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The Göttingen minipig for assessment of retinoid efficacy in the skin: comparison of results from topically treated animals with results from organ-cultured skin

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Abstract

Göttingen minipigs were treated topically for 6 d with a novel retinoid (MDI 301) at concentrations ranging from 0.3% to 30% in cream vehicle. Treatment of the minipigs did not adversely affect their health (hematological and necropsy parameters) or produce changes in the skin suggestive of retinoid-induced skin irritation. After killing the animals, skin samples from each treatment site were excised and maintained in organ culture for 6 d. In addition, untreated skin was also maintained in organ culture and treated with MDI 301 (0.1–5 µg/ml). After 3 d, the culture supernatants were collected and analyzed for levels of collagen type I and for matrix metalloproteinases (MMPs). Both skin samples treated *in vivo* and skin samples exposed to MDI 301 in culture demonstrated increased collagen production. Only slight changes in levels of MMP-2 (gelatinase A) or MMP-9 (gelatinase B) were seen. After 6 d, the organ-cultured skin was fixed in formalin and prepared for histology. The organ-cultured skin was compared to skin that was fixed at killing after *in vivo* treatment. Epidermal hyperplasia was quantified at various MDI 301 concentrations. *In vivo* and *in vitro* treatments showed similar results—although the thickness was not substantially changed on average, there were focal areas of hyperplasia at higher retinoid concentrations. Taken together, these data suggest that MDI 301 enhances collagen production in minipig skin, without irritation. Furthermore, these studies suggest that minipig skin exposed to the retinoid in organ culture is equally predictive as topically treated skin. The *in vitro* organ culture approach may provide a cost-effective alternative model to that of the intact animal for skin retinoid testing.

Keywords

Collagen; Minipig; Matrix metalloproteinase; Organ culture; Retinoid; Skin

Introduction

The Göttingen minipig has been extensively utilized for preclinical studies in the pharmaceutical industry. Many of the organ systems of the pig resemble their human counterpart quite well. Likewise, physiological and pathophysiological responses in the pig are similar to those in humans (Douglas 1972; Brown and Terris 1996; Reeds and Odle 1996; Qvist et al. 2000; Svendsen 2006). Dermal studies find the minipig especially useful, as

its skin is in many respects more comparable to human than rodent, or traditional non-rodents, such as dog and primates (Swindle and Smith 1998). The minipig model though, as with other large-animal systems, can be problematic. The cost of purchasing and maintaining these animals often precludes their wide use in early-stage drug development. In a recent study, we demonstrated that skin from Göttingen minipigs could be successfully maintained in organ culture for several days (Dame et al. 2008). As part of that study, it was demonstrated that the conditions required for maintaining pig skin in organ culture were similar to conditions that had previously been shown to work with human skin (Varani et al. 1993, 1994). It was also shown in our study that keratinocytes and fibroblasts could be readily isolated from Göttingen minipig skin and that responses of the pig skin cells to known contact-irritant and contact-sensitizing agents were quantitatively and qualitatively similar to responses previously characterized with isolated cells from human skin (Varani et al. 2007a). Based on these findings, we suggested that the use of organ-cultured skin from Göttingen minipigs, in conjunction with fibroblasts and keratinocytes from the same source, might provide a cost-effective alternative to the use of intact animals for early-stage safety assessment in drugs designed for topical use.

The earlier study provided evidence that organ-cultured minipig skin can provide a suitable platform for early-stage safety evaluation. Whether the same approach would prove useful for efficacy studies was not addressed. In the present report, we demonstrate, using a combination of in vivo–in vitro approaches, that the efficacy of a novel retinoid can be assessed utilizing skin from Göttingen minipigs.

Materials and Methods

MDI 301

MDI 301 is a 9-cis RA derivative in which the terminal carboxylic acid group has been replaced by a picolinic ester. MDI 301 was synthesized as described in the original patent (US Patent 5,837,728; Molecular Design International; Memphis, TN) and our previous report (Varani et al. 2003). For topical treatment, MDI 301 was applied in a standard vehicle cream and expressed as a percent (wt MDI 301/wt cream). For in vitro studies, MDI 301 was dissolved in dimethyl sulfoxide (DMSO) at 20 mg/ml and diluted in culture medium at the time of use.

The stability of the cream-based MDI 301 was verified by dissolving the 30% MDI 301 cream (7-mo old) into DMSO to make 20 mg/ml MDI 301. This was functionally compared to freshly made MDI 301 in DMSO. Both preparations produced an almost exactly equal dose-dependent proliferative response in human foreskin fibroblasts grown in KBM-low calcium (0.1 mM Ca^{2+}) medium (data not shown).

Culture media and reagents

Keratinocyte basal medium (KBM) was obtained from Lonza (Walkersville, MD) and supplemented with 50 µg/ml gentamycin sulfate (Invitrogen, Grand Island, NY). This basal medium (Ca^{2+} concentration of 0.15 mM) was further supplemented with calcium chloride (Aldrich-Sigma, St. Louis, MO) to a final Ca^{2+} concentration of 1.55 mM (KBM-1.55 Ca^{2+}). Dulbecco's modified minimal essential medium (Invitrogen) with 25 mM glucose [DMEM–10% fetal calf serum (FBS)] was supplemented with 1% non-essential amino acids (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), and 10% fetal bovine serum (Hyclone, Logan, UT).

Animals

The animals used in the present study included five female Göttingen minipigs (4 mo old) obtained from Marshall BioResources (North Rose, NY). Topical administration of MDI 301

in the cream vehicle was carried out by Ricerca Biosciences, LLC (Concord, OH) as part of an ongoing safety assessment study. Treatment of the animals was in accordance with regulations outlined in the USDA Animal Welfare Act (9 CFR Parts 1, 2, and 3) and the conditions specified in Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). A veterinarian or designated technician observed each animal at least once daily for signs of illness or distress. No such observations were reported. At the end of the studies, animals were killed using Fatal-Plus (pentobarbital sodium; Vortech Pharmaceutical, Dearborn, MI).

In vivo treatment protocol and assessment

MDI 301 was first assessed for potential toxicity and skin irritation in the Göttingen minipig whole animal model, with 6 d of repeated topical dosing of the agent. Each animal had six sites (4 cm² per site) along its back, treated with a control vehicle cream and five doses of MDI 301 (0.3, 1.0, 3.0, 10, and 30% wt/wt). Approximately 1-mm thickness of cream was applied to each site and covered with Tegaderm[®] foam (3 M, St Paul, MN) placed directly over the site and then held in place with gentle wrapping of the pig with Tensoplast[®] elastic tape (BSN Medical, Hamburg, Germany). Each day, the site was scored for irritation/redness, swelling/edema, flaking, cracking, and blanching prior to application of the dose for that day. Digital photographs were taken daily of the sites after removal of the wrapping and prior to treatment. Cage-side observations and clinical signs were recorded at 1 and 2.5 h post-dose daily during the study. Body weights were taken daily before treatment and prior to necropsy. Food consumption was measured daily for the duration of the study. Blood samples for clinical pathology (hematology, serum chemistry, and coagulation) and MDI 301 levels were collected prior to dosing and before termination of the 7-d study. The animals were euthanized at 7 d, and a necropsy was conducted on all animals with preservation of selected tissues.

Organ culture protocol and in vitro treatment/assessment

Immediately after animal killing, full-thickness skin was dissected from the test sites and from an untreated site on the back of each animal. The skin was transported (approximately 5 h) from Ricerca Biosciences to our laboratory at the University of Michigan in cold KBM-1.55 mM Ca²⁺, supplemented with 2.5 µg/ml (1X) amphotericin (Invitrogen). Upon arrival in the laboratory, the skin was prepared for culture as previously described (Dame et al. 2008) by first removing the subcutaneous tissue and cutting into 1×4-cm strips. The dermis was then carefully shaved with a scalpel until only a thin layer of it remained, reducing the total skin thickness from 20 mm to about 2–3 mm. The strips of skin were further divided into 3×3-mm pieces. The skin was then cultured as either whole skin organ culture or used as explant for outgrowth of keratinocytes and fibroblasts. For organ culture, two pieces were placed in individual wells of a 24-well dish with 0.5 ml of KBM-1.55 mM Ca²⁺. In addition to the organ culture of in vivo-treated skin, skin from the untreated site was also cultured and exposed in vitro to various concentrations of MDI 301 (0.1–5 µg/ml). Cultures were grown in a humidified incubator with an atmosphere of 95% air and 5% CO₂ at 37°C. At day 3 and day 6, the conditioned media were collected for analysis, and fresh culture medium was added at day 3. At day 6, the tissue was fixed in 10% buffered formalin for histology.

Cell outgrowth from tissue explants

From each dose site, 20 3×3-mm pieces were wetted with DMEM-10%FBS and spread over the surface of a collagen-coated 25-cm² flask (10 µg/cm² rat tail type I collagen; BD Biosciences, Bedford, MA). Briefly, culture dish surfaces were coated with collagen in 0.02 M acetic acid for 18 h, dried, and rinsed before use. After 1 d, the pieces adhered to the flask surface and 2.5 ml of media was carefully added. At 4 d, the volume was increased to 5 ml and

subsequently changed every 2 d. After 10 d, the outgrowth of keratinocytes and fibroblasts were separated by differential trypsinization and counted.

Epidermal thickness

The skin collected at necropsy and the organ-cultured skin were formalin-fixed, paraffin-embedded, and processed for hematoxylin/eosin histology. The stained tissue slides were scanned and digitized with a ScanScope XT (Aperio Technologies, Vista, CA). The epidermal thickness was quantified using Aperio ImageScope (v9.1.191571). The epidermis was outlined with the software *pen tool* and the area and length were presented. Epidermal thickness was expressed as micrometer square area/micrometer length, and fold changes were calculated. Each treatment condition was represented by two skin pieces, with an average length of 2,130 μm quantified per piece.

Type I collagen

Culture fluids were assayed for type I collagen by Western blotting (Varani et al. 2007a). Briefly, organ culture fluids representing equal quantity of protein were resolved using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk solution in Tris-buffered saline with 0.1% Tween (TTBS) for 1 h at room temperature. Following this, they were incubated overnight with a rabbit antibody to human type I collagen (1:10,000 dilution; Abcam Inc., Cambridge, MA) in the same buffer at 4°C. The membranes were then washed with TTBS and bound antibody was detected using the Phototope-HRP Western detection kit (Cell Signaling Technologies Inc., Danvers, MA). Images were scanned, digitized, and quantified using NIH image analysis software.

MMP production

Substrate embedded enzymography (zymography) was used to assess levels of latent and active MMP-2 and MMP-9 in organ culture fluids. As described previously (Lateef et al. 2004), SDS-PAGE gels were prepared with the incorporation of gelatin (1 mg/ml) at the time of casting. After electrophoresis under non-reducing conditions to separate proteins and overnight incubation to allow for substrate digestion, zones of hydrolysis were identified as “holes” in the stained gels and quantified. Values for latent and active MMP-2 and MMP-9 bands were obtained following digitization.

Results

Topical treatment of Göttingen minipig skin with MDI 301 under occlusion: general findings

Five Göttingen minipigs were treated for six consecutive days with MDI 301 (0.3% to 30%) under occlusion. The animals were examined daily during the treatment phase. Figure 1 demonstrates the appearance of skin sites on day 7 of one representative animal, treated with vehicle alone, and various concentrations of MDI 301. No demonstrable changes were observed in any treated site at any time during the treatment period. Blood samples for clinical pathology (serum chemistry, hematology, coagulation) were collected prior to dosing and at the termination of the study (day 7). Overall, the findings were unremarkable (data not shown). A rise in creatinine kinase and aspartate transaminase was observed in one of the animals, but hemolysis was also observed in the blood sample of this animal, possibly influencing the test results. There were no obvious changes in other hematological parameters, and the only finding at necropsy was a red-discolored thymus in one of the animals. These sporadic findings were thought to result from the stress associated with restraint and bleeding. It was concluded that topical application of MDI 301 at up to 30% for six consecutive days did not adversely affect the health of the Göttingen minipigs or cause skin irritation.

Further evidence for a lack of toxicity after in vivo MDI 301 treatment was the outgrowth of keratinocytes and fibroblasts from explanted tissue. Approximately equal numbers of cells were recovered from 10-d explant cultures from all sites (differences not statistically significant). With fibroblasts, recovery numbers ranged between 7.4 and 10.1×10^5 cells per flask. With keratinocytes, recovery numbers ranged between 1.8 and 2.3×10^5 cells per flask. The only consistent retinoid effect was a tendency toward rising metabolic activity with increasing concentrations of MDI 310, as evidenced by a more rapid lowering of the pH in these explants culture flasks (not shown).

Effects of MDI 301 on type I collagen production: in vivo–in vitro comparison

At the time of killing, skin from each of the treated sites (including the vehicle control site) was collected under aseptic conditions. Skin samples were prepared for organ culture as described in the “Materials and Methods” and incubated for 6 d in culture. Culture fluid collected at day 3 was analyzed for type I collagen by Western blotting. As shown in the left panel of Fig. 2, there was a dose-responsive increase in the level of type I collagen secreted into organ culture fluid.

Skin from a non-treated site was also obtained at the same time under aseptic conditions and prepared for organ culture with varying concentrations of MDI 301. The right panel of Fig. 2 demonstrates the in vitro effects of MDI 301 on collagen production. As seen in the panel, there was an increase in type I collagen secreted into the culture fluid collected at day 3. Of interest is that baseline values for type I collagen were substantially higher in the in vitro control than they were for the in vivo control. This suggests that the 6-d in vivo treatment with the vehicle cream alone, under occlusion, may actually depress collagen production. Having said that, MDI 301 was shown to significantly stimulate collagen production in a dose-dependent fashion in both the in vitro model and the in vivo model.

Effects of MDI 301 on MMP production: in vivo–in vitro comparison

The same culture fluids from the in vivo-and in vitro- treated pig skin were assessed for MMP-2 and MMP-9 by gelatin zymography. As seen in Fig. 3, there was a modest increase in the activity of these proteases with exposure to MDI 301, although these changes were much less than the fold increase in collagen production. This trend was seen in both the in vivo- and in vitro-treated skin. The untreated (in vivo) skin used for the in vitro treatments had a higher baseline protease activity, suggesting that the occlusion itself with cream vehicle reduced protease activity, as it did collagen production.

Effects of MDI 301 on epidermal thickness: in vivo–in vitro comparison

Tissue was fixed in 10% buffered formalin on day 6. After fixation for histology and staining with hematoxylin and eosin, epidermal thickness was assessed morphometrically. Areas of focal hyperplasia were observed in some of the skin samples, but except for a modest increase in thickness at the 10% MDI 301 treatment site, the differences were not consistent or significant (Fig. 4, upper and lower panels).

Discussion

Pig skin is structurally and functionally similar to human skin (Swindle and Smith 1998), and the pig is commonly used in the development of drugs for topical delivery. One drawback to the use of this species is expense. The cost of using inbred pigs, such as the Göttingen minipig as well as other large animals, can be prohibitively high. Except in cases where sustaining a whole animal is the only viable study method, alternate approaches are requisite. In a recent study (Dame et al. 2008), we demonstrated that skin from adult Göttingen minipigs could be maintained in organ culture for several days. Keratinocytes and fibroblasts from the same skin

could also be established and grown in monolayer culture. Finally, it was shown that the response of the pig skin cells to a panel of contact irritants and contact sensitizers was similar to the response reported earlier with human skin in organ culture and human skin cells in monolayer culture (Varani et al. 2007a). Based on those recent data, we concluded that skin from adult Göttingen pigs could be used in organ culture as part of a preclinical topical drug safety assessment strategy.

The present study continues our effort to determine the extent to which Göttingen minipig skin in organ culture might prove useful in drug development. The studies conducted here, using a combination of in vivo and in vitro treatment protocols, demonstrated that MDI 301 (an experimental retinoid) induces similar increases in type I collagen production following drug exposure in organ culture as seen following topical application to intact animals. Increased collagen production was seen without a significant epidermal hyperplasia (in vivo or in vitro) and without evidence of skin irritation or systemic toxicity (assessed in vivo). Our previous studies have demonstrated that MDI 301 is effective in stimulating new collagen production in human skin in organ culture (Varani et al. 2007b) as well as in rodent skin when applied topically (Warner et al. 2008). MDI 301 is not irritating in rodent skin (Varani et al. 2003; Warner et al. 2008) and does not induce surrogate markers of inflammation (pro-inflammatory cytokine and leukocyte adhesion molecule expression) in human skin organ culture (Varani et al. 2007b). The findings here are consistent with the past studies in human and in rodent skin.

The relationship between effective concentrations of MDI 301 in the intact pig versus skin organ culture is of interest. Topical treatment with a concentration as low as 0.3% MDI 301 stimulated measurable collagen production, while an in vitro concentration of 1 µg/ml (approximately 3 µM) produced an effect. With *all-trans* retinoic acid (RA), the prototypic retinoid for skin repair (Kligman et al. 1986, 1993; Weiss et al. 1988), topical preparations containing 0.025% to 0.1% are therapeutic. Penetration studies have found that when a therapeutic dose of RA is applied to the skin, concentrations of 1–2 µM are found in the viable portion of the epidermis and upper dermis (Duell et al. 1992). Similar RA concentrations (1–2 µM) are effective when utilized with human skin in organ culture (Varani et al. 2007b). The in vivo–in vitro dose relationship observed here with the Göttingen minipig is consistent with what would be expected from previous studies using RA.

In order to treat topically with MDI 301, the retinoid was applied in a cream vehicle under bandage occlusion. Logistically, this was necessary due to the nature of the animal study, but we also knew that percutaneous absorption of a compound is often increased under occlusion (Zhai and Maibach 2001). We observed, though, that occlusion alone with the cream vehicle caused a reduction in collagen production and a slight reduction in MMP-2/-9 activity. Other parameters were not measured in this study, but we may surmise that a general reduction in metabolic activity may result with occlusion. When MDI-301 was added to the cream (under occlusion), these functions returned to levels of untreated skin, and in the case of collagen, further increased many fold.

In summary, past work has made extensive use of human skin in organ culture for assessing both efficacy and safety with agents that are not yet approved for human use. In many respects, human skin is ideal. There are, however, inherent limitations with the use of human skin, most notably availability. This would not be the case with the Göttingen minipig, given the large amount of accessible skin from a single animal. Based on the findings we have presented here and previously, we conclude that skin from adult Göttingen minipigs can (1) be established in cell and organ culture, (2) provide an in vitro culture system that can be used for preclinical safety assessments, and, finally, (3) serve to study efficacy of agents designed for use in human skin. Employing this method, herein, this study has shown that the experimental retinoid MDI 301 is non-irritating even at high concentrations (30%), while stimulating collagen production

at lower concentrations. Organ-cultured skin from the pig, in conjunction with keratinocytes and fibroblasts from the same source, can provide a cost-effective in vitro culture system that can be used as a viable alternative to large animal or human use studies.

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Figure 1. Appearance of skin sites from one representative animal of five Göttingen minipigs. Animals were treated with vehicle alone and various concentrations of MDI-301. Photographs were taken on 1 d following the last of six daily treatments under occlusion.

Figure 2.

Type I collagen in organ culture fluid of skin from Göttingen minipig treated in vivo or in vitro with MDI 301. Collagen was assessed by Western blots followed by digitization and quantification. Values are means and standard errors based on organ cultures from three different minipigs. Statistical significance was determined using the Student's *t* test, comparing each treatment group to its control. Asterisk indicates statistical difference from the control group at $p < 0.05$ level. Inserts, Western blots from one minipig and one set of organ cultures.

Figure 3. MMP-2 and MMP-2 in organ culture fluid of skin from Göttingen minipig treated in vivo or in vitro with MDI 301. MMPs were assessed by gelatin zymography followed by digitization and quantification. Values are means and standard errors based on organ cultures from three different minipigs. Statistical significance was determined using the Student *t*-test, comparing each treatment group to its control. *Asterisk* indicates statistical difference from the control group at $p < 0.05$ level. *Inserts*, gelatin zymograms from one minipig and one set of organ cultures.

Figure 4.

Epidermal thickness in Göttingen minipig skin treated in vivo or in vitro with MDI 301. Skin organ cultures were incubated for six days. At the end of the incubation period, tissue sections were prepared for histology and stained with hematoxylin and eosin (magnification, $\times 250$). Epidermal thickness was assessed morphometrically. Values are means and standard errors based on measurements taken (micrometer square area/micrometer length) from two skin pieces per treatment for each animal, with an average length of 2,130 μm quantified per piece (in vivo from five animals, in vitro organ culture from three animals). Using the Student's *t* test, no significant difference was found comparing each treatment group to its control.