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Original Paper

Inhibition of Fibroblast Collagen Synthesis and Proliferation by Levamisole and 5-Fluorouracil

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Experimental studies indicate that anastomotic healing in the intestine is compromised by the immediate postoperative administration of 5-fluorouracil and levamisole. Since fibroblast functions are crucial to healing, we investigated the effects of (combinations of) both drugs on proliferation and collagen synthesis of rat skin fibroblasts *in vitro*. Proliferation was measured in actively dividing cells by cellular [^{3}H]thymidine uptake and collagen synthesis in non-dividing cells by [^{3}H]proline incorporation into collagenase-digestible protein. 5-Fluorouracil strongly and significantly ($P < 0.05$) reduced DNA synthesis and collagen synthesis at concentrations of 1 μM or more. The latter effect was not specific for collagen since total protein production was affected similarly. Both effects depended on the duration of exposure to the drugs. Levamisole also inhibited fibroblast proliferation dose-dependently, but less effectively than 5-fluorouracil: 50% inhibition was observed at approximately 0.1 mM. Collagen synthesis was unaffected by levamisole. If levamisole was added together with a low (0.1 μM) concentration of 5-fluorouracil, which in itself did not decrease thymidine incorporation, levamisole's antiproliferative effects became apparent at concentrations as low as 1 μM. A similar effect, but at a much higher concentration (1 mM) was noted on fibroblast collagen synthesis. These results indicate that levamisole potentiates 5-fluorouracil effects in fibroblast cultures and that direct effects of these drugs, alone or in combination, on fibroblast proliferation and collagen synthesis may be responsible for their negative influence on wound repair. © **1998 Elsevier Science Ltd.**

Key words: collagen, fibroblast, 5-fluorouracil, levamisole, proliferation
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INTRODUCTION

SURGERY REMAINS the only potentially curative treatment modality for early-stage colorectal cancer, but a majority of the patients presenting with this disease are candidates for adjuvant chemotherapy because of their limited chances of 5-year survival [1]. Massive preclinical and clinical research efforts have shown that 5-fluorouracil is the most effective single agent to be used as an adjunct to surgery for colorectal cancer and current research aims to improve its efficacy by combinations with other agents such as folinic acid (leucovorin) and interferon-α [2, 3]. In this respect, its combination with levamisole has yielded promising results [4], particularly in stage III colon carcinoma [5]. Although major questions still have to be resolved concerning timing and route of administration of cytostatic agents, there exists growing interest in the use of immediate postoperative adjuvant therapy after resection of intestinal malignancies [6–9].

While conventional adjuvant therapy in colorectal cancer routinely is withheld until weeks after surgery, there exists an excellent rationale to start therapy in the immediate post-operative period [10]. Theories of tumour-cell kinetics and drug resistance predict that cancer cells are more susceptible to anticancer therapy when the tumour burden is small [11] and numerous animal studies have demonstrated that a primary tumour can inhibit the growth of metastatic deposits and that its removal can increase the growth rate of metastases [12]. Also, tumour implantation and growth of metastases in animals is enhanced by operation and anaesthesia [13]. In addition, the peri-operative period is a logical time for adjuvant (immuno)therapy since the cellular antitumour responses, which are already depressed in patients with

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gastrointestinal cancer, are—transiently—decreased further by major surgery [14].

It thus becomes necessary to investigate the potential effects of such therapies on the healing of intestinal anastomoses. In previous experimental studies in the rat we have shown that peri-operative administration of 5-fluorouracil may severely impair the development of anastomotic strength [15]. This loss of strength is accompanied by a reduced collagen synthetic capacity and lowered accumulation of collagen in the wound area. Levamisole, alone or in combination with 5-fluorouracil, also appears to compromise intestinal repair [16].

The proliferation phase of the wound healing sequence is dominated by the fibroblasts. These cells are the primary source of collagen, on which protein wound strength essentially depends. In fact, the number of fibroblasts participating in repair is often an indirect measure of the development of wound strength [17]. Anastomotic repair in the intestine is characterised by a massive increase in collagen synthesis during the early postoperative period [18, 19]. This study describes the direct effects of 5-fluorouracil and levamisole, alone and in combination, on rat fibroblast proliferation and collagen synthesis measured *in vitro*. In addition, we collected serum from rats which had been treated with these drugs, and investigated whether it would impede these cellular functions.

MATERIALS AND METHODS

Materials

All supplies for cell culture were purchased from Life Technologies (Breda, The Netherlands). 5-Fluorouracil was obtained from Abic (Netanya, Israel) and levamisole from Janssen (Beerse, Belgium). L-[2,3-^{3}H]proline (1.41 TBq/mmol) and [6-^{3}H]thymidine (999 GBq/mmol) were purchased from Amersham International, Little Chalfont, U.K. Collagenase (type VII) was obtained from Sigma (St. Louis, Missouri, U.S.A.). All other reagents were of analytical grade (Merck, Darmstadt, Germany).

Animals

In order to obtain normal rat serum (NRS) for maintaining the rat fibroblast cultures, a serum pool was formed from blood collected from young adult male Wistar rats. Postoperative rat serum was obtained from animals used for parallel studies on intestinal anastomotic healing [16]. Rats underwent resection and anastomosis of both the small and large bowel and groups of animals received intraperitoneal 5-fluorouracil (20 mg/kg body weight/day), oral levamisole (5 mg/kg body weight/day) or both immediately after operation and on the next two days. A control group received saline only. Three days after operation all animals were killed and serum was collected and stored at −80°C.

The experiments were approved by the Animal Ethics Committee of the Medical Faculty of the University of Nijmegen.

Cell culture

Rat skin fibroblast lines were obtained from explants of skin biopsies from young adult male Wistar rats. They were grown in Roswell Park Memorial Institute medium (RPMI1640, Dutch modification) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, antibiotics (40 μg/ml gentamycin) and 10% heat-inactivated fetal calf serum (FCS) at 37°C in a 5% CO_2, 95% air humidified atmosphere. Cells were used before the 10th passage. Cell counting was performed on a Coulter Counter ZM (Coulter Electronics, Mijdrecht, The Netherlands).

Assay of fibroblast proliferation

Freshly trypsinised fibroblasts were plated in 96-well microtitre plates (6 wells for each experimental condition) at a density of approximately 2×10^3 cells/well in 0.1 ml RPMI plus 10% FCS. After a 4 h incubation the medium was replaced by 0.1 ml RPMI and the cells were incubated for another 18 h. Subsequently, the medium was replaced again by 0.1 ml RPMI plus 1% NRS and incubation continued for another 48 h. Thereafter, 18.5 kBq [^{3}H]thymidine (in 10 μl RPMI) was added and incubation continued for a final 24 h. The medium was then removed, the cells trypsinised and harvested (on a LKB Wallag cell harvester) and thymidine incorporation was measured. In various experiments the medium was supplemented with 5-fluorouracil or levamisole (or both) or the concentration or composition of the rat serum was varied during the last 72 h of incubation.

Assay of fibroblast collagen production

Assay of collagen production by steady-state, visually confluent fibroblasts was assessed over a 24 h period by [^{3}H]proline incorporation into collagenous protein. Freshly trypsinised fibroblasts were plated into 24-well plates at a density of approximately 3×10^4 cells/well in 1 ml RPMI plus 10% FCS. Three days after plating the medium was removed from the confluent cells and replaced, after washing the cells twice with phosphate-buffered saline, by 0.4 ml RPMI plus ascorbic acid (50 μg/ml) with 1% NRS. 24 h later the medium was replaced by 0.4 ml of the same medium plus β-aminopropionitrile (50 μg/ml) and 74 kBq/ml [2,3-^{3}H]proline for the final 24 h of culture. In various experiments the medium was supplemented with 5-fluorouracil or levamisole (or both) or the concentration or composition of the rat serum was varied during the final 48 h of culture.

After the labelling period, 0.2 ml of 50 mM Tris-HCl pH 7.6 containing 0.25 M ethylenediaminetetraacetic acid (EDTA), 0.1 M *N*-ethylmaleimide (NEM) and 10 mM phenylmethylsulphonylfluoride (PMSF) was added to each well and the total content was removed. The wells were washed twice with 1 ml 50 mM Tris-HCl pH 7.6 containing 1 mM proline. The wash solutions were combined with the suspension containing cells, and medium and 0.2 ml bovine serum albumin (4.25% w/v) was added. The final suspension was freeze/thawed three times and the proteins were precipitated with trichloroacetic acid (TCA; final concentration 10%). The radioactive protein was separated from free [^{3}H]proline by repeated (3×) washes with 5% TCA containing 1 mM proline at 4°C.

The final sediment was dissolved in 0.75 ml 0.2 M NaOH and neutralised by the addition of 0.3 ml 1 M Hepes and 0.3 ml 0.15 M HCl. Aliquots from this solution (0.1 ml) were counted to determine the incorporation in total protein. In order to quantitate proline incorporation into collagen, 0.2 ml 20 mM Tris-HCl pH 7.6 containing 50 mM $CaCl_2$ and 0.1 ml (27 IU) collagenase were added to a 0.5 ml aliquot of the solubilised sample and the mixture was incubated for 3 h at 37°C. The digestion was terminated by the addition of TCA and tannic acid up to final concentrations of 0.3 and 1.5 mM, respectively. After centrifugation (10 min 10 000*g*) a 1 ml aliquot of the supernatant was counted in a liquid scintillation analyser. The same procedure was followed without

the addition of collagenase. Subtraction of the counts released during this control incubation from those released in the presence of collagenase yielded the collagen-specific incorporation, which will be referred to as collagenase-digestible protein (CDP). Subtraction of the radioactivity in the CDP fraction from that in total protein yields the incorporation into non-collagenous protein (NCP). Incorporation into CDP and NCP was quantified per well.

The relative collagen synthesis was calculated with the formula [20] that takes into account the enrichment of proline in collagen compared to other proteins:

$$\text{RCS} = \%\ \text{relative collagen synthesis}$$

$$= \frac{\text{CDP}}{(\text{NCP} \times 5.4) + \text{CDP}} \times 100\%$$

For each experimental condition, six wells were used: four wells for the actual measurement of [^{3}H]proline incorporation and two wells for a cell count at completion of the incubation period.

Statistical analysis

Differences between control and drug-treated cultures were tested for significance ($P < 0.05$) using two-sided Student's *t*-test.

RESULTS

Addition of 5-fluorouracil to the culture medium for 72 h resulted in a marked, dose-dependent suppression of DNA synthesis in actively dividing cultures of rat skin fibroblasts in the logarithmic growth phase (Figure 1). Inhibition was significant from concentrations of 1 μM upwards and 50% inhibition was observed at a concentration of approximately 20 μM.

Figure 2(a) shows that levamisole, if present for 72 h, also suppressed fibroblast proliferation, but from a concentration of 10 μM upwards: 50% inhibition was found at a concentration of approximately 100 μM. However, if levamisole was added together with a low (0.1 μM) concentration of 5-fluorouracil, which in itself did not decrease thymidine incorporation, its antiproliferative effects became apparent at concentrations as low as 1 μM (Figure 2(b)). The same phenomenon was observed if levamisole was added together with 10 μM 5-fluorouracil (Figure 2(c)). Apparently, a small dose of levamisole was able to strongly potentiate 5-fluorouracil-induced suppression of fibroblast proliferation (or, alternatively, 5-fluorouracil enhanced the levamisole effect).

The experiments on collagen synthesis were performed with cultures of confluent, non-dividing fibroblasts. Under these conditions, the addition of neither 5-fluorouracil nor levamisole (nor their combination) significantly affected the number of viable cells present. The effect of 5-fluorouracil on fibroblast collagen synthesis is illustrated in Figure 3.

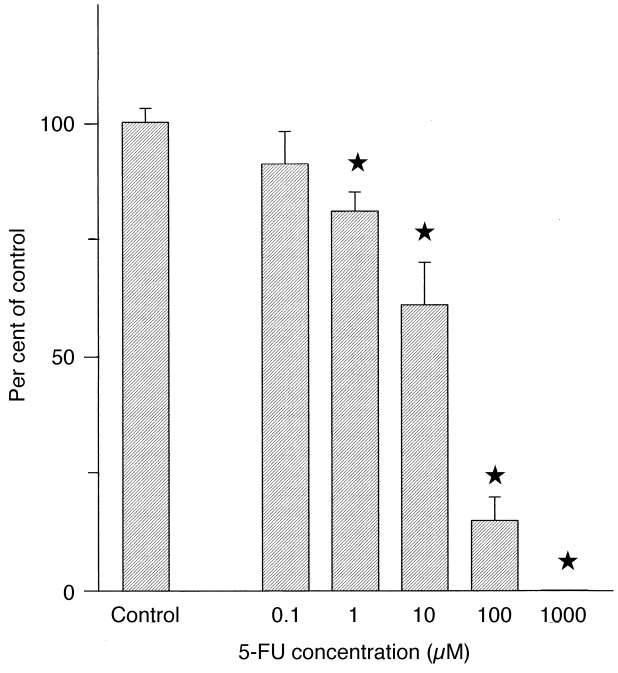

Figure 1. Effect of 5-fluorouracil on fibroblast proliferation. 5-Fluorouracil was present for 72 h. The average (± S.D.) of six measurements is given as percentage of [^{3}H]thymidine incorporation in the absence of 5-fluorouracil (control). *$P < 0.05$ versus control.

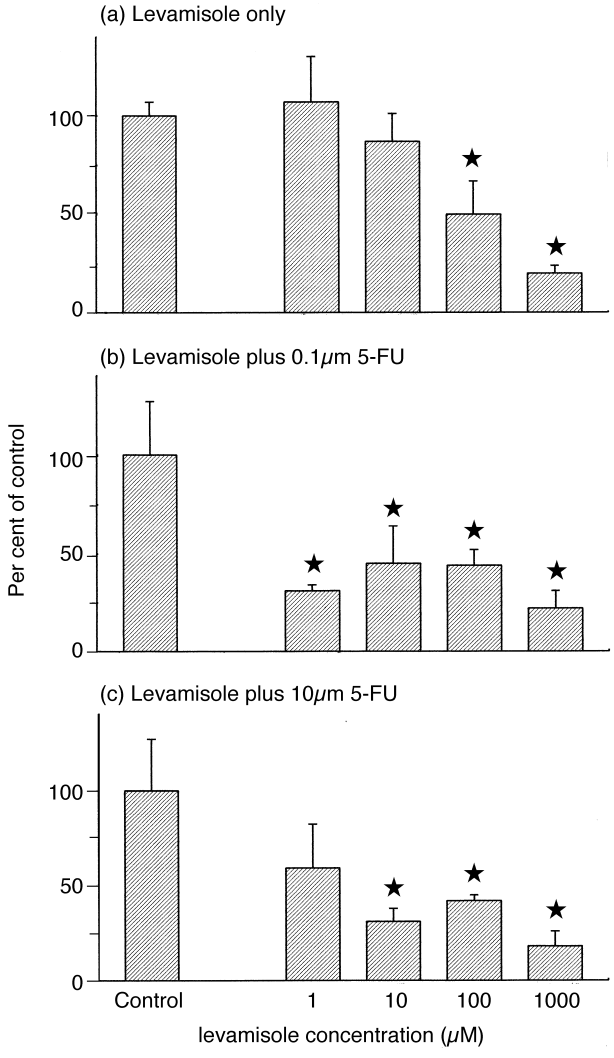

Figure 2. Effect of levamisole on fibroblast proliferation. Results are given for the effect of levamisole alone (a) or in the presence of 0.1 μM (b) or 10 μM (c) 5-fluorouracil. The drugs were present for 72 h. The average (± S.D.) of six measurements is given as percentage of the control. The latter constitutes the [^{3}H]thymidine incorporation in the absence of levamisole (with or without 5-fluorouracil). *$P < 0.05$ versus control.

If 5-fluorouracil was added only during the final 24 h of incubation, collagen synthesis was inhibited by 20% in the 1–10 μM range; this effect did not persist at higher concentrations. However, if 5-fluorouracil was present over a 48 h period, all concentrations from 1 μM upwards significantly reduced the production of collagenase-digestible protein per cell. This effect was not specific for collagen since the percentage relative collagen synthesis remained unaltered (Figure 3(b)).

Collagen synthesis in the presence, over a 48 h period, of increasing concentrations of levamisole is shown in Figure 4(a). Neither collagen production nor the production of non-collagenous protein (data not shown) was affected by levamisole in concentrations up to the millimolar range. If levamisole was added in the presence of 0.1 μM 5-fluorouracil (which itself did not affect synthesis), the highest levamisole concentration tested seemed to have an inhibitory effect: at 1 mM collagen synthesis was slightly, but significantly, reduced (Figure 4(b)). This potentiating effect of levamisole was also found if the drug was added in the presence of 10 μM 5-fluorouracil (Figure 4(c)). This concentration of 10 μM 5-fluorouracil alone reduced synthesis by 50%, but addition of 1 mM levamisole resulted in a further 30% inhibition of fibroblast collagen synthesis. The reduction appeared to be specific for collagen since the relative collagen synthesis decreased from 6.5 ± 0.4 to 4.8 ± 1.2%.

In order to investigate whether postoperative administration of levamisole, alone or in combination with 5-fluorouracil, would induce the sustained presence of circulating compounds which could interfere with fibroblast functions, we examined the effects of increasing concentrations of rat serum on fibroblast proliferation and collagen synthesis. In Figure 5 the effects of serum obtained from control (operated) rats are compared with those of serum from rats which had received levamisole plus 5-fluorouracil on the day of operation and the next two days. Figure 5(a) shows that collagen synthesis increased with serum concentration but that there was no significant difference between the two sera (present for 48 h). Figure 5(b) indicates that serum from both groups of rats, present for 72 h, equally affected fibroblast proliferation. Serum collected from rats which had received levamisole (or 5-fluorouracil) alone showed similar effects (results not shown).

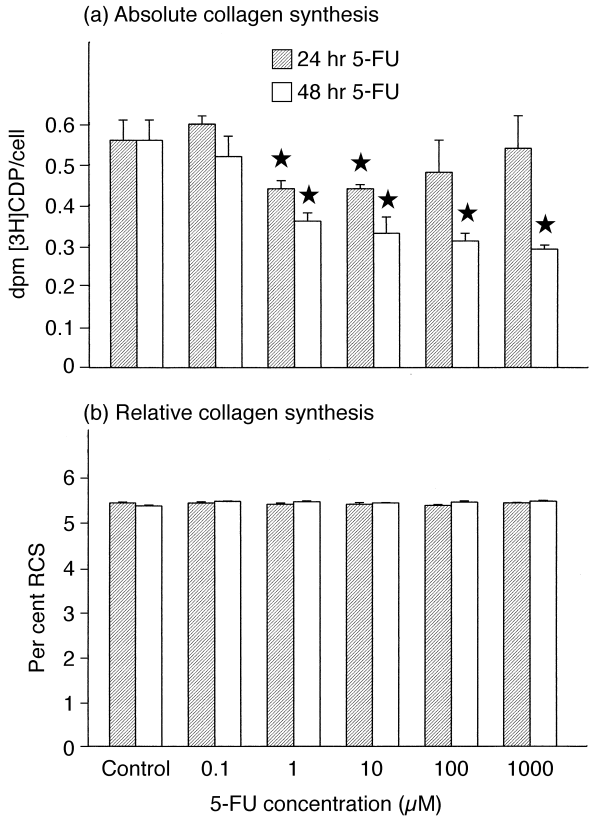

Figure 3. Effect of 5-fluorouracil on fibroblast collagen synthesis. Results are the average (± S.D.) of four cultures. (a) Represents the absolute collagen synthesis, calculated as radioactivity incorporated in collagenase-digestible protein (CDP); (b) the relative collagen synthesis (RCS) calculated as a percentage of total protein synthesis. Shaded bars represent results after 24 h incubation with 5-fluorouracil and open bars results after 48 h incubation with 5-fluorouracil. *$P < 0.05$ versus control.

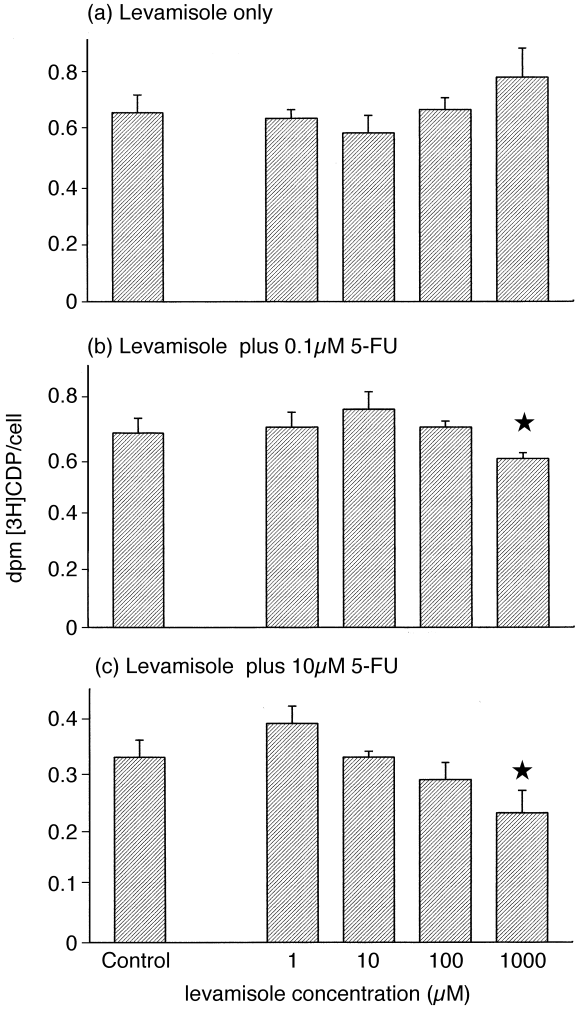

Figure 4. Effect of levamisole on fibroblast collagen synthesis. Results are given for the effect of levamisole alone (a) or in the presence of 0.1 μM (b) or 10 μM (c) 5-fluorouracil. The drugs were present for 48 h of incubation. The average (± S.D.) of four measurements is given for the absolute collagen synthesis calculated as radioactivity incorporated in collagenase-digestible protein (CDP). Control values were measured in the absence of levamisole (with or without 5-fluorouracil). *$P < 0.05$ versus control.

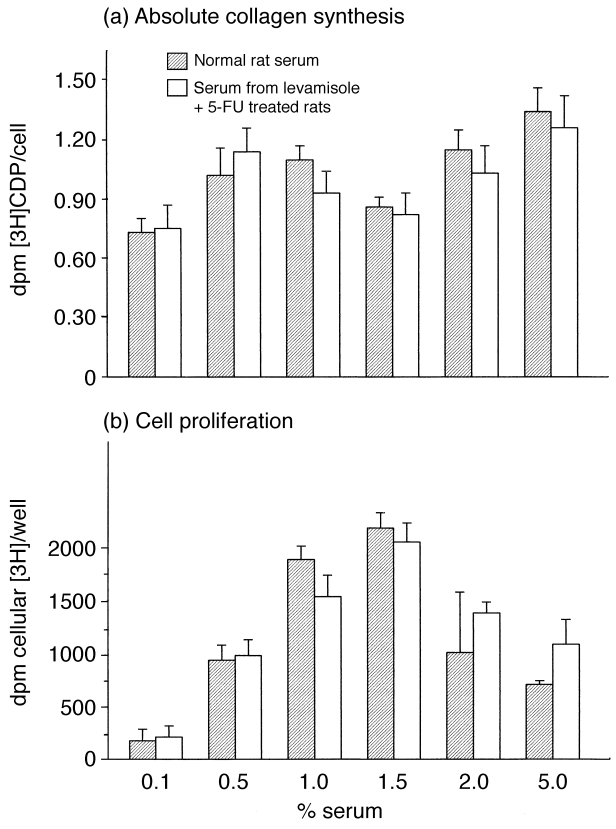

Figure 5. Effect of serum concentration on fibroblast collagen synthesis and proliferation. (a) Average (± S.D.) of four wells for measurement of absolute collagen synthesis. (b) Average (± S.D.) of six wells for measurement of proliferation. Shaded bars: pooled serum from operated (cf. Materials and Methods) rats; open bars: pooled serum from operated rats treated with levamisole plus 5-fluorouracil. Sera were present for 48 h in the collagen synthesis assay and for 72 h in the proliferation assay.

DISCUSSION

5-Fluorouracil and levamisole, if administered alone or together in the immediate postoperative period, may impair the development of strength in experimental intestinal anastomoses and thus increase the chances of anastomotic dehiscence [16]. Elucidation of the mechanisms involved is necessary in order to find ways to avoid this unwanted phenomenon. Fibroblasts are essential to the repair sequence, especially in terms of the deposition of the new extracellular matrix [21]. Thus, fibroblast migration, proliferation and production of matrix components are fundamental to adequate healing. The present results indicate that direct inhibition of essential fibroblast functions may be responsible for the negative effects of these drugs on wound repair.

It is to be expected that an antiproliferative agent such as 5-fluorouracil will inhibit proliferation in actively dividing cell cultures, although in earlier studies we found the proliferation of human skin fibroblasts to be refractory to 5-fluorouracil concentrations up to 50 μM [22]. In these experiments the cells were only exposed to 5-fluorouracil for 24 h. The present results, obtained after 72 h exposure to 5-fluorouracil, showed 50% inhibition of proliferation at a concentration of approximately 20 μM. Apparently, the inhibition of proliferative activity strongly depends on the duration of exposure, which has also been reported for ocular (subconjunctival) fibroblasts [23].

Very little has been reported on the possible effects of 5-fluorouracil on matrix production. In our previous study [22], non-dividing cultures of human skin fibroblasts were exposed for 24 h to 5-fluorouracil concentrations up to 50 μM without affecting basal collagen synthesis, while transforming growth factor-β-induced collagen synthesis was inhibited at—pharmacologically relevant—drug concentrations between 1 and 10 μM. However, the present results show that, like inhibition of proliferation, suppression of collagen synthesis appears dependent on duration of exposure: incubation of fibroblasts for 48 h with 5-fluorouracil at concentrations of 1 μM or more results in a consistent reduction in the amount of collagenase-digestible protein per cell. Since these results were observed in confluent, non-dividing cell cultures, it seems likely that the inhibitory effect is the result of an RNA-directed, rather than a DNA-directed, action of 5-fluorouracil [24].

5-Fluorouracil, administered daily as an intraperitoneal bolus of 20 mg/kg body weight from the day of surgery onwards, strongly suppresses the healing of intestinal anastomoses in the rat [15, 16, 25]. Such a drug regimen does not appear to lead to a sustained presence of circulating compounds which affect fibroblast functions most relevant to repair. While both fibroblast collagen synthesis and proliferation, as measured *in vitro*, depend on the presence of serum, we found no indications that these processes are compromised by serum collected from the 5-fluorouracil-treated animals. This may not be surprising since 5-fluorouracil, which is not taken up by cells, is inactivated and eliminated rapidly [26]. However, although we do not know what concentrations of 5-fluorouracil are actually achieved in the wound area, the current data suggest that the detrimental effects to repair are caused solely by direct inhibition of fibroblast proliferation and collagen synthesis.

The use of levamisole, together with 5-fluorouracil, is recommended for stage III, node-positive, patients with colon cancer [5, 27]. In stage II patients, its use has demonstrated a trend toward a lower rate of recurrence, but no improvement in overall survival [28]. However, the optimal sequence and schedule of this drug combination has yet to be established. There are excellent reasons to start treatment immediately after surgery [10] and a small study has already been reported where levamisole, together with 5-fluorouracil, was given on the first three postoperative days [29]. Thus, its potential detrimental effects on intestinal repair, which we reported recently [16], are of great interest.

It has been proposed that the substantial therapeutic advances of levamisole are due to enhancement of the body's immune response rather than the biochemical modulation of 5-fluorouracil [30, 31], although the mechanisms of interaction between these two agents largely remain to be elucidated.

The present results indicate that levamisole in itself can inhibit fibroblast proliferation. A significant reduction is achieved at a concentration of 0.1 mM which is much higher than those achieved clinically [31]. However, if levamisole is combined with a dose of 5-fluorouracil, which is so low as not to affect fibroblast proliferation, addition of only 1 μM levamisole reduces proliferation by 70%. Thus, in this respect, levamisole, at a pharmacologically relevant concentration, appears to potentiate 5-fluorouracil fibroblast toxicity. It is

noteworthy that levamisole, up to concentrations of 3 μM, reportedly has no demonstrable effects on 5-fluorouracil cytotoxicity in human colon cancer cells *in vitro* [32].

It has been reported that 2 mM levamisole strongly inhibits the production of type X collagen, but not that of type II collagen, in chick chondrocytes by reducing the gene transcription rate [33]. However, our data show that levamisole, in concentrations up to 1 mM, does not appear to affect collagen synthesis in skin fibroblasts. Interestingly, the combination of 1 mM levamisole with either 0.1 or 10 μM 5-fluorouracil results in a limited, but significant, lowering of fibroblast collagen production. Although this concentration presumably far surpasses the clinically relevant range, it remains noticeable that in this respect levamisole is also able to potentiate the effect of 5-fluorouracil.

In summary, our data suggest that the impairment of wound healing by (combinations of) 5-fluorouracil and levamisole, as previously observed by us in animal studies, may largely be explained by direct effects on fibroblast function. While its mechanism has yet to be elucidated, a synergistic effect between 5-fluorouracil and levamisole with regard to both proliferative and synthetic functions of the fibroblast has been demonstrated. Thus, in the context of wound repair, concurrent administration of these two drugs in the immediate postoperative period may, although improving cytotoxicity for tumour cells, certainly be harmful for essential reparative functions of other cells.

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