Non-Invasive Visualization of Epidermal Responses to Injury Using a Fluorescent Transgenic Reporter

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We describe transgenic mice in which expression of a reporter, the yellow fluorescent protein (YFP), is locally activated in epidermal cells at sites of injury. YFP is detectable a day after injury; its levels peak within 3 d, and then decline over the subsequent week. Expression is also activated by a chemical irritant, and is suppressed by topical administration of hydrocortisone. These mice permit non-invasive time-lapse monitoring of responses to injury in vivo. They can be used to detect epidermal activation and to test agents that may provoke or attenuate epidermal responses.

Key words: epidermis/fluorescent/in vivo/keratinocytes/skin trauma/transgenic mice

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Physical or chemical insults to the skin initiate a complex series of responses that include overlapping phases of hemostasis, degeneration, inflammation, activation, re-epithelialization, granule tissue formation, and remodeling (reviewed in Clark, 1996; Singer and Clark, 1999; Mehendale and Martin, 2001). These events have been studied in detail, both as means of elucidating basic principles of cell–cell and cell–matrix interactions and as promising therapeutic targets. The early phases are of additional importance in assessing the safety of numerous commercial products, ranging from soaps to cosmetics to occupational hazards. Mice have been frequently used in these studies (e.g., Patrick et al., 1987; Wilmer et al., 1994; Smith et al., 1997), and their use has increased recently due to the availability of numerous transgenic and knockout mouse strains in which responses to injury are perturbed (reviewed in Scheid et al., 2000; Mehendale and Martin, 2001). These studies have been limited, however, by the fact that non-invasive assays, such as measurement of ear thickness following topical application of a drug, are somewhat crude and non-specific, whereas more discriminating histological or molecular measurements are cumbersome and can only be performed following sacrifice.

Here, we describe a novel assay of epithelial responses to cutaneous injury that circumvents some of these limitations. It is based on the use of transgenic mice that express yellow fluorescent protein (YFP) in the cytoplasm of neurons under the control of regulatory elements derived from the thy1 gene (Feng et al., 2000; Lichtman and Sanes, 2003). During the course of studying peripheral nerve regeneration in these mice (Pan et al., 2003), we observed a transient local upregulation of YFP following full depth skin incision. This response was so strong that it was readily visible in live mice, using a dissecting microscope equipped with fluorescent optics. In this report, we characterize the YFP response, show that it reflects a local injury-induced upregulation of endogenous Thy1 in keratinocytes (Scheinuius et al., 1986; Rheins et al., 1987), and demonstrate that it can also be used to monitor responses to chemicals that cause or attenuate cutaneous inflammatory responses.

Results and Discussion

The thy1 gene is expressed by numerous cell types, including thymocytes, peripheral T cells, and neurons (reviewed in Morris, 1985). An ~8 kb genome fragment that included upstream and intronic sequences was capable of replicating most features of the thy1 expression pattern in transgenic mice. Sequences in introns 3 and 4 of the human and mouse genes are required for expression in most non-neuronal cells, so that genomic sequences lacking these introns drive nominally neuron-specific expression of reporters (Vidal et al., 1990; Kelley et al., 1994; Caroni, 1997). We generated transgenic mice in which these neuron-specific elements were linked to the YFP and found, as expected, that many central and peripheral neurons were YFP-positive whereas most non-neuronal cells were YFP-negative (Feng et al., 2000). Recently, we used these mice to analyze degeneration and regeneration of peripheral axons (Pan et al., 2003). We imaged the nerve transcutaneously using a dissecting microscope equipped with fluorescence optics, made an incision through which the nerve was damaged, then followed axonal responses. During the course of these experiments, we were surprised to observe a dramatic increase in YFP fluorescence in a restricted area bordering the incision site (Fig 1a, b). Additional experiments showed that the injury-related increase in YFP fluorescence occurred even when no nerve was injured, and even when no nerve trunk was present in the area. Realizing that this response might be a useful surrogate marker of

Abbreviation: YFP, yellow fluorescent protein
injury-induced changes in or under the skin, we characterized it further.

First, to determine which cells expressed YFP following injury, we cut sections through the wounded area and stained them with an antibody to green fluorescent protein (GFP) (that also recognizes YFP) plus the nuclear dye, DAPI. After wounding, YFP was expressed in keratinocytes of all epidermal layers, including the strata basale, spinosum, and granulosum (Fig 1c). In contrast, no increase in YFP expression was detected in dermal cells following injury, in epidermal cells distant from a wound site, or at wound sites in wild-type (non-transgenic) mice (Fig 1d, e). Therefore, fluorescence detected in the skin of transgenic mice following injury represents upregulation of YFP in keratinocytes.

Next, we documented the time course of the response. A patch of thigh skin was shaved, a 2 mm incision (*) was made in the skin of the hindlimb, over the saphenous nerve (n). The area was then photographed in a fluorescence dissecting microscope at indicated times. Localized YFP fluorescence was visible 1 d after incision (c), reached maximum intensity around 4 days (e), declined gradually (f, g) and was gone by day 13 (h). Scale bar = 1 mm.

Figure 1
Yellow fluorescent protein (YFP) induction in keratinocytes near wound sites. (a) Bright field image of mouse back skin, 3 d after hair removal and full depth incision. Scar tissue and swelling are seen at the center. (b) Fluorescent image of the same field. Skin surrounding the incision is intensely fluorescent. (c–e) Sections of skin, doubly stained with anti-GFP and nuclear dye, DAPI (left panels show GFP alone; GFP is green and DAPI is blue in right panels). (c) Skin adjacent to the incision from YFP transgenic, examined 3 d after cutaneous injury. YFP is strongly and selectively expressed in epidermal cells. (d) Skin from an uninjured YFP transgenic mouse. No epidermal YFP is detectable, but a few dermal cells (arrowhead) are YFP-positive. (e) Wild-type (C57) mouse, 3 d after cutaneous injury. Dotted line indicates the border between the epidermis (epi) and the dermis (der). Scale bars = 1 mm (a, b) and 50 μm (c–e).

Figure 2
Time course of yellow fluorescent protein (YFP) expression following injury. A 2 mm incision (*) was made in the skin of the hindlimb, over the saphenous nerve (n). The area was then photographed in a fluorescence dissecting microscope at indicated times. Localized YFP fluorescence was visible 1 d after incision (c), reached maximum intensity around 4 days (e), declined gradually (f, g) and was gone by day 13 (h). Scale bar = 1 mm.
injury (Fig 2d–h). Similar responses were observed in ear, tail, and toes (data not shown). The time course of YFP expression closely mirrors that of the re-epithelialization phase of wound healing (Viziam et al., 1996; Blomme et al., 2003). In addition, it has been reported that keratinocyte proliferation is increased in a broad area surrounding the incision, similar to the circle of fluorescence in YFP transgenics (Garlick and Taichman, 1994). Thus YFP expression likely marks keratinocytes that are actively participating in re-epithelialization.

Because expression of YFP in our transgenic mice is controlled by regulatory elements of the thy1 gene, we asked whether injury-induced fluorescence reflected up-regulation of the endogenous thy1 gene. Immunohistochemical studies have shown that endogenous thy1 is expressed at low levels in normal epidermis, but induced in keratinocytes after physical or chemical trauma (Scheynius et al., 1986; Rheins et al., 1987). Double labeling with antibodies to Thy1 and to YFP showed that levels of both proteins increased together in both space and time (Fig 3). We also considered an alternative possibility, that YFP expression was controlled by genomic elements near the site of transgene insertion (integration site-dependent expression; discussed in Feng et al., 2000). A second line of Thy1-YFP transgenic mice (YFP16), in which the transgene shows a distinct integration site-dependent pattern of expression within the nervous system, also showed injury-induced expression in keratinocytes (data not shown). This result argues strongly that injury-induced expression is controlled by sequences within the transgene. Thy1 is also found in the dermis and in epidermal dendritic cells of uninjured skin (Scheynius et al., 1986; Havran et al., 1991), but YFP was not detectably expressed in these cells (Figs 1c and 3a). Thus, in addition to the regulatory elements that promote largely neuron-specific expression, the transgene contains sequences that promote high level of expression in keratinocytes following injury.

Finally, we asked whether YFP might be useful for monitoring responses to chemicals that cause injury or attenuate inflammatory responses to injury. In one set of experiments, we tested a hair removal cream, NAIR (Cartier Products, New York, New York), which contains calcium hydroxide and is known to be corrosive when applied for extended period of time. When NAIR was applied for 3 min and then washed off, only low levels of YFP were visible the following day (data not shown). In contrast, application for 10 min led to significant YFP upregulation in keratinocytes (Fig 4a, b). In a second set of experiments, we tested the anti-inflammatory glucocorticoid, hydrocortisone. Hydrocortisone prevents leukocyte infiltration following injury; this effect, and possibly also direct effects on keratinocytes, suppress gene upregulation in the wounded epidermis (Bator et al., 1998). Topical application of 1% hydrocortisone cream immediately after wounding almost completely suppressed expression of YFP in the skin (Fig 4c, d).

**Figure 3**
Yellow fluorescent protein (YFP) and endogenous Thy1 are co-regulated following injury. (a) In uninjured skin, Thy1 and GFP are expressed at low levels. (b) Three days after cutaneous injury, Thy1 and YFP are co-expressed at high levels. Dotted line indicates the border between the epidermis (epi) and the dermis. Sections were triply labeled with anti-Thy1 (red: a, a’, b, b’), anti-GFP (green: a”, a”, b”, b”) and DAPI (blue: a”, b”). Each set of micrographs shows a single field. Scale bar = 10 μm.

**Figure 4**
Yellow fluorescent protein (YFP) reports on chemicals that cause or attenuate cutaneous injury. (a) Bright field and (b) fluorescent image of a region of skin to which NAIR had been applied for 10 min 3 d earlier, then rinsed away. This chemical irritant led to YFP induction. (c, d) Fluorescent images of skin that had been incised 3 d earlier. Animal in c was not further treated; 1% hydrocortisone cream was applied locally to the wound site in d, immediately after skin incision. Hydrocortisone prevented induction of YFP. Arrowhead: nerve. Scale bars = 1 mm (a, b) and 500 μm (c, d).

**Conclusions**

Epidermal keratinocytes are centrally involved in cutaneous wound healing (Clark, 1996; Singer and Clark, 1999). In
addition to being the direct targets of injury and the basis for regeneration, keratinocytes sense environmental stress, secrete factors that promote the inflammatory response, and respond to signals secreted by nearby cells such as dermal fibroblasts (Werner and Smola, 2001). Several markers that distinguish activated from resting keratinocytes have been described (Martin, 1997; Szabowski et al, 2000), perhaps including Thy1 (Scheynius et al, 1986). To date, however, none have been used extensively for assaying epidermal responses to trauma. Our serendipitous discovery that YFP is transiently upregulated in keratinocytes near sites of physical or chemical damage provides a new tool for analyzing such responses in vivo. Moreover, these mice provide a new reagent for assaying genes and treatments that might affect epidermal wound healing.

Material and Methods

Mice Generation of transgenic mice expressing YFP under the control of regulatory elements from the murine thy1 gene is described in Feng et al (2000). In most of the experiments described here, we used adult mice of line YFP-H weighing 20–30 g; similar results were obtained with the YFP-16 line. Both lines are available to academic researchers from the Induced Mutant Resource at Jackson Laboratory (Bar Harbor, Maine; strain designations: B6.Cg-Tg(Thy1-YFP)16Jrs/J and B6.Cg-Tg(Thy1-YFP-H)2Jrs/J).

Live imaging Mice were anesthetized by subcutaneous injection of ketamine and xylazine. The back or hind leg was depilated by shaving. In some cases, NAIR lotion or 1% hydrocortisone cream (Water-Jel Technologies, Carlstadt, New Jersey) was also applied. A small incision (~2 mm) was made in the skin under a dissecting microscope equipped with fluorescence optics (SZX12; Olympus Optical, Tokyo, Japan). Incisions in hind-leg and ear were made with an iroectomy scissor. Incisions on the back were made with a surgical scalpel. Images were acquired with a cooled CCD camera. After surgery and imaging, animals were allowed to recover and were returned to their cages. The Animal Studies Committee (IACUC) at Washington University approved the described procedures.

Immunofluorescence For histological analysis, mice were killed with an injection of sodium pentobarbital. Skin from ears or hind legs was fixed by immersion in 4% paraformaldehyde, infiltrated with sucrose, frozen, and sectioned in a cryostat. Sections were stained with rabbit anti-GFP polyclonal antibody (1/1000, Chemicon, Temecula, California) and rat anti-Thy1.2 antibody (1/50, BD Biosciences, Palo Alto, California), followed by species-specific Alexa-conjugated secondary antibodies (1/500, Molecular Probes, Eugene, Oregon).

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References


Morris R: Thy-1 in developing nervous tissue. Dev Neurosci 7:133–160, 1985


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