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THE MIGRATION OF FIBROBLASTS INTO AN *IN VITRO* WOUND

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Summary.—A method for the study *in vitro* of fibroblast migration in wounds is described. This consists of producing standard fibroblast monolayers (derived from subcultures of explants of flank skin from young male rats) in microtitre plates, wounding the monolayer in a linear fashion with a razor blade, removing the monolayer from one side of the linear wound and retaining the other side for migration studies. Preliminary results using the method are described which illustrate its reproducibility and ease of performance.

FIBROBLAST MIGRATION is of importance to a number of biological processes including wound healing. The phenomenon may be studied in the laboratory using an *in vitro* "wound" model, *i.e.* the movement of cultured fibroblasts into a cell-free area. By this means, information has been obtained about the time course of migration (Wolf and Lipton, 1973), the influence of serum (Lipton *et al.*, 1971) and the relationship between migration and cell division (Dulbecco and Stoker, 1970). However, the techniques employed have often resulted in a ragged and ill-defined cell-free area, unsuitable for accurate quantitative assessment (Bürk, 1973). Other criticisms relate to the use of established cell lines, which may show behaviour uncharacteristic of normal cells (Boone, 1975; Shields, 1976), and to the use of heterologous sera such as foetal calf serum, in spite of evidence that these may influence cells differently from autologous sera (Irie, Irie and Morton, 1974; Holley and Rubin, 1971).

We have developed a technique, adapted from that of Bürk (1973), for reliably wounding monolayers of cells in flat-bottomed microtitre wells. We present in this paper a description of the technique and its use in examining some factors which affect the migration of normal rat skin fibroblasts in the presence of autologous serum.

MATERIALS AND METHODS

Source of cultured fibroblasts.—The fibroblasts (*i.e.* the fibroblast-like cells of tissue culture) were obtained from cultured explants of flank skin from young (3–6 months), male, B.S. strain inbred rats (Heslop, 1968). Skin explants (approximately 1 mm in diameter) were cultured in Falcon (Oxnard, Ca.) tissue-culture flasks (growth area 25 cm^2) containing Eagle's Minimal Essential Medium (G.I.B.Co. powdered MEM supplemented with 2·2g/l $NaHCO_3$; 12·5mM HEPES buffer; 50,000 u/l benzyl penicillin and streptomycin; 10 mg/l gentamicin sulphate) supplemented with 10% foetal calf serum (FCS: supplied by Laboratory Services Ltd, Auckland, N.Z.), according to the method of Tom, Jakstys and Kahan (1974). If substantial outgrowth had not occurred within 14 days, the cultures were discarded. When confluent at approximately 3 weeks, the primary cultures were trypsinized (0·25% trypsin; G.I.B.Co.), divided into two and subcultured. These secondary cultures were used for the experiments within 2 weeks of preparation. All cultures were maintained at 37° in a moist atmosphere containing 10% CO_2 in air.

Experimental system.—Secondary cultures were gently trypsinized, washed twice with isotonic Dulbecco's phosphate-buffered saline and the cells counted, in duplicate, with a Neubauer haemocytometer. Unless otherwise specified, 20,000 cells, suspended in 200 μl of MEM supplemented with 10% freshly prepared autologous normal rat serum (NRS), were

placed into each well of a flat-bottomed Sterilin (Middlesex, U.K.) microtitre tissue-culture plate. This produced a confluent monolayer in the presence of 10% NRS. Ten or 12 replicate samples per group were prepared.

After 48 h, the fibroblast monolayer in each well was wounded and 200 μl of test medium added. The experiment was stopped after a further 48 h (unless otherwise specified) and the cells fixed and stained by replacing the medium in each well with Paragon stain (Searle Laboratories, High Wycombe, Bucks, England).

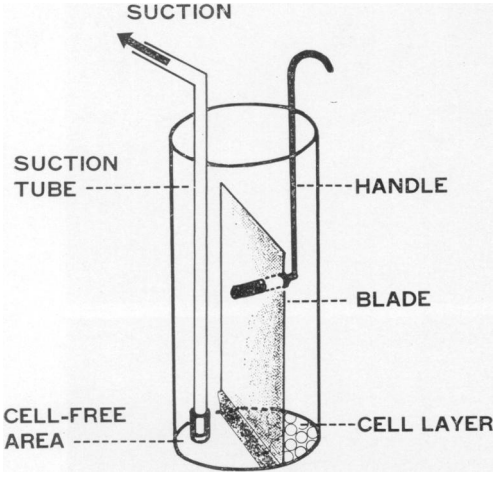


FIG. 1.—Diagram of wounding technique. A single microtitre well is depicted diagrammatically. The modified razor blade is placed vertically into the well in such a way that the cutting edge lies along a diameter, then the cell layer on one side of the blade is sucked off. See text for details.

In vitro *wound*.—Fig. 1 diagrammatically depicts the method used to produce an *in vitro* wound in the fibroblast monolayer adherent to the bottom of each microtitre well. The technique employs a blade combined with the aspiration of cells from one half of each well to create a marked edge (henceforth termed starting-line) and a cell-free area (henceforth termed wound); Fig. 2.

The blade was cut with heavy scissors from the edge of a Schick Super stainless steel razor blade, so that it was 6 mm broad and 10 mm long. The ends of the cutting edge were rounded off, reducing the effective blade breadth to 5 mm.

To produce a wound in each microtitre well, the blade was hand-held with the aid of an appropriately bent paper-clip inserted in a hole drilled centrally through the blade 7 mm above the cutting edge. This hole was fitted with a bush cut from silicone rubber tubing so that the blade was held securely, but could pivot on the paper clip as downward pressure was applied, thus ensuring an evenly scored starting-line. The suction tube, consisting of a bent, amputated 18-gauge syringe needle with a bevelled silicone rubber tip, was then used to dislodge the cells from one side of the blade as the well was emptied of medium. Because of the risk of cell damage by drying, test medium was added to the wells immediately after the wounding procedure. The wells were then checked using an inverted stage phase-contrast microscope (Nikon Model M, Nippon Kogaku K.K., Tokyo 100, Japan).

Assessment and analysis.—After fixing and staining, the microtitre wells were examined using an Olympus binocular microscope (Olympus, Tokyo, Japan) and those wounds considered technically unsatisfactory were excluded (criteria for exclusion are given in the Discussion). Cellular migration was assessed at $\times 100$ magnification with the aid of a 10×10 eye-piece grid (apparent dimensions = 1 mm $\times$ 1 mm). Two measurements were made: firstly, the total number of cells which had moved across the central 3 mm of each starting-line was counted; secondly, the maximum distance migrated by the cells in each wound was recorded. Distribution-free (non-parametric) methods of statistical analysis were employed because counts of migrating cells are not by nature normally distributed, nor in the case of our data was there an approximation to a normal distribution. In consequence, the median (Md) of each group is given, together with the semi-interquartile range (Q), rather than the more familiar mean and standard deviation (Phillips, 1978). Where appropriate, Kruskal–Wallis analysis of variance (Kruskal and Wallis, 1952) was used for comparing 3 or more groups and the Mann–Whitney *U* test (Mann and Whitney, 1947) for comparing any two groups. The 1% level of significance has been adopted.

RESULTS

Using this micro-wounding technique, assessments were made of the time course of migration and the influence of cell density and serum concentration on migration. The inhibition of migration by lowered temperature and two drugs (colchicine and mitomycin C) was also examined.

Time course of migration

In this experiment migration (in the presence of MEM with 20% NRS) was assessed at 2, 4, 6, 8, 10, 12, 18, 24 and

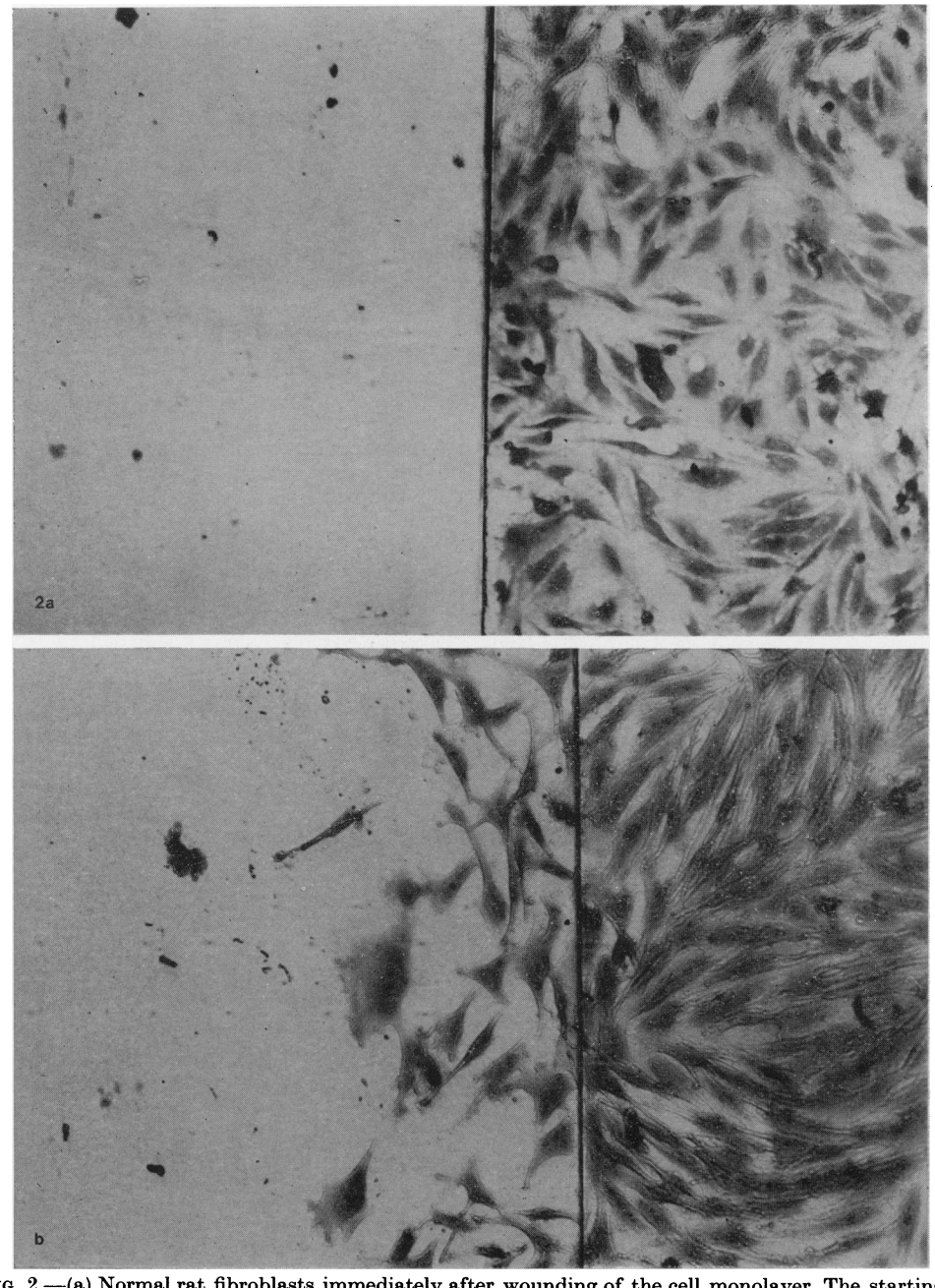


FIG. 2.—(a) Normal rat fibroblasts immediately after wounding of the cell monolayer. The starting-line, marked by the blade on the micro-well bottom, divides the undisturbed cells from the cell-free wound. (b) After 48 h incubation, in the presence of 20% NRS, numerous cells have migrated into the wound, Paragon stain, × 80.

48 h after wounding. The results are depicted graphically in Fig. 3.

There was no visible evidence of fibroblast migration until 6 h after wounding. At that time, whole cells had migrated across the starting-line in 3 out of 7 wells.

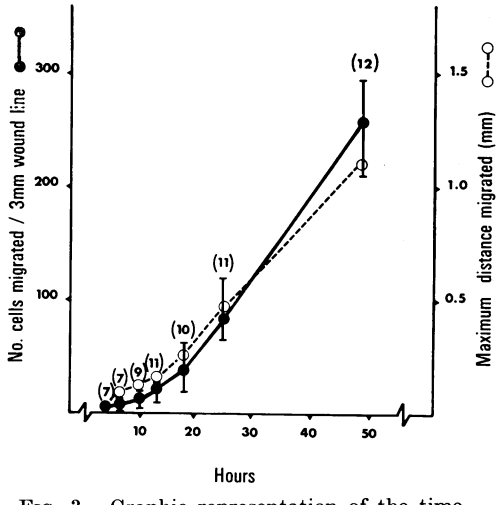


FIG. 3.—Graphic representation of the time course of fibroblast migration into an *in vitro* wound. The median number of cells migrated per 3 mm wound line is shown (—●—) ±Q. The median maximum distance migrated is also shown (--○--); Q is omitted for clarity, although it was of similar magnitude to that for the number of cells migrated. The figures in parentheses indicate the number of satisfactory replicates assessed for each point.

Subsequently, there was a steady increase in the number of cells across the line. The maximum distance migrated correlated closely with the total cell number migrated (Fig. 3). Mitotic figures were not observed in the wound area until 24 h after wounding.

Influence of cell density on migration

The next experiment was designed to determine if the number of cells migrating across a given length of starting-line was related to the cell density behind the line. Two experimental groups were used, having either 5,000 or 20,000 cells per well at the start of the experiment. The cell density (No. cells/mm^2) behind the line was estimated when the experiment was stopped.

TABLE I.—*The Influence of Cell Density on Migration*

Original No. cells per well	No. cells per mm^2* in cell layer at time assessment (Md ± Q)	No. cells migrated† per 3mm line (Md ± Q)	Max. distance migrated† in mm (Md ± Q)
5,000	181 ± 19	81 ± 35	0·65 ± 0·15
20,000	642 ± 15	280 ± 46	0·90 ± 0·10

* The density of the cell layer was estimated by direct counting at least 0·5 mm behind the starting-line (the area of a microtitre well is approximately 33 mm^2).

† Mann–Whitney *U* test confirms significant differences between the two groups for both parameters. Ten replicates were assessed in each group.

The results are given in Table I. The number of cells and the maximum distance migrated were significantly lower for the wells originally containing 5,000 cells than for those containing 20,000 cells.

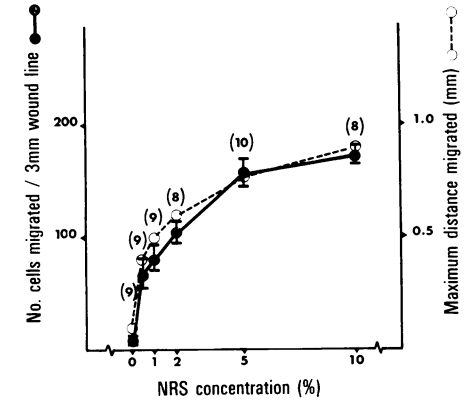
Influence of serum concentration on migration

In this experiment, migration was assessed in the presence of varying concentrations of NRS in MEM: 0·5%, 1%, 2%, 5%, 10%, 20%, 30%, 50%, 100%. The results are depicted graphically in Fig. 4 (*a* and *b*). There was a rapid rise in both number of cells migrating and distance migrated with increasing concentrations of serum, reaching a maximum at 30%. It will be noted that slight migration occurred in the absence of serum. This may have been due to incomplete washing of cells, for in two earlier experiments the absence of serum was associated with no migration and some cell death. Migration was completely inhibited by 100% serum.

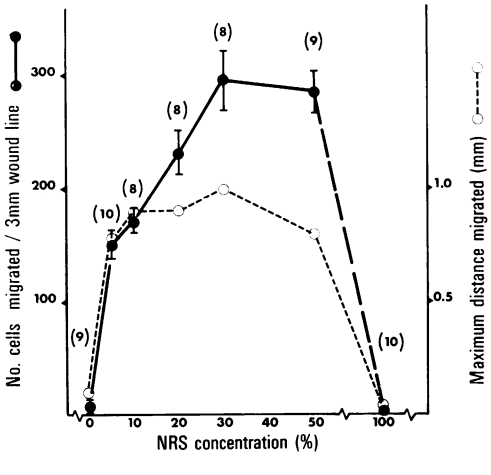
Similar results were obtained in a parallel experiment using FCS, except that maximal migration was achieved at a concentration of 20%.

Inhibition of migration

First, the influence of lowered ambient temperature on migration (in the presence of 20% NRS in MEM) was examined. At



(a)



(b)

FIG. 4.—Graphic representation of the influence of NRS concentration on fibroblast migration into an *in vitro* wound. The median number of cells migrated per 3 mm wound line is shown (—●—) ±Q. The median maximum distance migrated is also shown (--○--); Q is omitted for clarity, although it was of similar magnitude to that for the number of cells migrated. The figures in parentheses indicate the number of satisfactory replicates assessed for each point. (a) NRS concentration 0–10%, (b) NRS concentration 0–100%.

37° normal migration was observed (No. cells migrated/3mm line; Md = 266, Q = 29; maximum distance migrated in mm: Md = 1·0, Q = 0·10); at 22° in 2 out of 10 wells, cell processes had moved over the line, but not whole cells; at 4° there was no migration and the undisturbed cells had become rounded and more densely stained.

Next, the influence of two drugs which inhibit cellular behaviour were compared (in the presence of 20% NRS in MEM). Mitomycin C (BDH Chemicals, Poole, England), which specifically inhibits cell division (Szybalski and Iyer, 1964), was used at a concentration of 0·25 μg/ml. Colchicine, which inhibits both cell division (Deysson, 1968) and migration (Wolf and Lipton, 1973), was used at a concentration of 0·0125 μg/ml. The observed effects of these agents are summarized in Table II.

TABLE II.—*The Effect of Mitomycin C and Colchicine on Migration*

Groups	No. cells migrated* per 3mm line (Md ± Q)	Max. distance migrated* in mm (Md ± Q)
Control	276 ± 48	1·00 ± 0·05
Mitomycin C	75 ± 15	0·50 ± 0·05
Colchicine	0	0

* Kruskal–Wallis analysis of variance confirms significant differences between the groups for each parameter (2 d.f.; H = 23·1 and 24·4). Nine or 10 replicates were assessed in each group.

The migration of fibroblasts grown in colchicine at this non-lethal concentration was completely inhibited. In contrast, mitomycin C, used at a concentration sufficient to cause some cell death (the cell populations in replicate groups declined from 20,000 to 10,000 over 48 h) allowed considerable migration, though there was significant inhibition compared to controls. These results indicate that cell division in the monolayer behind the starting-line is not a necessary condition for migration.

DISCUSSION

The wounding method, which was adopted from that of Bürk (1973), enables accurate definition of the cell-free area and direct quantitative assessment of migration. The technique is easily learned and

can be carried out by a single operator with reasonable reproducibility. This is demonstrated by comparing similar groups in separate experiments, *e.g.* No. of cells migrated/3mm line in presence of MEM with 20% NRS at 48 h (Md ± Q): 266 ± 29; 231 ± 20; 280 ± 46; 224 ± 27; 276 ± 48. Another advantage is the more economical use of reagents, sera and cells, thus allowing a larger number of replicate tests to be performed.

The most common technical problems producing unsatisfactory wounds were: (1) The wound starting-line being cut too deeply into the bottom of the plastic microwells — this substantially impairs migration; (2) suction damage to the intact layer around the ends of the blade; (3) the intact layer being swept back slightly when the blade is lifted. It is noteworthy that there was no evidence of active retraction by the cell sheet after wounding as found by Raff and Houck (1969). These imperfections were readily identified, using the inverted-stage microscope, immediately after wounding. The occurrence of unsatisfactory wounds averaged 15%.

Using this method it was found that the *in vitro* wound begins to be populated by migrating cells after 6 h. This result is consistent with the findings of other workers (Sholley, Gimbrone and Cotran, 1977). That cell division is not a necessary accompaniment is shown by the experiment with mitomycin C and also the absence of mitotic figures until 24 h after wounding.

Previous workers, using 3T3 cells, have found that cell proliferation in the wound is maximal at around 36 h after wounding (Todaro, Lazar and Green, 1965; Dulbecco and Stoker, 1970). The total number of cells present in the wound area at 48 h might therefore be somewhat increased by proliferation.

The requirement for serum (or factors contained in it) to promote migration is in accord with the findings of Wolf and Lipton (1973) and Lipton *et al.* (1971), though not with those of Raff and Houck (1969). There was substantial migration at low serum concentrations although maximal migration was observed at the relatively high concentrations of 30% NRS and 20% FCS. The decline in migration at high serum concentrations may be caused by either the dilution of the MEM by serum or by active inhibitory factors contained in the serum. The other experiments demonstrated that migration is influenced by modifications in the culture conditions, such as the cell density adjacent to the wound, a low ambient temperature and the presence of inhibitory drugs. The results obtained were consistent with expectations.

This economical and reliable method is enabling us to extend our studies of fibroblast function in wound healing and neoplastic disease.

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