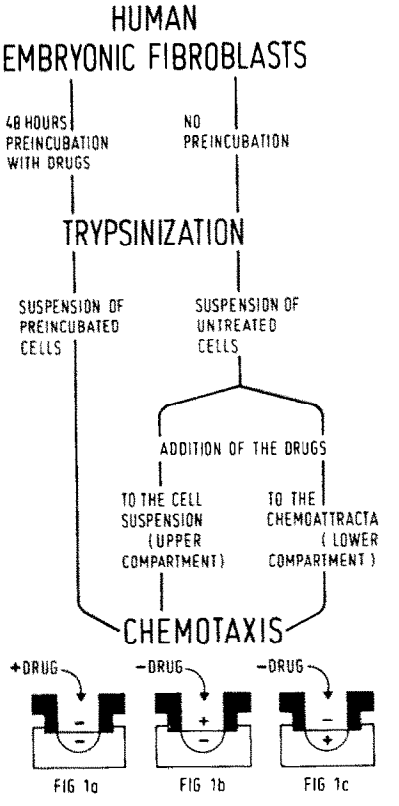

Fig. 1. Schematic representation of the experimental design of the chemotaxis assays. (a) The cells are preincubated with the drug. (b, c) The cells have contact with the drug only during the chemotaxis assay.

given to the attractant (lower compartment of the Boyden chamber, see Fig. 1c) or, alternatively, in a second set of experiments, we added the drug to the cell suspension (upper compartment of the Boyden chamber, see Fig. 1b). Under the latter conditions we observed the immediate impacts of the chosen drugs on cell migration.

RESULTS

All experiments were carried out with human embryonic skin fibroblasts which showed under the experimental conditions used a constant rate of collagen production and also a reproducible migratory response to chemoattractants such as fibronectin or conditioned medium [19].

Biochemical analysis

Influence of malotilate on collagen synthesis and cell-proliferation. Human embryonic fibroblasts were grown to confluency ($2–4 \times 10^5$ cells/cm^2), trypsinized and reseeded at the same cell density. During the 24 hr of preincubation the cells were grown in a proline-free medium to deprive them of this amino-acid prior to pulse labeling with radioactive proline. Malotilate was added to the incubation medium in concentrations ranging from 3.5 μM to 70 μM.

After 24 hr of incubation medium and cells were separately collected and prepared for polyacrylamide gel-electrophoresis and for hydroxyproline/proline determination. While the banding pattern upon electrophoretic separation did not change under the influence of malotilate a slight decrease in protein synthesis was observed as the concentration of malotilate increased (as judged from total incorporation of ^{3}H-proline in nondialysable protein).

More interestingly, malotilate causes a substantial reduction of collagen relative to the overall protein synthesis. This is more obvious in the medium than in the cell layer (Fig. 2a).

In parallel experiments hydrocortisone, colonicin or cyanidanol, respectively, were added to fibroblast cultures in concentrations published previously [20–23]. These drugs have widely been studied and are in general discussion for their beneficial inhibitory potential on collagen synthesis in fibrotic processes.

Under the same incubation conditions as for malotilate we analysed the influence of these drugs on collagen synthesis. In these experiments medium and cells were pooled and prepared for gel electrophoresis (not shown) and aminoacid analysis for determination of radioactive hydroxyproline and proline.

Figure 3a shows a dose dependent decrease of collagen relative to the overall protein synthesis after incubation of human embryonic fibroblasts with four drugs. The effect of cyanidanol is most prominent while the remaining drugs are less effective.

This reduction is fairly specific for collagen synthesis. The overall synthesis of non-collagenous proteins is virtually unaltered. Under the experimental conditions malotilate and hydrocortisone show no cytotoxic effect in contrast to cyanidanol and colchicin (Fig. 3b).

It is interesting to notice that malotilate had virtually no effect on collagen synthesis when the drug

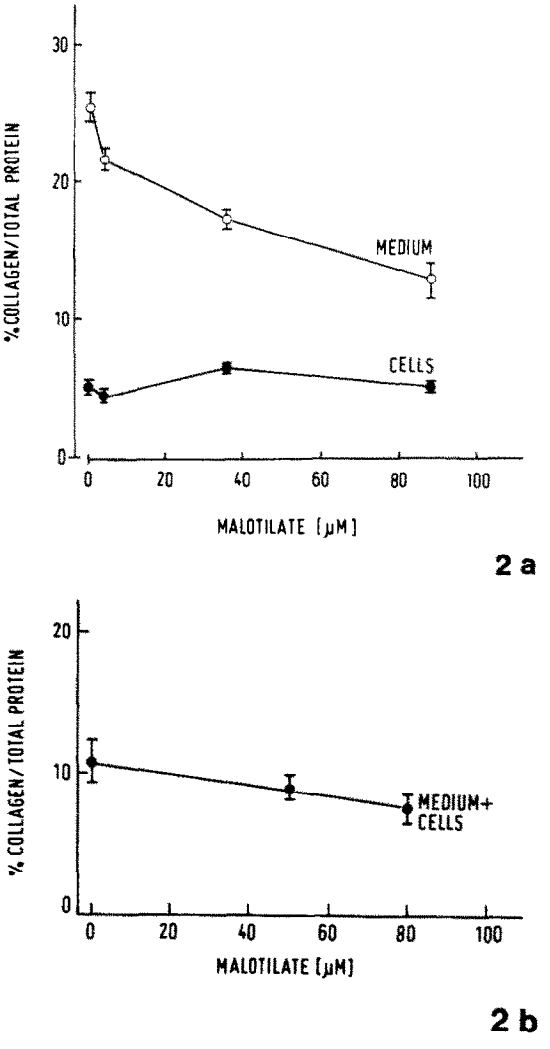

Fig. 2. Influence of increasing doses of malotilate on collagen production and secretion (calculated from the amino-acid analysis). Secreted undialysable collagen in the medium, unsecreted collagen within the cells: (a) 2 days after trypsinization, (b) 4 days after trypsinization. Each point represents the mean value and standard deviation of three independent analyses.

was given to fibroblasts at high cell density which had reconstituted their extracellular matrix yet by maintenance of the cells for prolonged times (more than 3 days following trypsinisation, Fig. 2b).

There is experimental evidence that in some fibrotic conditions the ratio between type I and type III collagen is altered while in others there is no change [24, 25]. Here we analysed the influence of malotilate on the ratio between type I and type III collagen using slab gel electrophoresis in SDS–polyacrylamide. Gels were run under reducing and non-reducing conditions which allows to distinguish between the two collagen types [13]. There was no obvious difference in the collagen pattern of malotilate treated fibroblasts and controls neither in cell-layers nor in medium. This suggests that malotilate has a simultaneous impact on synthesis of both collagen types (data not shown).

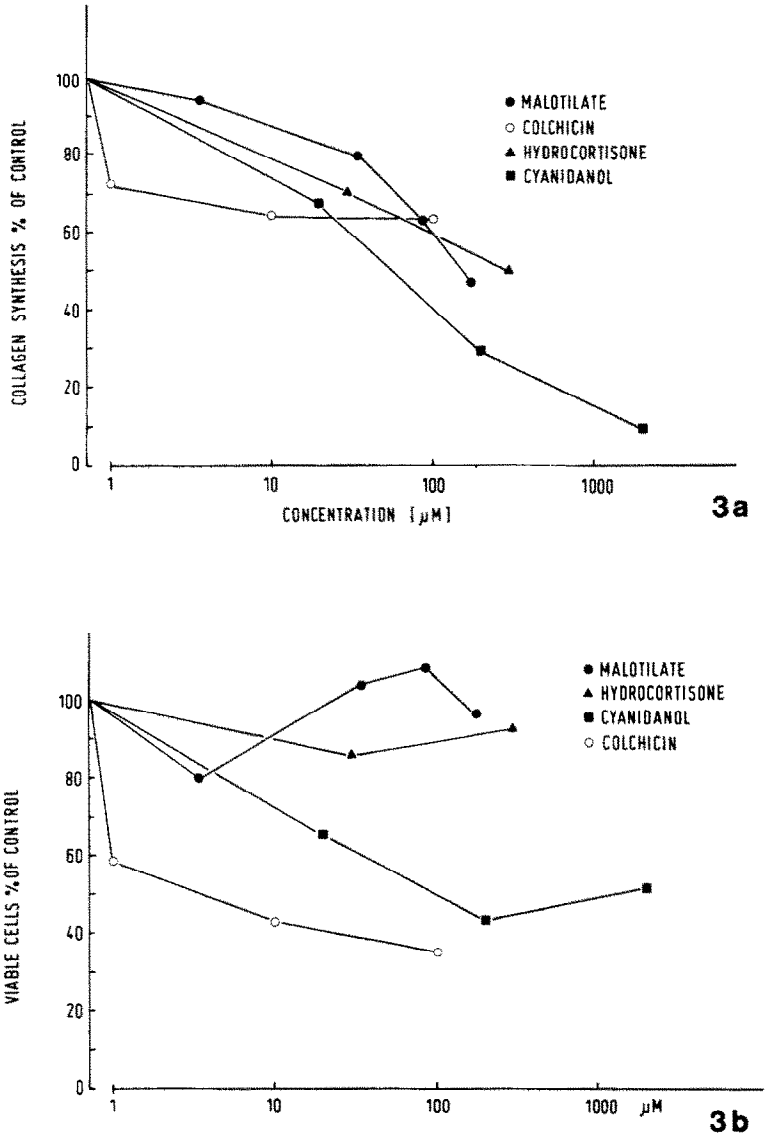

Fig. 3. Comparison of four drugs commonly studied for their inhibitory potential on collagen synthesis in fibrotic processes: malotilate, colchicin, hydrocortisone, cyanidanol. (a) Dose-dependency of collagen synthesis. Values show the decrease of collagen synthesis under the respective drug. 100% is obtained from untreated cells. (b) Dose-dependency of viability of the fibroblasts. The viability was tested by the Trypan blue exclusion assay by which dead cells stain intracellularly. The data were obtained from two independent experiments.

Indirect immunofluorescence staining

This experimental approach should demonstrate if malotilate has a major effect on cell shape or on the overall deposition of the extracellular matrix.

In order to see whether malotilate is able to change the pattern and distribution of the extracellular matrix we stained malotilate treated fibroblast cultures with matrix specific antibodies. Human embryonic fibroblasts were incubated over 1–3 days with malotilate (70 μM). After incubation the cultures were washed, fixed and stained with antibodies directed against distinctive collagen types and against fibronectin. Figures 4a–f summarise a representative experiment showing treated and untreated cell cultures stained with antibodies against fibronectin (Figs. 4a and b), type I (Figs. 4c and d) and type III collagen (Fig. 4e and f).

In no instance could we find substantial alterations in the distribution nor in the pattern of collagen types nor in that of fibronectin. Moreover, malotilate did not cause any alteration in cell shape nor in the structure of the cytoskeleton (not shown).

Chemotaxis

Chemotaxis is defined as a directed movement of cells in a gradient of a chemoattractant. This cellular property may also play a role in the repair phase of fibrotic processes [5].

Our experiments were designed in such a way as to find out if malotilate influences the migratory

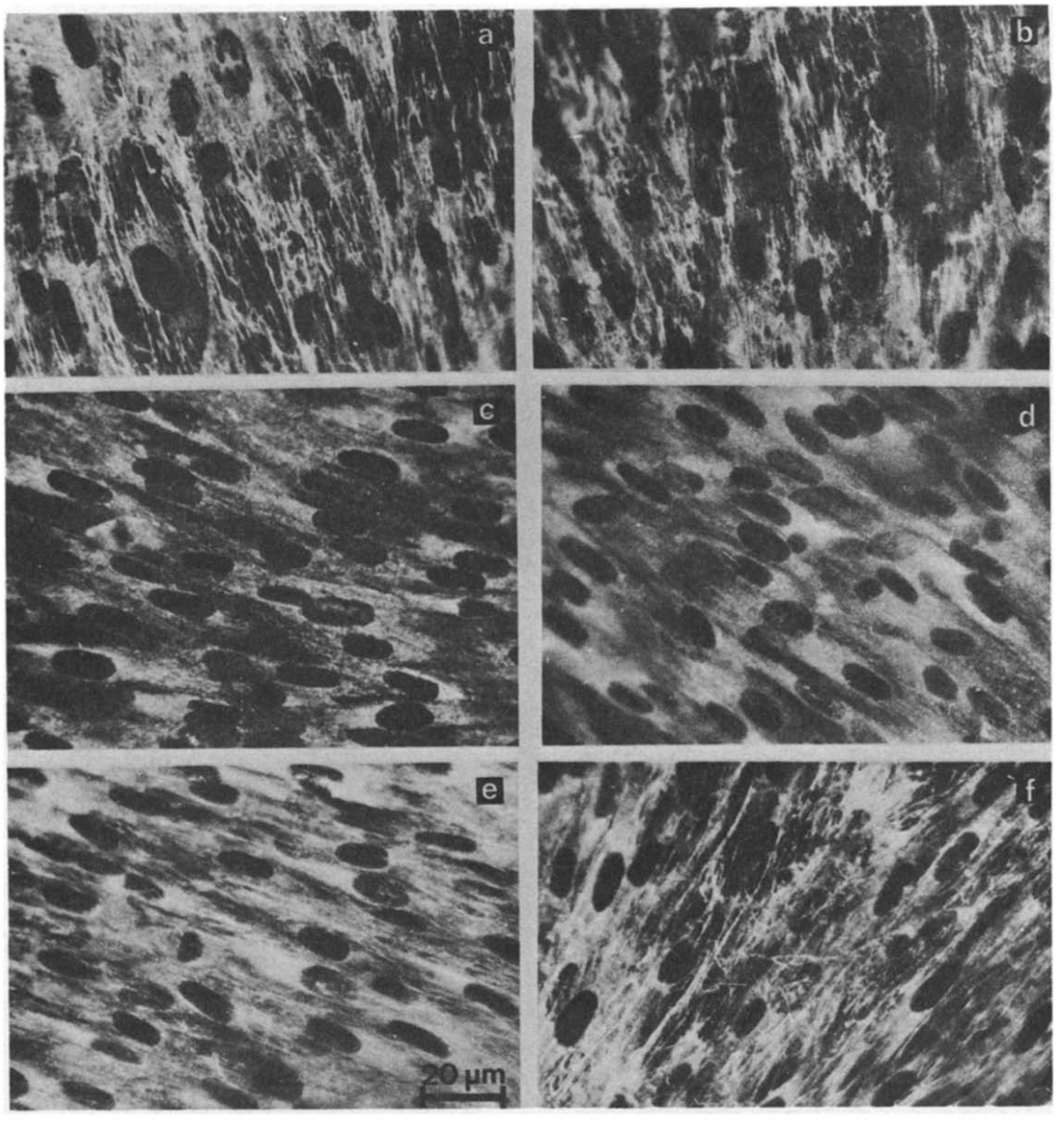

Fig. 4. Immunofluorescence staining of malotilate-pretreated human embryonic skin fibroblasts (52 hr treatment) with antibodies to: (a, b) fibronectin; (c, d) collagen type I; (e, f) collagen type III; (a, c, e) controls; (b, d, f) malotilate-incubated cells.

behaviour of fibroblasts following long term exposure to the drug (see Fig. 1a) or being exposed only during the assay (see Fig. 1b and c). Furthermore, we analysed whether malotilate interacts with the chemoattractants by synergistic or by repressive/suppressive action.

Long time incubation. Fibroblasts pretreated with malotilate for 72 hr showed a reduced migratory potential. For example, in a concentration of 90 µM malotilate, only about 70% of the cells are still able to migrate. Other cell strains (Dermatosparactic sheep cells, an Hepatoma cell line) showed a similar behaviour (data not shown).

Short time incubation. In this instance two experimental designs were chosen, one by which fibroblasts had direct contact to the agent (Fig. 1b), and an other by which the drug was together with the chemoattractant (Fig. 1c).

In both sets of experiments we could demonstrate that cells exposed to malotilate only during the chemotaxis assay exhibit a low chemotactic response (Fig. 5), while the addition of malotilate to the attractant had minor effects (about 85% of the control cells are still migrating). In the latter instance one may assume that the impact of malotilate is only seen after the drug has diffused to the upper compartment.

In a similar experiment as outlined in Fig. 1b we tested the influence of hydrocortisone, colchicin and malotilate on the chemotaxis of fibroblasts. The results are summarized in Fig. 6. It is quite obvious th :t malotilate, cyanidanol and colchicin inhibit the

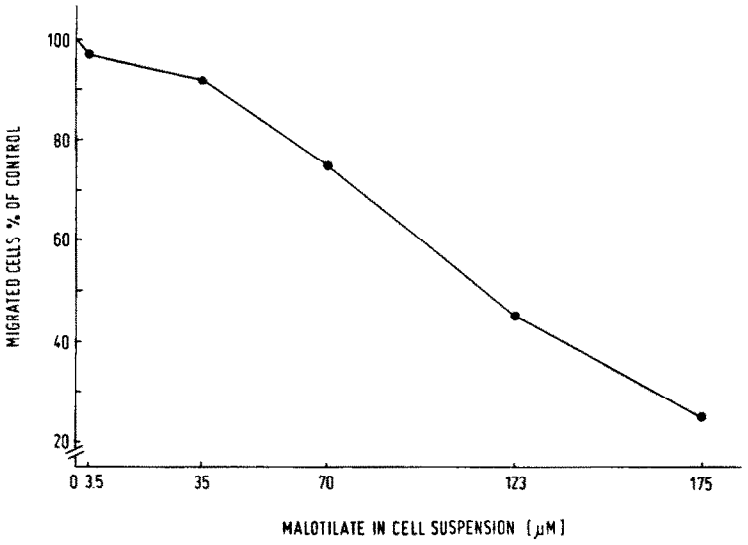

Fig. 5. Inhibitory effect of malotilate on the chemotactic activity of fibroblasts. Increasing concentrations of malotilate were added to the cell suspension (see Fig. 1b). The data were obtained from three independent experiments. The 100% value is obtained from fibroblasts which were incubated in the absence of any drug.

chemotactic migration of fibroblasts. Hydrocortisone in contrast has little effect. A similar pattern of an inhibition of migration was observed when fibronectin (20 μg/ml) was used instead of fibroblast conditioned medium (not shown).

DISCUSSION

There is considerable interest in the understanding of what mechanisms are involved in collagen regulation and of how collagen synthesis can be suppressed. There is hope that a better understanding of the basic mechanisms would help to tackle specific aspects of treatment of fibrotic processes in liver and other parenchymal tissues. In this instance one faces the experimental problem of what biological system to choose for the study of potentially antifibrotic drugs. Here we used the *in vitro* culturing system of human embryonic fibroblasts which we analysed for their capacity to synthesize collagen and for their migration in response to a chemoattractant. We focused our interest on these cellular properties

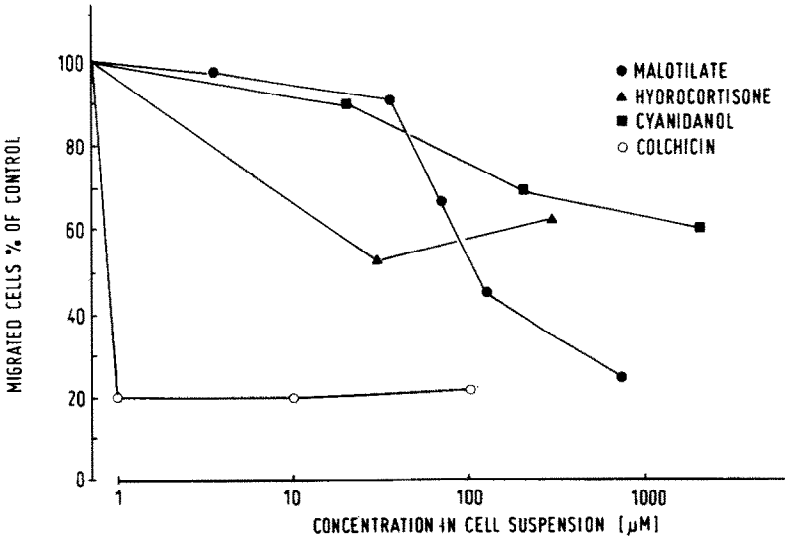

Fig. 6. Chemotactic activity of fibroblasts under the influence of four drugs: malotilate, hydrocortisone, cyanidanol, colchicin. The drugs were added to the cell suspension in increasing concentrations (see Fig. 1b). The data were collected from a single set of experiments by which triplicate values were analysed for each concentration shown. 100% migration of fibroblasts was seen when cells were not exposed to any of the drugs specified.

because excessive accumulation of matrix components [24] and immigration of fibroblasts from surrounding tissues into the injured tissue may be a major hallmark of a fibrotic process in which parenchymal tissue is progressively replaced by connective tissue [7].

By the present experiments we could show that malotilate under *in vitro* conditions specifically inhibits synthesis of collagen while synthesis of other proteins is less affected.

It is of special interest that this inhibitory effect is seen only on those cells which were trypsinized 2 days prior to the *in vitro* labelling. One can assume that these monolayers have not fully reconstituted their extracellular matrix yet. These fibroblasts possibly represent a model situation of an injured tissue analogous to that seen during wound healing or in fibrotic processes. If malotilate influences only such "injured tissues", it would show inhibitory features which are certainly desirable for a so called antifibrotic drug.

Compared to other compounds studied here or used in the treatment of fibrotic processes, malotilate is of moderate efficacy showing a distinctive reduction of collagen synthesis (Fig. 3a) combined with a relatively high tolerance for cells (Fig. 3b).

Furthermore, malotilate was highly effective to inhibit directed migration of fibroblasts as analysed by a chemotaxis assay using conditioned medium or fibronectin as a chemoattractant. At present, it is not clear whether malotilate disturbs the cellular recognition of the attractant of the motile apparatus of the cell. In any case, when fewer fibroblasts were recruited from neighbouring tissues, it is less likely that excessive amounts of collagen are produced and deposited.

In addition there is considerable interest to understand the mechanisms by which malotilate interferes with collagen production. Since intercellular degradation of collagen was the same in controls and malotilate-treated cells (data not shown), we can assume that malotilate is active on the biosynthetic pathway. In order to obtain further insight into the question whether malotilate exerts a translational, a transcriptional or a more complex control mechanism, further experiments are under way.

Our data provide circumstantial evidence that malotilate combines a unique set of properties the impact of which may result in a beneficial influence on the course of fibrotic processes. Whether or not this drug is indeed suited for human application will only be shown by studies of clinical trials.

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