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A comparison of newborn versus old skin fibroblasts, their potential for tissue repair

Y. Horiuchi and T. J. Ryan

Department of Dermatology, Slade Hospital, Oxford, UK

SUMMARY. **An assessment was made of the ability of human skin fibroblasts from newborn and old subjects to produce intracellular urokinase inhibitor and urokinase-plasminogen activator when exposed to phorbol myristate acetate. Urokinase inhibitor produced by human skin fibroblasts may be essential for cell growth regulation. The expression of urokinase inhibitor and/or urokinase-plasminogen activator may possibly be age-related. The principal observation that newborn skin fibroblasts show a low level and poorly sustained production of intracellular inhibitors of urokinase could help to explain the flexibility of the wound healing process in the foetus.**

Contemporary interest in foetal surgery has led to a search for an explanation of rapid wound healing (Siebert *et al.*, 1990). Wound healing requires rapid remodelling of the tissue, which is an inherent feature of the developing embryo but not a feature of old tissues, when the flexibility of youth is exchanged for the stability of age.

One important mechanism for such a change in behaviour is control of the remodelling process of proteolytic enzymes in the foetus. The serine proteases urokinase (UK) and tissue-plasminogen activator (t-PA), which catalyse the conversion of plasminogen to plasmin, have been shown to be essential for the proliferation of normal cells, transformed cells and malignant cells (Diamond *et al.*, 1980), and for embryo implantation (Strickland *et al.*, 1976). Plasminogen activator inhibitors (PA-Is) (Kruithof, 1988) are present in fibroblasts (Crutchley *et al.*, 1981; Masuzawa *et al.*, 1988), endothelial cells, macrophages, and placental tissue. One method of investigating cell function relevant to wound healing is the use of the tumour promoter phorbol myristate acetate (PMA) (Diamond *et al.*, 1980). This agent elicits a variety of biological responses in many different cells and tissues through the calcium and phospholipid sensitive protein kinase-C (Nishizuka, 1984). It has been previously shown that proteolytic activity in the normal chick embryo fibroblast can be induced by low concentrations of phorbol esters (Wigler and Weinstein, 1976), and more recent studies have indicated that the secretion of protease-nexin, a protease inhibitor from human fibroblasts is stimulated by phorbol ester (Eaton and Baker, 1983). As an extension of that study (Eaton and Baker, 1983), the observations which follow in this paper were made on some other effects of phorbol esters in newborn versus adult fibroblasts.

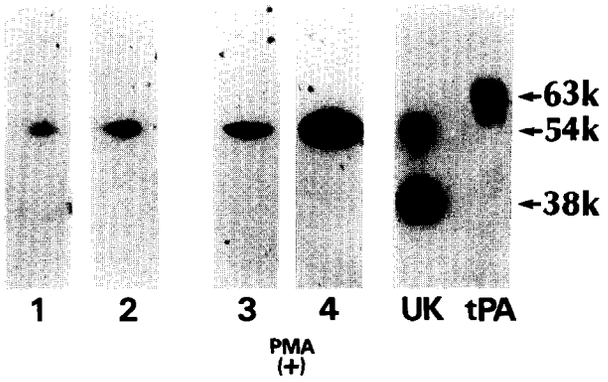

Fig. 1

Figure 1—Zymography showing intracellular urokinase-plasminogen activator (u-PA) expression and stimulation of newborn FBs. Lane 1. Production of a very small amount of u-PA of non-treated newborn FBs at day 6 of culture, Lane 2. Slight increase in expression of u-PA of non-treated newborn FBs at day 28 of culture, Lane 3. Slight stimulation of u-PA of PMA-treated newborn FBs following exposure to PMA for 48 h, Lane 4. Stimulation of u-PA of PMA-treated newborn FBs following exposure to PMA for 24 days, UK, urokinase (u-PA); t-PA, tissue-plasminogen activator. (t-PA used was purified from Bowes melanoma conditioned medium.) (A plus sign indicates addition of PMA in Lanes 3, 4) (The molecular weights of UK and t-PA are shown in kilodaltons (kD) on the right.)

Materials and methods

Fibroblast cultures

Human fibroblasts (FBs) derived by explant culture of slices of epidermis including upper dermis, cut into 2 mm cubes from healthy adult skin from different sites of the body and healthy newborn foreskin, were grown in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Gibco, UK), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco, UK) in a humidified atmosphere of 5% CO_2: 95% air. The cells were seeded 40×10^4 cells/dish in a 6 cm (20 cm^2) plastic dish (Falcon, Beckton Dickinson, USA). The medium was changed twice a week. The cell strains used were 5 cell strains from old subjects (59 yr, passage 7; 60 yr, passage 6; 60 yr, passage 7;

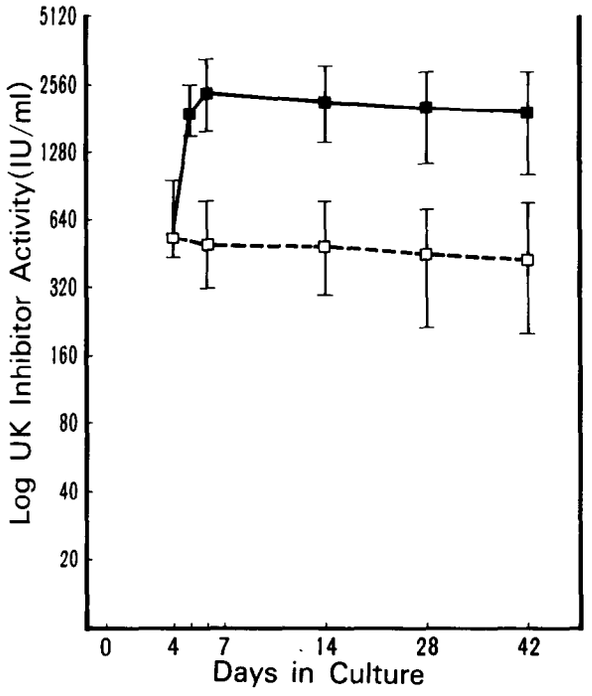

Fig. 2

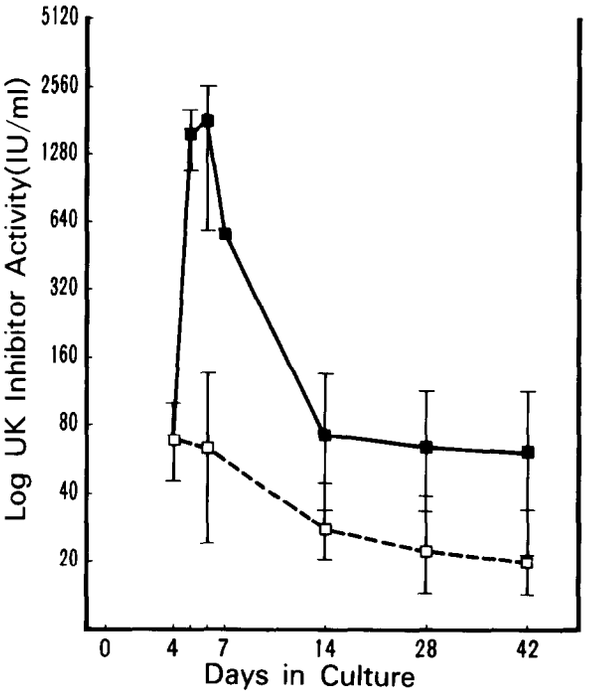

Fig. 3

Figure 2—Time course of UK inhibitor activity of old skin FBs. Open square (□), non-treated cells; Closed square (■), PMA-treated cells.
Figure 3—Time course of UK inhibitor activity of newborn FBs. Open square (□), non-treated cells; Closed square (■), PMA-treated cells.

62 yr, passage 4; 74 yr, passage 8), and 7 cell strains from newborn foreskin (passage 3, 4, 5, 6, 6, 8 and 10).

PMA treated cultures

In duplicate cultures reaching confluency (4th day of culture), medium was completely replaced twice a week by medium containing 10 ng/ml (16 nM) phorbol 12-myristate 13-acetate (PMA) (Sigma, UK) dissolved in demethyl sulphoxide (DMSO) (Sigma, UK) at a final concentration of 0.001%.

Measurement of cell growth

Cultured cells were detached from the dishes using 0.05% (W/V) trypsin/0.02% (W/V) EDTA (ethylene-diaminetetraacetic acid) on specified culture days (days 4, 6, 14, 28 and 42 of culture in non-treated cells, and 24, 48 and 72 hours and days 10, 24 and 38 of culture in PMA-treated cells), and washed twice in cold PBS (phosphate buffered salt solution, pH 7.4). Extracts were made at a concentration of 10^4 cells per 1 μl of extract buffer, 0.5% (W/V) Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, and 0.01% (W/V) NaN_3, at pH 7.4. They were subsequently centrifuged at 966 g for 20 min for removal of cell debris, and stored at −20°C until use. The cells were counted on a Neubauer plate.

Assay of urokinase inhibitor activity

Urokinase (Leo Laboratories Ltd, Denmark) was dissolved with extract buffer and applied to a fibrin-agar plate to obtain the standard curve of UK activity (expressed as International Units (IU)/ml). A fibrin-agar plate was prepared as previously described with some modification (Cederholm-Williams *et al.*, 1985; Masuzawa *et al.*, 1988). The titre of UK-I activity in cell extract was defined in terms of equivalent UK IU/ml inhibited.

Polyacrylamide gel electrophoresis and zymography

Samples (50 μl of each cell extract) were electrophoresed with sodium dodecyl sulphate (SDS) through a 10–15% gradient polyacrylamide as previously described (Cederholm-Williams *et al.*, 1985). Plasminogen enriched fibrin-agar overlays were used to detect plasminogen activators, and reverse fibrin-agar overlays, to detect urokinase inhibitors (Cederholm-Williams *et al.*, 1985).

Results

Intracellular urokinase plasminogen activator

Using this relatively insensitive technique of fibrinolysis autography, no intracellular PA activity was detected in old skin FBs, even in the presence of PMA. By contrast, newborn skin FBs showed very small amounts of activity, which were increased by the exposure to PMA (Fig. 1).

Intracellular urokinase inhibitor

Using the fibrin-agar plate assay, UK-I activity (Fig. 2) in PMA-treated old skin FBs at 48 h, increased 3–5 times (mean, 2520 IU/ml; range, 1560 to 3120 IU/ml) compared to control old skin FBs (mean, 580 IU/ml; range, 460 to 980 IU/ml). This increase was sustained during prolonged exposure to PMA (Fig. 2). By contrast, levels of UK-I in untreated newborn FBs

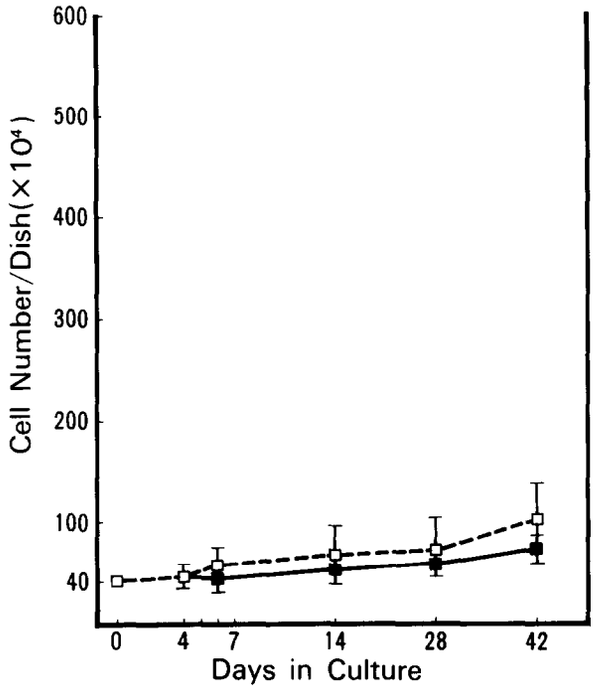

Fig. 4

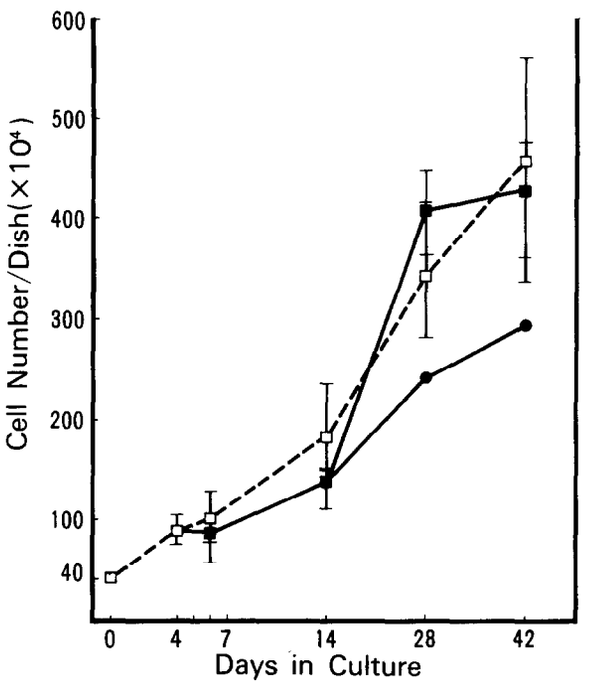

Fig. 5

Figure 4—Time course of cell growth of old skin FBs. Open square (□), non-treated cells; Closed square (■), PMA-treated cells. **Figure 5**—Time course of cell growth of newborn FBs. Open square (□), non-treated cells; Closed square (■), PMA-treated cells; Closed circle (●), growth inhibited cells* following exposure to PMA. (*The growth inhibited cells were from a strain which had been passaged six times.)

(Fig. 3) at a comparable 4 days of confluency were low (mean, 72 IU/ml; range, 46 to 98 IU/ml). After 48 h of PMA exposure, newborn FBs produced levels of UK-I in the range 600 to 2640 IU/ml; mean, 1920 IU/ml (Fig. 3). This represents a 5–50-fold increase over control levels. This increase was not sustained during prolonged exposure to PMA.

Cell growth

Old skin FBs (Fig. 4) grew slowly and showed further growth inhibition during PMA exposure. Fast-growing newborn skin FBs (Fig. 5) showed inhibition of growth for 10 days following exposure to PMA. However, even in the continued presence of PMA, newborn FBs recovered their rapid growth pattern after 10 days in six out of seven cell strains.

Discussion

Extracellular proteolysis uses enzymes which are often also active intracellularly; these play an important role in the control of cell shape and the remodelling processes of the cytoskeleton necessary for migration and mitosis. One such activator, urokinase, is co-localised with the adhesion protein vinculin and is believed to have a role in the control of the adhesion of the actin cytoskeleton within the cell membrane (Hebert and Baker, 1988). It has been postulated that inhibitors of urokinase act as a fine control of adhesion (Ryan, 1989). In order for cells to show features of shape change, migration and mitosis at the speed that characterises foetal tissue, it is necessary that this fine control shows exquisite flexibility (Siebert *et al.*, 1990). By contrast in the adult, the maintenance of the cytoskeleton using a whole range of fibrillar materials requires that they show considerable stability and resist proteolysis. While stability and flexibility are not mutually exclusive, the difference in behaviour of adult tissues from foetal tissues in response to wounding could be explained if injury stimulates the production of both proteolytic enzymes and their inhibitors and if the levels of the latter are greater in adult tissues and their production less easily switched off.

We have observed that newborn fibroblasts stimulated by phorbol esters show an initial expression of plasminogen activator and inhibitor. They are able to respond by an increase in both but the production of the inhibitor is soon switched off. The striking feature of the response of the adult fibroblasts is that production of the inhibitor is sustained. This reminds us of our observation of the effects of the mechanical stimulus of centrifugation and *in vivo* skin expansion on fibroblasts in which there is also a difference between young and old (Masuzawa and Ryan, 1985), characterised by a more sustained production of inhibitor in the older cells. In those studies, we showed a correlation between inhibition of the growth fibroblasts and a high inhibitor level. Stability we suppose is manifested by a more permanent cytoskeleton and less easily lysed attachments. This would be expected to have a growth inhibitory effect. Urokinase-plasminogen activator expression in young adult cells can be quite small. Old cells showing even less expression will be more sensitive to the effects of an inhibitor. Environmental changes, such as the presence of phorbol ester, will then incite a qualitatively different

response in older cells from that observed in the very young.

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The Authors

Yasuhiro Horiuchi, MD, Research Fellow.
Terence J. Ryan, DM, FRCP, Consultant Dermatologist

Department of Dermatology, Slade Hospital, Oxford, UK.

Requests for reprints to: Dr Y. Horiuchi, Department of Dermatology, Kitasato University School of Medicine, Sagamihara 228, Japan.

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