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ORIGINAL ARTICLE
Research

Effect of All-trans Retinoic Acid on Panniculus Carnosus Muscle Regeneration in Fetal Mouse Wound Healing

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Background: The dermal panniculus carnosus (PC) muscle is critical for wound contraction in lower mammals and is a useful model of muscle regeneration owing to its high cellular metabolic turnover. During wound healing in mice, skin structures, including PC, are completely regenerated up to embryonic day (E) 13, but PC is only partially regenerated in fetuses or adult animals after E14. Nevertheless, the mechanisms underlying wound repair for complete regeneration in PC have not been fully elucidated. We hypothesized that retinoic acid (RA) signaling, which is involved in muscle differentiation, regulates PC regeneration.

Methods: Surgical injury was induced in ICR mice on E13 and E14. RA receptor alpha (RARα) expression in tissue samples from embryos was evaluated using immunohistochemistry and reverse transcription-quantitative polymerase chain reaction. To evaluate the effects of RA on PC regeneration, beads soaked in all-trans RA (ATRA) were implanted in E13 wounds, and tissues were observed. The effects of RA on myoblast migration were evaluated using a cell migration assay.

Results: During wound healing, RARα expression was enhanced at the cut surface in PCs of E13 wounds but was attenuated at the cut edge of E14 PCs. Implantation of ATRA-containing beads inhibited PC regeneration on E13 in a concentration-dependent manner. Treatment of myoblasts with ATRA inhibited cell migration.

Conclusions: ATRA inhibits PC regeneration, and decreased RARα expression in wounds after E14 inhibits myoblast migration. Our findings may contribute to the development of therapies to promote complete wound regeneration, even in the muscle. *(Plast Reconstr Surg Glob Open 2022;10:e4533; doi: 10.1097/GOX.0000000000004533; Published online 28 September 2022.)*

INTRODUCTION

The muscles of the dermal tissue layer panniculus carnosus (PC) lie beneath the dermal fat layer and above the subcutaneous fatty tissue and fascia.[1] PC is a trace organ in humans, but is widely present in the dermis of quadrupeds, including rodents.[2] Compared with other muscles, PC generally contains small fibers, with considerable heterogeneity in size and muscle fiber regenerative capacity.[3] The PC muscle is primarily composed of type II fibers, which are considered to assist in the ability of rodents' loose skin to spasm and thermoregulate[4] and to promote wound contraction[5] and revascularization.[6] In addition to its well-established functional roles, a deeper understanding of PC in animal models and humans may be useful for elucidating the hemodynamics of skin flaps and principles of wound healing, and for performing subcutaneous drug delivery studies. Owing to mammals' migratory properties, PC has traditionally been considered critical in most mammalian wound healing and burn repair processes, that is, wound contraction.[7,8] The roles of PC in wound contraction include the promotion of collagen secretion and cell viability[9] and provision of a low-resistance plane between fascial layers.[10] Alternatively, changes in the tensile strength of a wound due to contraction of the transected PC are also considered to play a key role.[11] Indeed, wounds with PC removed reportedly exhibited prolonged contraction, resulting in irregular scarring.[12] As such,

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Received for publication May 5, 2022; accepted July 27, 2022.

DOI: 10.1097/GOX.0000000000004533

Disclosure: *The authors have no financial interest to declare in relation to the content of this article. This work was supported by JSPS KAKENHI (grant number: 19H03815).*

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elucidating the roles of PC in wound healing may facilitate the development of therapies to prevent scar formation.

Considering the mechanisms of skin regeneration, the wound healing process in the mammalian fetus has received considerable interest owing to the switch from an intact state in early development to a typical adult-like scar-forming phenotype in late development. During wound healing in adult animals, PC regeneration terminates and leaves a gap, whereby opposing ends do not merge. Although regeneration is incomplete after E14, fetal skin wounds formed at E13 demonstrate complete PC regeneration by 72 hours after wounding.[13] As the timing of this switch coincides with the switch between E13, in which the skin regenerates completely, and E14, which leaves visible and persistent scarring, we hypothesized that the regulation of PC regeneration could be involved in scarless wound healing.

All-trans retinoic acid (ATRA), a metabolite of vitamin A, is a ligand for nuclear receptors, including retinoic acid receptors (RARα, β, γ).[14] ATRA regulates muscle differentiation through transcriptional regulation of MyoD and myogenin.[15,16] Beneficial effects of retinoic acid (RA) signaling on tissue regeneration have been reported in both cardiac and skeletal muscles.[17,18] However, to date, no reports are available on the role of ATRA in the healing or regeneration of PC, a specialized rhabdomyolysis muscle. We hypothesized that exogenous ATRA is regulated by the stimulation of RA signaling in PC regeneration during fetal wound healing. In this study, we aimed to investigate the role of ATRA in PC regeneration using an originally developed mouse fetal wound healing model.

Takeaways

Question: What is the effect of exogenous retinoic acid (RA) on the regeneration of panniculus carnosus (PC) in mouse fetuses?

Findings: Retinoic acid inhibits PC regeneration, and decreased expression of RARα in wounds after embryonic day 14 inhibits myoblast migration.

Meaning: Regulation of retinoic acid may enable regeneration of wounds containing muscle.

MATERIALS AND METHODS

Ethics Approval

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Keio University School of Medicine [approval number: 13072-(2)]. All experiments were performed in accordance with Institutional Guidelines on Animal Experimentation at Keio University.

Fetal Wounding and Skin Harvesting

We used 8-week-old female ICR mice. The mice were obtained from Sankyo Laboratories, Japan. All mice were provided water ad libitum while cohoused in an environmentally controlled facility maintained at 24 °C under a 12-hour light–dark cycle from 0700 to 1900 hours. Individual mice were kept in individual cages until sampling. Fetuses were designated as E0 when a plug was observed in the vagina of dams. Fetuses were subjected to surgery on E13 and E14. To create a fetal wound, pregnant mice were administered general 3% isoflurane anesthesia via inhalation, and their abdomens were disinfected with ethanol. Laparotomy was performed to incise the myometrium and amniotic membrane. A 2-mm-thick incision was made on each flank of the fetus using a pair of microsurgical scissors (n = 12 fetuses per time point). A 9-0 nylon purse-string suture was performed on the myometrium to separate the fetus from the abdominal fluid. The dam's abdominal wall was sutured with 4-0 nylon. At multiple time points after the injury, the animals were killed via cervical dislocation. Fetal wounds were harvested at 3, 6, 9, 12, 36, and 72 hours after wounding. Tissue sections were fixed by immersing in 4% paraformaldehyde overnight and then fixed in 20% sucrose overnight. The tissue was snap-frozen in the Tissue-Tek O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) and stored at −70 °C until the preparation of frozen sections.

Immunohistochemistry

The frozen samples were cut to 7-μm-thick sections and fixed in dry acetone at room temperature (15 °C–25 °C) for 10 minutes. Immunohistochemistry was performed using a rabbit anti-RA receptor α antibody (sc-515796; Santa Cruz Biotechnology, Inc., Dallas, Tex.). The slides were incubated with 2% normal goat serum in phosphate-buffered saline (PBS) at room temperature for 30 minutes for blocking after heat-induced antigen retrieval. The primary antibody was diluted 1:100 with PBS, applied overnight to the slides at 4 °C, and then washed three times with PBS. Subsequently, Alexa Fluor 488-conjugated goat antirabbit antibody (ab150077; Thermo Fisher Scientific, Waltham, Mass.) diluted 1:200 with PBS was applied at room temperature for 1 hour and washed three times with PBS. Nuclear counterstaining was performed with DAPI (4',6-diamidino-2-phenylindole), enclosed with ProLong gold (9071; Thermo Fisher Scientific). The slides were observed using an all-in-one stereomicroscope (BZ-X800; KEYENCE, Osaka, Japan).

RNA Extraction and Reverse Transcription

The total RNA was extracted from the skin using a single-phase solution of phenol and guanidine isothiocyanate (311-02501; ISOGEN, Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. A region containing PC was collected from the edge of the wound under a microscope. The collected total RNA was mixed with PrimeScript IV 1st strand complementary DNA (cDNA) Synthesis Mix (6215A, Takara Bio, Shiga, Japan), and was promptly reverse transcribed into cDNA.

Quantitative Real-time PCR

Reverse transcriptase-polymerase chain reaction and expression quantification were performed as previously reported.[13] Gene expression of RARα was quantitatively compared with real-time PCR in triplicate using the TaqMan Gene Expression Master Mix (4369016; ThermoFisher Scientific) on Applied Biosystems 7500

Fast. *ACTB* was used as an endogenous control for normalization. An unpaired bilateral Student *t* test was performed to detect statistically significant differences in gene expression. Results with *P* values less than 0.05 were considered statistically significant.

ATRA Bead Transplantation

DOWAX ion-exchange beads (diameter, 200 μm; Sigma Chemical, St. Louis, Mo.) were immersed in 5-, 1-, 0.1-, or 0.01-mg/mL ATRA (188-01113; FUJIFILM Wako Chemicals Co. Osaka, Japan) for 20 minutes at room temperature. Beads immersed in dimethyl sulfoxide (DMSO) were used as controls. The beads were washed twice in Dulbecco's essential medium containing 10% fetal bovine serum and implanted under the PC during fetal surgery on E13. The fetuses were collected using the same procedure described above, tissues were observed, and RNA was collected. The area of the reproduced PC was measured using ImageJ software (version 1.53p). Each experiment was repeated three times.

Boyden Chamber Assay

C2C12 (CRL-1772) was obtained from ATCC as myoblasts (Manassas, Va.). Before the assay, the cells were treated with mitomycin C (Nakarai Tesque, Inc., Kyoto, Japan) to remove proliferative effects (10 μg/mL, 37 °C for 3 hours). To prepare the Boyden chamber (Sigma-Aldrich, St. Louis, Mo.), a 12-μm polycarbonate membrane was coated with 1.2-μg/mL fibronectin (356008; Corning Inc., Corning, N.Y.) or 0.1- (low) or 5- (high) mg/mL ATRA on the lower surface. The cells (1×10^4) were placed on the upper membrane surface and incubated for 2 hours at 37 °C, 5% CO_2, and 95% relative humidity. The number of cells migrating through the membrane pores was quantified under a microscope.

Statistical Analysis

A Mann–Whitney U test was performed to determine the significance of differences in migration or gene expression using Statistica software version 9.0 (StatSoft, Tulsa, Okla.). The results of descriptive statistics are presented as mean ± standard deviation. The threshold for statistical significance was set at a *P* value less than 0.05. Each experiment was conducted in triplicate.

RESULTS

Regeneration of PC and Expression of RARα in E13 and E14 Wounds

At 72 hours after wounding, skin structures, including PC, were completely regenerated in E13 wounds, but PC had not regenerated in the wound area on E14. (**See figure, Supplemental Digital Content 1**, which displays regeneration of PC during skin wound healing in E13 and E14 mouse fetuses. A, PC regenerates with wound healing at 72 hours after wounding in E13 fetuses, whereas on E14, the wound heals without regeneration (red arrows). Yellow arrow: wound region. Green dotted line: PC. Scale bar: 100 μm. B, RARα expression in E13 and E14 mouse fetuses at 6 hours after wounding. In E13 fetuses, RARα **http://links.lww.com/PRSGO/C161**.)

Immunostaining revealed RARα expression at the cut edge of PC in unhealed wounds at 6 hours after wounding on E13, whereas no RARα expression was observed in PC in the unwounded area. In contrast, on E14, RARα expression was noted in the woundless area, but this expression decreased near the PC transection edge. (**See figure 1B, Supplemental Digital Content 1**, which displays regeneration of PC during skin wound healing in E13 and E14 mouse fetuses. B, RARα expression in E13 and E14 mouse fetuses at 6 hours after wounding. In E13 fetuses, RARα is expressed at the cut surface of PC (white arrows), but not in PC of the unwounded area. In E14 fetuses, RARα expression is decreased at the cut edges of PC (red arrows), whereas RARα is expressed in unwounded PC. Epi: epidermis. Der: dermis. Yellow arrows: wound region. White dotted line: PC. Scale bar: 100 μm. **http://links.lww.com/PRSGO/C161**.) Real-time PCR confirmed that RARα expression was significantly higher in the wound than in the unwounded area on E13 ($P = 0.01$). Conversely, RARα expression was lower in the wound than in the unwounded area on E14 ($P = 0.006$). (**See figure 1C, Supplemental Digital Content 1**, C, Gene expression of RARα at 6 hours after E13 and E14 wounds. $^*P < 0.05$. **http://links.lww.com/PRSGO/C161**.)

An observation of temporal changes in the wound revealed RARα expression at the cut edge of PC in the wound 3 hours after wounding on both E13 and E14. The expression trends were maintained during wound healing in E13 wounds, whereas the wound healed with reduced RARα expression at the PC wound edge after 6 hours in E14 wounds. (**See figure 1D, Supplemental Digital Content 1**, which displays regeneration of PC during skin wound healing in E13 and E14 mouse fetuses. D, RARα expression in PC during the wound healing process in E13 and E14 fetuses. Yellow arrows: wound region. White arrows: regions of RARα expression. Red arrows: areas of attenuated RARα expression. White dotted line: PC. Scale bar: 100 μm, **http://links.lww.com/PRSGO/C161**.) E14 wounds exhibited wound healing trends that were similar to those of E13-type regeneration until the middle of the wound healing process, indicating that the changes in retinoid X receptor (RXR)α expression in PC occurred during the wound healing process on E14.

Alterations in PC Regeneration With the Transplantation of ATRA-Immersed Beads

ATRA-immersed beads (ATRA beads, 5 mg/mL) or control beads were transplanted into the layer underneath PC in the wound of E13 fetuses, and the tissue was collected after 72 hours. Epithelialization, but not PC regeneration, occurred following ATRA bead transplantation. (**See figure 2A, Supplemental Digital Content 2**, which displays implantation of ATRA-immersed beads into E13 fetal wounds and regeneration of PC. A, Schematic of the experiment. **http://links.lww.com/PRSGO/C162**.)

In contrast, PC around transplanted control beads regenerated completely, indicating that ATRA inhibited PC regeneration. (**See figure 2B, Supplemental Digital Content 2**, B, PC regeneration at 72 hours after bead implantation visualized with hematoxylin-eosin staining.

ATRA-immersed beads suppress PC regeneration. Yellow arrows: wound region. Green dotted line: PC. Scale bar: 100 μm. **http://links.lww.com/PRSGO/C162**.) This inhibition of regeneration occurred in a concentration-dependent manner. (**See figure 2C, Supplemental Digital Content 2**, which displays implantation of ATRA-immersed beads into E13 fetal wounds and regeneration of PC. C, ATRA concentration versus area of PC regeneration. PC regeneration is inhibited in a concentration-dependent manner. *P < 0.05. **http://links.lww.com/PRSGO/C162**.) Furthermore, the expression of RARα in the PC area was decreased by ATRA bead implantation (P = 0.009), in contrast to that in unwounded areas on E13. (**See figure 2D and E, Supplemental Digital Content 2**, which displays implantation of ATRA-immersed beads into E13 fetal wounds and regeneration of PC. D, RARα expression in the wound at the bead implantation site. RARα expression is decreased at the ATRA-soaked bead implantation site. White dotted line: PC. E, RARα gene expression in bead-implanted wounds. *P < 0.05, **http://links.lww.com/PRSGO/C162**.)

Inhibition of Myoblast Migration by ATRA

To examine the link between ATRA inhibition of PC regeneration and myoblast migration, we investigated the migratory ability of myoblasts in the presence and absence of ATRA.

In a Boyden chamber assay, myoblast migration was significantly inhibited in the presence of ATRA (control versus high concentration of ATRA; P = 0.023) (Fig. 1).

In conclusion, inhibition of myofibroblast migration by ATRA is involved in the regeneration of PC in fetal mouse wounds (Fig. 2).

DISCUSSION

In adults, skeletal muscle possesses regenerative capacity that is primarily mediated by satellite cells, the stem cells of skeletal muscle. Known mediators of activation include hepatocyte growth factor, insulin-like growth factor-1, fibroblast growth factors, platelet-derived growth factors, and interleukin-6.[19,20] However, complete regeneration is not possible after cutting muscle bodies in adults; PC exhibits minimal regeneration around muscle wound edges in adult mice.[21] During developmental stages, an injured PC on E13 regenerates completely; however, in wound healing after E14, complete PC regeneration is not observed, and gaps remain after cutaneous wound healing. Nevertheless, dermal structures regenerate completely 72 hours after wounding.

Among the various signaling pathways that regulate skeletal muscle development and growth, we focused on the RAR signaling pathway. Two classes of RARs have been described: RARs and RXRs, each comprising three isoforms α, β, and γ, encoded by separate genes. RARs mediate the effects of ATRA, the active metabolite of vitamin A. RARs form heterodimers with RXRs, bind to RA response elements in the promoter regions of target genes, and activate gene transcription upon ligand binding.[14] In the absence of a ligand, RAR–RXR heterodimers bind to RA response elements associated with the corepressor and exert repressor activity on target gene expression.[21] Retinoids (vitamins A and RA) have been demonstrated to regulate skeletal muscle differentiation and metabolism in mouse, zebrafish, and chicken skeletal muscle cells.[22–24]

Based on the literature, we hypothesized that RA and downstream signals are involved in PC regeneration during wound healing in E13 fetuses. As we were unable to measure endogenous RA levels, we could not directly determine whether endogenous RA levels changed with development. To circumvent this, beads containing ATRA were implanted in contact with the wound. As previously reported, ATRA-soaked beads gradually released ATRA for 24 hours after implantation.[25] In E13 wounds, PC regeneration was altered by ATRA, exhibiting dose-dependent inhibition of regeneration. However, the perturbation of PC regeneration in E14 fetuses was not affected by ATRA treatment (data not shown).

Although the mechanisms by which ATRA alters the regenerative pattern of PC remain obscure, our study revealed distinct expression patterns of RARα in E13 and E14 wounds and reduced migratory capacity of myoblasts in the presence of ATRA. Furthermore, a recent study reported that RA signaling is more active in quiescent than in activated satellite cells and that stimulation of RA signaling impairs the differentiation process of mouse muscle satellite cells.[26] In summary, on E13, RA ligand expression is low, and RAR expression is elevated only at the wound edge, which promotes myoblast migration and differentiation as well as muscle regeneration in the absence of a ligand. Conversely, from E14 onward, RAR expression is low, and muscle does not regenerate. We conjecture that in the presence of RA, myoblast migration and differentiation are inhibited, and PC regeneration does not occur, even on E13.

RA acts differently on myoblasts derived from normal limbs and limb blasts of amphibians. The effects of RA on proliferation, differentiation, and adhesion also differ between these cell types.[27] In addition, RA affects the arrest of myoblast proliferation and differentiation in MyoD-expressing cells.[28] These results suggest that RA acts differently depending on the differentiation stage of myogenic cells. We observed that RA-soaked beads differentially affected PC regeneration. We speculate that the PC muscle in E13 and E14 fetuses may be at distinct stages of differentiation and responds differently to retinoids.

While PC is a vestigial organ in humans, the superficial fascial system is considered to be an evolutionary remnant of PC.[29] Moreover, PC is reportedly present in some body parts, such as the heel.[30] Based on the reported importance of PC regeneration in the prevention, healing, and recurrence of chronic ulcers, the effect of RA on PC regeneration demonstrated in this study may be useful in chronic ulcer treatment in the future.[31]

A limitation of this study is that we only evaluated the effects of exogenous RA and did not address the regulation of endogenous RA and RAR expression during embryonic development. This point may be addressed in future studies by generating knockout mice and conducting wound-specific knockdown experiments using genetic manipulation.

In summary, our study demonstrated that PC regeneration during embryonic development switches between E13

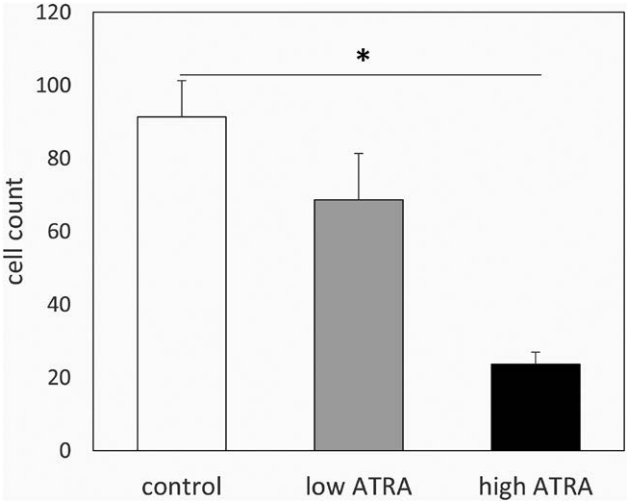

Fig. 1. Migratory ability of C2C12 cells in the presence of ATRA. *$P < 0.05$.

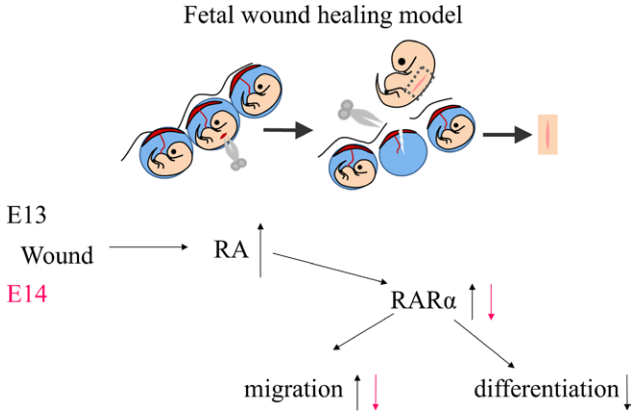

Fig. 2. Schema of the relationship between RA and RAR and PC regeneration in mouse fetuses.

and E14 and may involve RAR expression. Furthermore, our results indicate that exogenous ATRA exerts inhibitory effects on PC by acting on myoblasts. These findings may facilitate the development of therapies for the complete regeneration of wounds involving skeletal muscle.

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ACKNOWLEDGMENT

We acknowledge past and present members of our laboratory for their foundational work, helpful discussions, and comments to improve the manuscript.

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