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Effects of Y27632 on keratinocyte procurement and wound healing

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Summary

A number of Rho-kinase inhibitors have been developed for various clinical applications. We examined the effects of the Rho-kinase inhibitor Y27632 on keratinocyte proliferation and migration, and found it promoted primary human keratinocyte proliferation and migration in both monolayer and skin explant cultures. In addition, topical application of Y27632 enhanced cutaneous wound closure in the majority of wounds in mice. The growth and migration effects of Y27632 appeared to be specific to keratinocytes compared with dermal fibroblasts, and required intact Jun kinase function. Y27632 seems to be a promising agent for keratinocyte procurement and wounding healing.

A number of pharmacological inhibitors of the Rho signalling cascade have been developed for biological and clinical applications. One of these, Y27632, an inhibitor of Rho-associated protein kinase (ROCK),^{1,2} has been shown to increase embryonic stem-cell survival and keratinocyte stem-cell procurement in culture.^{3–6} The aim of this study was to examine how Y27632 affects keratinocyte and dermal fibroblast cell growth and migration, and to evaluate the *in vivo* effects of this reagent on cutaneous wound healing.

Report

The study was approved by (the Department of Dermatology of Duke University) and all animal experiments were conducted in accordance with (an animal protocol approved by Duke animal use committee).

Cells were cultured in the presence or absence of varying concentrations of Y27632. Treatment with Y27632 at concentrations as low as 2.5–20 $\mu\text{mol/L}$ reduced the levels of phosphorylated myosin light chain (p-MLC), a known target of Rho kinases, in both keratinocytes and dermal fibroblasts (supplementary Fig. S1a). Interestingly, keratinocytes displayed increased cell proliferation in response to treatment with 5, 10 or 20 $\mu\text{mol/L}$ Y27632, as indicated by higher cell counts and increased incorporation of bromodeoxyuridine (Fig. 1a; supplementary Fig. S1b). By contrast, fibroblast cell numbers were decreased by Y27632 (Fig. 1a), and this was not accompanied by an increase in cells positive for Trypan blue (data not shown). Morphologically, the treated keratinocytes appeared to be smaller than control cells and were sometimes elongated (supplementary Fig. S1c).

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The effects of Y27632 on keratinocyte wounding were assessed. Near-confluent cultures of KC and Fb were starved of epidermal growth factor (EGF) and serum, respectively, for 16 h, and then the cell layer was scratch-wounded with a 10 μ L fine pipette tip, and then fed with complete media along with 0 or 10 μ mol/L Y27632. Y27632 markedly reduced the wound-closure time, whereas it had no apparent effect on the migration of fibroblasts (Fig. 1b). These results indicate that Y27632 promotes cell growth and migration in a keratinocyte-specific manner.

Keratinocyte outgrowth from the wound edge is one of the cellular processes central to wound healing. To reproduce this process *in vitro*, we used human skin explant cultures. Split-thickness human skin tissues were cultured in the presence of 0 or 10 μ mol/L Y27632. We found that Y27632 markedly increased keratinocyte outgrowth, as indicated by the increased number of cell counts on post-treatment days 2 and 5 (Fig. 1c). The treated cells appeared small and shiny compared with the dark and flattened cell appearance of the control cells (Fig. 1d). Immunostaining showed that the basal cell-specific cytokeratin (CK)14 and the wound-responsive CK6 were strongly expressed in both treated and untreated cells (supplementary Fig. S2a,b), whereas the cell-cycle regulator cyclin D1 was markedly increased in the treated cells (supplementary Fig. S2c). Thus, Y27632 promotes keratinocyte outgrowth from skin explants while preserving the molecular markers of the wound-healing responses.

To further test whether Y27632 can promote cutaneous wounding healing *in vivo*, we created full-thickness punch-biopsy wounds 4 mm in diameter on the dorsal skin of 20 mice, and then covered the wounds with Telfa sponges (Kendall Health Care, Mansfield, MA, USA) soaked with phosphate-buffered saline or 20 μ mol/L Y27632. Animals were killed with CO₂ days 3 (two animals/group), 5 (two per group), 7 (three per group) and 10 (three per group). None of the animals exhibited obvious adverse responses during treatment, but the wounds treated with Y27632 were visibly smaller than the control wounds by day 3 (Fig. 1e,f). By day 10, five of the six treated wounds appeared nicely healed, whereas four of the six control wounds still had remnants of scabs (Fig. 1e,f). These data indicate that topical application of Y27632 can expedite cutaneous wound healing in mice.

The c-Jun amino-terminal kinase (JNK) and extracellular regulated kinase (ERK) signalling pathways have important roles in cell growth and migration. Protein lysates were collected from keratinocytes at 1 h after scratch-wounding or treatment with 20 ng/mL EGF in the presence or absence of 10 μ mol/L Y27632. We found that pERK was decreased by Y27632 in keratinocytes stimulated with scratch wounding or treatment with epidermal growth factor (Fig. 2a), whereas pJNK was not reduced by Y27632 in either case.

To further examine whether JNK and ERK are important for Y27632-induced cellular responses, we treated keratinocytes with Y27632 together with pharmacological inhibitors of JNK and ERK. Targeted inhibition of JNK with BI-78D3 (Tocris Bioscience, Bristol, UK) resulted in increased levels of the JNK target c-Jun in the cytoplasm, but no change in the subcellular localization of the nuclear factor- κ B RelA subunit (supplementary Fig. S3a,b).

Keratinocytes were also cultured with 10 μ mol/L Y27632 with or without 10 μ mol/L BI-78D3 or 20 μ mol/L PD98059 (Tocris Bioscience). A scratch-wounding assay was carried out as before, but this time cells were treated with 10 μ mol/L Y27632 with or without 10 μ mol/L BI-78D3 or 20 μ mol/L PD98059. Co-treatment with BI-78D3 and Y27632 reduced cell counts and inhibited cell migration into scratch wounds (Fig. 2b,c). Additionally, BI-78D3 abolished the fusiform cell morphology induced by Y27632, as visualized by immunostaining for CK6 and RelA (Fig. 2d and supplementary Fig. S3b). By

contrast, although the mitogen-activated protein kinase kinase inhibitor PD98059 reduced cell proliferation, it had only a slight effect on the migratory and fusiform cell phenotype induced by Y27632 as assessed by immunofluorescent staining (Fig. 2d; supplementary Fig. S3b). Thus, unlike ERK, the JNK signalling pathway is not impaired by Y27632, and is required for the Y27632-promotion of keratinocyte growth and migration.

Findings to date indicate that Y27632 is a useful reagent for keratinocyte procurement and possibly wound healing in clinical uses. These findings emphasize the importance of further mechanistic and preclinical studies to determine how Y27632 promotes keratinocyte cell proliferation and migration and wound healing. Specially, future studies are needed to address whether the keratinocyte effects of Y27632 are attributable to inhibition of Rho kinase or nonspecific targeting (possibly of other kinases), and whether topical Y27632 will induce adverse clinical outcomes such as skin cancer. It is interesting to note that H1152, another Rho kinase inhibitor, decreased keratinocyte proliferation in the present study (data not shown). In addition, genetic inhibition of either *RhoA* or ROCK1 did not enhance keratinocyte migration or wound healing in previous studies.^{7,8} These results indicate that Rho–ROCK inhibition may not be sufficient for the observed phenotypes, and suggest that Y27632 may involve actions of hitherto unidentified direct or indirect targets such as those involved in the JNK and ERK signalling pathways. The role of Y27632 in keratinocyte immortalization raised a concern about cancer;⁵ however, keratinocytes treated with Y27632 were able to differentiate when tested in three-dimensional skin culture.⁵ In addition, we found in this study that Y27632 inhibits cell proliferation of the human squamous cell carcinoma (SCC) cells A431, SCC25 and Cal27 (supplementary Fig. S4). Similarly, others have shown previously that Y27632 inhibits melanoma growth and invasion.⁹ These data indicate that topical Y27632 is unlikely to induce SCC or melanoma, but further *in vivo* testing is required to confirm this. Of further interest, Y27632 inhibits fibroblast-mediated collagen contraction,¹⁰ indicating that it may modulate scar formation. Such a possibility is worth testing in human scar tissue or diabetic animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Learning points

- The Rho kinase inhibitor Y27632 promotes proliferation of human keratinocytes but not fibroblasts or SCC cells.
- Y27632 enhances human keratinocyte outgrowth from skin explants, which is accompanied by increased expression cyclin D1 and normal expression of cytokeratin 14 and 6.
- Topical application of Y27632 on mice expedited cutaneous wound healing with no apparent toxicity to the animal.
- JNK signalling is required for the fusiform cell morphology and the enhanced keratinocyte cell proliferation and migration induced by Y27632.

Figure 1.

(a) Cell counts for keratinocytes (KC) and fibroblasts (Fb) after 2 days of culture in the presence or absence of various concentrations of Y27632. Experiments were performed in triplicate, and graphs represent the mean $\pm$ SD of data obtained from three independent experiments. (b) Scratch-wounding assay of KC and Fb treated with phosphate-buffered saline or 10 μ mol/L Y27632. Images were taken at 0 h (dashed lines indicate wound size) and 18 h after wounding and are representative of triplicate experiments. (c) Keratinocyte outgrowth assessed by microscopy on days 2 and 5 in split-thickness human skin tissues cultured in the presence of 0 or 10 μ mol/L Y27632. (d) Cells in tissue explants; graph represents the cells (mean $\pm$ SD) counted along the radius of 3–5 explants starting from the edge of the explant. A two-tailed *P*-value is presented for each time point. (e,f) Cutaneous wounds were created on the backs of mice that were then treated with phosphate-buffered saline 20 μ mol/L Y27632: (e) representative images of the wounds and (f) graphs of wound

size are shown for each time-point (days 3, 5, 7 and 10). A one-tailed P -value is also presented for each time-point.

Figure 2.

(a) Immunoblotting for phosphorylated Jun kinase (pJNK), phosphorylated extracellular regulated kinase (pERK) and actin in protein lysates from keratinocytes after scratch-wounding or treatment with epidermal growth factor in the presence or absence of Y27632. Relative densitometry is presented below each pJNK and pERK band, and data from two independent experiments are shown. (b) Cell counts (mean $\pm$ SD) of keratinocytes 2 days after Y27632 with or without BI-78D3 or PD98059. (c) Images of cell cultures taken immediately (dotted lines) and at 18 h after wounding. (d) Immunofluorescent staining of keratinocytes treated as in (b) showing staining for cytokeratin 6 (green) and nuclei (blue).