Stimulation of the proliferation of human dermal fibroblasts *in vitro* by a lipidocolloid dressing

• **Objective:** The effect of Urgotul on normal human dermal fibroblast proliferation was studied *in vitro* and compared with that of two other dressings: Mepitel and Tulle Gras.
• **Method:** Proliferation was measured by the extent of thymidine incorporation into the replicating DNA of proliferative fibroblasts in contact with the complete dressing. Additional cell viability and metabolism were evaluated using MTT assay. Morphology and ultrastructure analysis were based on immunolabelling and confocal laser microscopy.
• **Results:** Only Urgotul significantly stimulated thymidine incorporation, generally with a maximal proliferative effect at a contact time of 48 hours. This was confirmed by the observation of a greater number of dividing cells (mitotic cells) than in the control cultures. No cytotoxicity was observed following treatment with this dressing. Cells exhibited normal structural and ultrastructural features.
• **Conclusion:** Fibroblasts play a key role in dermal wound repair. The ability of Urgotul to promote fibroblast proliferation could explain its efficiency in the healing process of acute and chronic wounds.

• **Declaration of interest:** This study was supported by Urgo Laboratories.

lipidocolloid technology; proliferation; fibroblasts

Wound healing is a complex biological process that involves interactions between epidermal and dermal cells and the extracellular matrix, coordinated by an array of cytokines and growth factors. These cells restore the integrity of the injured tissue. This process has three main phases: inflammation; repair; remodelling.

During repair, the formation of granulation tissue is required to allow epidermal cells to migrate across the wound bed to close the injury site. The formation of granulation tissue observed clinically corresponds to the production of the extracellular matrix. Fibroblasts are key cells in the production of this extracellular matrix, which comprises proteins such as collagens and fibronectin, proteoglycans, hyaluronic acid and other miscellaneous molecular species. Fibroblasts migrate into the injury site, proliferate and fill the wound with newly synthetised matrix.[1-3]

Wound management requires selection of the most appropriate dressing for the wound and its environment.[4] Dressings can be divided into several types, based on their structure and composition. Wound contact dressings are used during the phases of granulation tissue formation and re-epithelialisation.

Urgotul (Urgo Laboratories) is a wound contact dressing developed from lipidocolloid technology. It is a non-occlusive hydrocolloid dressing comprising a 100% polyester net impregnated with hydrocolloid particles dispersed in a petrolatum jelly matrix. It is indicated for acute and chronic wounds.[5] A previous study showed that it did not induce a cytotoxic effect on fibroblast monolayer cultures.[6]

The present *in vitro* study examines the effect of Urgotul on normal human dermal fibroblast proliferation in culture, and then compares this effect with that induced by Tulle Gras (Solvay Pharma, France) and a non-adherent silicone dressing, Mepitel (Mölnlycke Health Care, Sweden). The two comparator dressings were chosen because they have the same indications as Urgotul.

Data on the effects on cell viability, metabolism, morphology and ultra-structure are also presented.

Materials and method

Fibroblast cultures

All standard cell culture reagents were purchased from Invitrogen (Cergy Pontoise, France). Normal human dermal fibroblasts were isolated from breast explant validated cell pools and cultivated at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2mM glutamine, 50UI/ml penicillin, 50µg/ml streptomycin and 10% foetal calf serum. Cell density and culture format/support are described for each type of experiment. For one series of experiments, cell proliferation was blocked by treating cells with 10µg/ml mitomycin C (Sigma, final concentration in culture medium) for two hours, at 37°C, followed by two washes in culture medium. This shows whether or not there is an increase in thymidine incorporation in fibroblasts that are unable to proliferate.

F.X. Bernard, PhD, Scientific Director,
C. Barrault, PhD, Unit Manager, Dermato-Cosmetics,
BIOalternatives SAS, Gençay, France;
F. Juchaux, MSc, Study Director, Dermato-Cosmetics,
BIOalternatives SAS, Gençay, France;
C. Laurensou, PhD, Formulation Department, Urgo Laboratories, Chenôve, France;
L. Apert, PhD, Urgo Laboratories, Chenôve, France.
Email: c.laurensou@urgo.fr

Dressings

The three dressings tested were provided in sterile packaging. Square pieces measuring exactly 1.5cm x 1.5cm of each dressing were aseptically prepared before each experiment and applied directly onto cell layers. Small sterile caps from plastic tubes, previously soaked twice in culture medium, were used to maintain the dressings at the surface of the cell layers. Controls with caps alone showed no significant influence on the parameters analysed.

Urgotul extracts were compared with other physical forms of Urgotul dressing only, particularly intact Urogul. Extracts were prepared by soaking 1.5cm x 1.5cm pieces in 400µl fibroblast culture medium in a 12-well microplate for 48 hours at 37°C. This determines if diffusible compounds from Urgotul are directly responsible for the stimulation of proliferation.

Urgotul is composed from polyester net, carboxymethylcellulose and vaseline; Mepitel is composed of polyamide net and silicone; Tulle Gras is composed of viscose net and vaseline.

Cell viability and metabolism

Normal human dermal fibroblasts were precultivated to confluency in 12-well microplates (one set of plates per incubation time). Equivalent pieces of dressings were then applied onto the cell layers, and the cells were cultivated for 24 hours, 48 hours, 72 hours, 96 hours or 168 hours. Controls comprised cultures without any dressing (with or without plastic caps). Each condition was repeated in triplicate.

At the end of each incubation time, the dressings were removed and the overall metabolic activity of the cultures was measured using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) assay,[7] which evidences the activity of mitochondrial dehydrogenases. The absorbance of formazan was recorded at 540nm with a ThermoMax microplate reader controlled by SoftMax software (Molecular Devices).

Images of the culture wells after dressing removal and MTT staining were taken, as well as microphotographs of cells before MTT staining.

Proliferation assays

Normal human dermal fibroblasts were seeded at low density (3 x 10^4 cells per well; 30% confluency) in 12-well microplates (one set of plates per incubation time) and pre-cultivated for 24 hours before application of the dressing, the Urgotul extract, or epidermal growth factor (EGF, Sigma; 10ng/ml, final) as a proliferation-inducing reference compound. Controls comprised cultures with no dressing (with or without plastic caps). Each condition was repeated in triplicate.

Cultures were incubated for 24 hours, 48 hours or 72 hours, with or without removal of the culture medium, and dividing cells were labelled by the addition of 10µCi per well (methyl-3H)-thymidine (Amersham) for the last 24 hours.

Cells were lysed and DNA was collected onto Skatron glass fibre filters (Molecular Devices, St Grégoire, France) using a Combi Cell Harvester (Molecular Devices). After extensive washing, thymidine incorporation was measured by scintillation counting using a LKB1211 Rackbeta counter.

Statistical analysis

The raw experimental data were transferred to and analysed using Prism software (Graph Pad Software). The inter-group comparisons were performed by variance analysis (Anova) with Dunnett's multiple comparisons test.

Visualisation by confocal laser microscopy

Normal human dermal fibroblasts were seeded to medium density in Labteck chambers (NUNC 177445), covered with dressings as described above and cultivated for 72 hours or 168 hours. Controls were cultures with no dressing (with or without plastic caps). Each condition was in duplicate.

At the end of each incubation time, the dressings were removed, the cell layers washed with serum-free DMEM medium, and the cells fixed with 4% paraformaldehyde (Sigma) in 200mM Tris-buffered saline (TBS) containing 1.54mM NaCl, 20mM $MgCl_2$, 20mM EGTA, pH 7.5 (TBS) for 20 minutes at room temperature. Cells were then rinsed in TBS and permeabilised using TBS/0.1% Triton X-100 (Sigma) for five minutes at room temperature.

Slides were finally washed in TBS/0.5% bovine serum albumin (TBSA) and sites from cell layers that could non-specifically bind the antibodies were saturated by incubation for 10 minutes in the same buffer. Tubulin and actin were respectively recognised by an anti-tubulin monoclonal antibody (Sigma T9026) and by a phalloidin Alexa Fluor 680 complex (molecular Probes A-22286; red fluorescence), mixed in TBSA.

Monoclonal antibody bound to tubulin was labelled using a fluorescein isothiocyanate-goat anti-mouse antibody conjugate (GAM-FITC; TEBU France M30801; green fluorescence).

After TBS washing and final processing, slides were observed by confocal laser microscopy using a Bio-Rad MRC 1024 confocal system (laser argon-krypton 15mW) coupled to an Olympus IX70 inverted microscope.

Results

Cell viability and metabolism

In a first set of experiments, the effects of the dressings on the overall normal human dermal fibroblast viability were analysed using a quantitative MTT reduction assay coupled to a direct visualisation of

cell layers. The results from the MMT assay were expressed as a percentage of the values of the untreated controls (controls with plastic cap, without dressing). The controls with or without a cap gave the same values (Fig 1).

Urgotul, Mepitel and Tulle Gras did not significantly modify the overall metabolic activity of the cultures (there were no significant differences in MTT reduction values).

Visual observation of the cell layers, however, showed clear differences between the treatments. Very few lesions, reflecting the 'print' pattern of the net of the dressing, were observed with Urgotul and Mepitel. In the case of Tulle Gras, the cell layer appeared to be significantly damaged on incubation with the dressing. The area in close contact with Tulle Gras exhibited large lesions after removal. In fact, differences appeared according to the location in the culture well. A clear decrease in cell number and metabolic activity was observed in the central area of the culture well, which was in contact with the dressing, whereas an increase in MTT staining (blue) of cells was observed in the periphery of the well. Tulle Gras was clearly cytotoxic for cells in contact with the dressing. However, the peripheral cells exhibited a stronger blue staining with MTT than the control cells (especially at 48 hours).

Fig 1. Effect of dressings on the viability of normal human dermal fibroblasts in confluent monolayers. Absorption values (%)

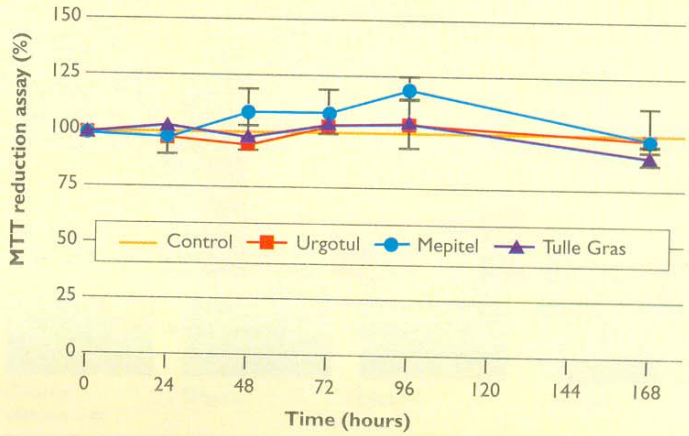
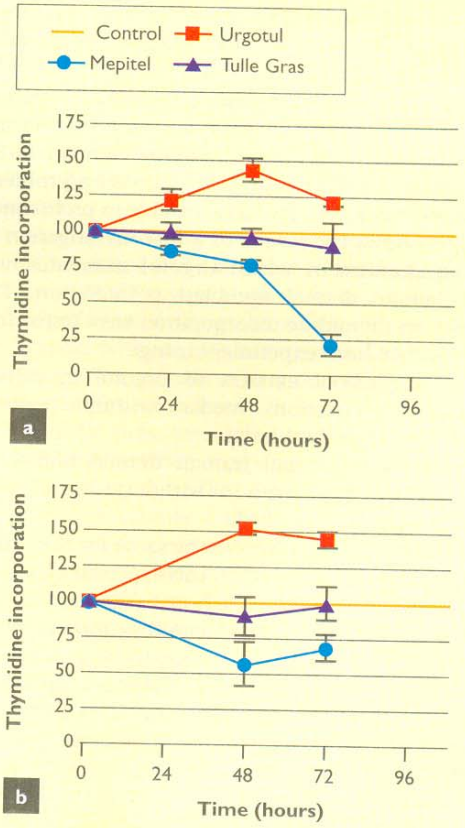


Fig 2. Effect of dressings on normal human dermal fibroblast proliferation: thymidine incorporation. Experiment without medium change (a) or with medium changed every 24 hours (b)



Cell proliferation

In non-confluent monolayer cultures, there was a significant proliferation of normal human dermal fibroblasts (the population doubled approximately every 48 hours), and replication rates can be precisely evidenced by the incorporation of thymidine into the DNA of replicating cells.

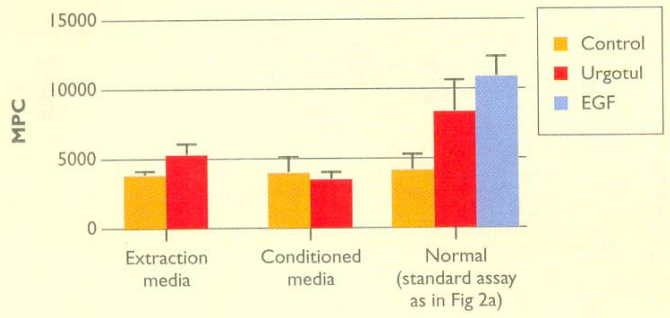
Fig 2 shows the effects of each dressing on normal human dermal fibroblast proliferation in a 'continuous' (standard) protocol (a) and in a protocol in which culture media were renewed every 24 hours (b). In both protocols Urgotul induced a significant increase of thymidine incorporation by normal human dermal fibroblast, with an apparent optimum at 48 hours (24 hours' incubation and 24 hours' thymidine labelling; $p<0.01$). Stimulation at 48 hours was 45% greater than in the controls in all the independent experiments performed (up to a maximal value of 70% for an experiment).

Mepitel exhibited an overall (non-significant) tendency to reduce thymidine incorporation. However, in the case of the long-term application without medium renewal (Fig 2a), a significant confirmed reduction of cell proliferation was observed, with no cell toxicity (moderate cytostatic effect).

As shown for metabolic activity (Fig 1), Tulle Gras had similar thymidine incorporation values to the control, despite having a clear toxic effect on the cells in its immediate environment.

To verify the specificity of the stimulation induced

Fig 3. Effects of different Urgotul 'preparations' on normal human dermal fibroblast proliferation: thymidine incorporation in 48-hour assay



by Urgotul and understand why the Tulle Gras-treated cultures did not exhibit reduced thymidine incorporation, thymidine incorporation was measured in normal cell layers and in equivalent cell layers previously treated with the cytostatic reagent mitomycin. As expected, mitomycin-treated cultures showed only a residual proliferation rate (8% thymidine incorporation), after 48 hours.

In this experiment, Urgotul, in normal conditions, increased thymidine incorporation by 9000 counts per minute (cpm) (170% of control, $p<0.01$) and by 800cpm in mitomycin-treated cells (175% of control, $p<0.01$). Tulle Gras did not induce a significant increase of thymidine incorporation compared with the control. Mitomcycin blocks division/proliferation, so only a very few cells are able to proliferate

Fig 3 shows the results of a first investigation of the mechanism by which Urgotul stimulates normal human dermal fibroblast proliferation. The effects on thymidine incorporation were tested in a standard 48-hour experiment using:

- Forty-eight-hour extracts of Urgotul in culture medium (extraction media without normal human dermal fibroblasts)
- Media from normal human dermal fibroblasts cultivated 48 hours with (or without) Urgotul
- Urgotul directly applied.

Incorporation levels were the same for the different control media tested. In this experiment, Urgotul applied directly for 48 hours (standard conditions) strongly stimulated thymidine incorporation (200% of control). EGF, tested as a reference proliferative compound, stimulated incorporation to 260% of the control. Only a low (1.35-fold, non-statistically significant) stimulation of proliferation was observed with Urgotul extract (much lower than the stimulation observed when Urgotul was tested in standard conditions).

To test the importance on the proliferative effect of direct contact between Urgotul and the cells, a model was designed in which Urgotul is tested both in direct contact with the cells (standard conditions) and at a sub-millimetre distance from the cell layer. A standard 48 hours thymidine incorporation assay showed that both presentations of Urgotul induced the same stimulation (150–160% of the control).

Cell morphology and structure

The effects of the different dressing applications on the morphology and ultrastructure of normal human dermal fibroblasts were evaluated by fluorescence confocal laser microscopy after immunolabeling of the cytoskeletal components β-actin and β-tubulin. The rearrangement of these proteins is a key parameter of cell behaviour modification, especially upon stress conditions.

The morphology of the normal human dermal fibroblasts treated by Urgotul and Mepitel were normal in terms of size and shape (fusiform) and tubulin and actin distribution. In some cells in contact with Urgotul, the actin network seemed denser (objective x 20), especially at focal adhesion sites (periphery, endings; objective x 60). In contrast, cells from Tulle Gras samples were abnormal, often rounded; many areas were completely devoid of cells. At the periphery of Tulle Gras, cells were rounded, possibly resembling dividing cells, but no special mitosis figure could be observed.

Urgotul showed a clear increased density of dividing cells (see arrows) when compared with the controls and the other dressings (Fig 4), confirming that this dressing stimulates proliferation in these experimental conditions.

Discussion

When a dressing is applied to a full-thickness wound in the early stages of healing, it comes into intimate contact with cells involved in the healing process. Urgotul, Tulle Gras and Mepitel are used in the granulation phase of healing,[5,8,9] so have direct contact with non-epithelialised tissue. The most evident cell model for investigation of the *in vitro* effects of these products was thus the human dermal fibroblast.

The selected testing protocol exposed cell monolayers to complete unmodified dressings, rather than extracts, for one to three days. These conditions were closer to standard clinical practice conditions.

Use of the intact dressing was also interesting as parameters such as mechanical contact/stress/stimulation or progressive/localised delivery of eventual substance from the solid/gel phase of the dressing could be key factors in a dressing's toxicity or efficacy. In other words, it was thought possible that a solid dressing could physically interact with underlying cells to produce effects that might not be detectable using soluble preparations of the dressing.

In vivo, wound contact dressings are usually cov-

ered with a secondary dressing and/or padding, leading to an optimal contact with the wound tissue. To mimic and stabilise this close contact and standardise conditions in all tested samples, a sterile plastic tube cap was loaded and centred on top of each piece of dressing. This prevented the sample from floating in the culture medium. In the absence of a dressing, this cap did not significantly modify the overall responses from the different tests.

This study focused on the differential effects of the wound dressings on fibroblast proliferation. *In vitro* proliferation of cells usually occurs in non-confluent cell layers because cell contact inhibits cell multiplication. *In vivo,* proliferation occurs following specific activation signalling — for example, following injury. In such *in vitro* experimental conditions as in this study, non-confluent fibroblasts proliferated without producing significant amounts of extracellular matrix. As these low cell density/unprotected layers are physically fragile, it was considered important to perform preliminary experiments to check the effects of the dressings on viability/cell metabolism. Detection of cytotoxic effects or any clear metabolic impacts would obviously impair such a cell proliferation study.

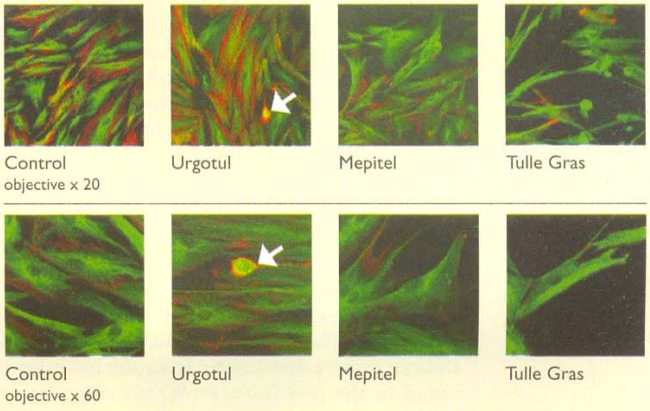
The behaviour of confluent, non-proliferative normal human dermal fibroblasts coated with dressings was evaluated using a MTT reduction-based methodology, which evidences the overall mitochondrial activity of the cells (activity of mitochondrial dehydrogenase). MTT labelling is a convenient method of studying cell viablity/toxicity, but is an imprecise indicator of cell metabolic activity.

Quantitative data did not show evident differences between the dressings. None had obvious effects on cell metabolism and viability, even after prolonged periods of contact (one week). These data confirmed the non-cytotoxic effect of the dressings previously described by Viennet et al.[6]

Interestingly, differences between the three dressings were observed through the additional visual analysis of the MTT-stained layers. A large hole almost devoid of stained cells in the centre of the culture well was observed in the Tulle Gras samples, reflecting a clear and prompt cytotoxic effect on the cells that had direct contact with the dressing. In contrast, the peripheral cells not covered by the Tulle Gras exhibited a significant increased blue (formazan) staining, which was even greater than in the control cultures (a moderate increase in the number of these peripheral cells is also not excluded). This could indicate an enhanced metabolic activity or at least a boost in the mitochondrial activity in these peripheral cells, and explain the close-to-control MTT reduction values measured with this dressing.

While the results of the MTT reduction assay did not really show any differences between the dressings, the proliferation assay performed on low cell density cultures was expected to be more discriminating. Thymidine incorporation into the replicating DNA of proliferative normal human dermal fibroblasts is a fully validated assay that gives a good evaluation of fibroblast proliferation rate. Growth factors such as EGF and other reference molecules clearly and reproducibly stimulate thymidine incorporation in this type of assay.

Fig 4. Morphology/ultrastructural organisation of cells treated by the dressings (72 hours): confocal laser microscopy



Immunodetection of tubulin (green) and actin (red) networks

In all the individual experiments, Urgotul significantly stimulated thymidine incorporation compared with the control, generally with a maximal effect when in contact for 48 hours. With Mepitel, fibroblast proliferation did not increase. Indeed, the overall tendency was a decrease in fibroblast proliferation at 24–48 hours, progressing to a statistically significant reduction in proliferation after long-term application without medium renewal, suggesting the progressive release of a moderately cytostatic component from the dressing.

With Tulle Gras, thymidine incorporation values did not decrease when compared with the untreated controls, as in the MTT assay. In previous non-reported experiments, a formulation of Tulle Gras, Lumiere, which contains balsam of Peru, had clear cytostatic effects, with a significant decrease in thymidine incorporation. The elimination of this mild antiseptic from the new formulation of Tulle Gras[10] appears to restore/enhance the metabolic/proliferative activity of the fibroblasts in the zones away from the dressing.

In this study solid samples of dressings were used in an adapted thymidine incorporation assay, which was originally designed to evaluate soluble compounds or drugs. Because of this exceptional physical form, together with the chemical (more or less

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lipophilic) composition of the samples, one could question the specificity of the proliferative effects observed with Urgotul, as well as a potential artefact obtained with Tulle Gras. For example, solid material from dressings may trap radioactivity from the medium and adhere to filters used for DNA recovery, leading to an increased non-specific signal. In addition, in an *in vitro* study, Dover[11] reported that treatment with a set of wound contact layers including Jelonet (Smith & Nephew), Bactigras (Smith & Nephew) or Silicone N/A (Johnson & Johnson) dressings did not stimulate fibroblast proliferation.

In the present study, a specific experiment was designed, based on the use of the same normal human dermal fibroblast cultures briefly treated (or not) with the cytostatic reagent mitomycin C. The cultures had the same confluency. If, in a standard 48-hour assay, a dressing led to strong incorporation values in the proliferation-blocked cells, then this signal would be artefactual. Urgotul, in normal conditions, increased thymidine incorporation (by 9000cpm and 800cpm in mitomycin-treated cells), indicating that the effects are dependent on the cells' ability to proliferate. Thus, the stimulation induced by Urgotul does not come from an artefactual trapping of radioactivity in potential Urgotul-diffusible particles. This effect is also confirmed by the proportional stimulation observed in the proliferation-limited cultures.

For Tulle Gras, no apparent physical artefact can explain the incorporation observed (despite the presence of an important grease residue observed on the cell surface). We hypothesise that thymidine incorporation increased in the peripheral cells away from the dressing, compensating for the loss of cell proliferation under the dressing. Further experiments with the dressing covering the entire well surface could confirm this hypothesis.

The effects of Urgotul on cell proliferation are confirmed by the observation of a greater number of dividing cells (mitotic cells) than in the control non-confluent cultures and any of the other dressings tested. Urgotul and Mepitel-treated cells exhibited a normal fusiform shape and normal tubulin and actin distribution, whereas application of Tulle Gras led to only few living cells, most of which were morphologically abnormal. In some cells in contact with Urgotul, the actin network seemed denser, especially at focal adhesion sites. This could indicate an enhanced migration potential of these cells.

EGF is a growth factor that stimulates the proliferation and migration of many cell types (for example, fibroblasts) and has been found to play an important role in the healing of ulcers.[12,13] As the level of skin cell activation obtained with Urgotul was the same as that obtained with EGF, it can be assumed that the potential effect of Urgotul should result in a clinical effect.

Urgotul's composition enables it to interact with the wound environment, particularly exudate. The lipidocolloid interface maintains a moist environment that not only helps preserve the viability and proliferative potential of the cells but also potentiates interactions between growth factors and their target cells.[14] Its non-adherent properties, demonstrated in clinical trials as pain-free and atraumatic removal,[15] were confirmed *in vitro* by the absence of significant lesions on cell layer; they depend on the formation of the lipidocolloid interface. *In vitro*, proliferative effects were observed with Urgotul and occurred both when the dressing was directly applied onto the cells and when it was at a sub-millimetre distance from the cells. A direct mechanical effect therefore did not seem to be involved.

Additionally, a trial using extracts of Urgotul was undertaken to check whether or not the proliferative effect observed came from the release of a freely soluble factor by Urgotul or by the fibroblasts conditioned in the presence of Urgotul. These extracts failed to significantly stimulate the incorporation of thymidine in a subsequent assay, indicating that the proliferative effect did not come from the release of a freely soluble stable factor.

Among the possible explanations for the behaviour of Urgotul in these experiments, the progressive formation of the lipidocolloid interface from the dressing remains a key factor. The specificity of the lipidocolloid interface (polymer matrix-carboxymethylcellulose particles and vaseline) developed by Urgo Laboratories could explain the absence of activity with the extracts and allow an interaction between the dressing and the cell layers even at a sub-millimetre distance. Further studies are required to elucidate the intimate mechanism by which Urgotul enhances the growth of fibroblasts.

As with any *in vitro* study, these data have a limited predictivity potential. Obviously, the model used is limited to a simple culture of human cells, whereas wound healing occurs in a complex, multicellular and inflammatory three-dimensional environment. In spite of these limitations, the experiment, under durations similar to those in clinical practice, produced evidence of differential responses of the underlying cells to the different dressings.

Conclusion

The favourable wound healing results obtained clinically on both acute and chronic wounds with Urgotul seem to be due not only to the non-adherent and atraumatic characteristics of its composition but also to local interactions with the cells in the wound environment. Interestingly, the results show that fibroblast proliferation was promoted in the presence of Urgotul, unlike the two other tested interface dressings, and confirmed the non-cytotoxicity of Urgotul on fibroblasts. ■