The role of the activin system in keloid pathogenesis

Anandaroop Mukhopadhyay,1 Sui Yung Chan,1 Ivor J. Lim,2 David J. Phillips,5 and Thang T. Phan2,3,4

1Departments of Pharmacy, 2Surgery, and 3Bioengineering, and 4NUS Tissue Engineering and Stem Cell Research Program, National University of Singapore, Singapore; and 5Monash Institute of Medical Research, Monash University, Victoria, Australia

Submitted 10 July 2006; accepted in final form 4 September 2006

Mukhopadhyay A, Chan SY, Lim LJ, Phillips DJ, Phan TT. The role of the activin system in keloid pathogenesis. Am J Physiol Cell Physiol 292: C1331–C1338, 2007. First published September 13, 2006; doi:10.1152/ajpcell.00373.2006.—Keloid scars represent a pathological response to cutaneous injury under the regulation of many growth factors. Activin-A, a dimeric protein and a member of the transforming growth factor-β superfamily, has been shown to regulate various aspects of cell growth and differentiation in the repair of the skin mesenchyme and the epidermis. Thus our aim was to study the role of activin and its antagonist, follistatin, in keloid pathogenesis. Increased mRNA expression for activin was observed in keloid scar tissue by performing RNase protection assay. Immunohistochemistry showed increased localization of both activin-A and follistatin in the basal layer of epidermis of keloid tissue compared with normal tissue. ELISA demonstrated a 29-fold increase in concentration of activin-A and an ~5-fold increase in follistatin in conditioned media in keloid fibroblasts compared with normal fibroblasts. Although keloid keratinoctyes produced 25% more follistatin than normal keratinocytes, the amounts of activin-A, in contrast, was ~77% lower. Proliferation of fibroblasts was stimulated when treated with exogenous activin-A (46% increase in keloids fibroblasts) or following co-culture with hHAaCaT cells (66% increase). Activin-A upregulated key extracellular matrix components, namely collagen, fibronectin, and α-smooth muscle actin, in normal and keloid fibroblasts. Co-treatment of follistatin with activin-A blocked the stimulatory effects of activin on extracellular matrix components. These findings emphasize the importance of the activin system in keloid biology and pathogenesis and suggest a possible therapeutic potential of follistatin in the prevention and treatment of keloids.

Co-treatment of follistatin with activin-A blocked the stimulatory effects of activin on extracellular matrix components, namely collagen, fibronec-}

Keloid scarring is a dermal fibroproliferative disorder formed as a result of an aberration in the normal progression of healing. Keloids and hypertrophic scars may follow local skin trauma or inflammatory skin disorders like laceration, tattoos, burns, injections, ear piercing, vaccination, bites, acne, abscess, or surgery (28). A keloid scar extends beyond the confines of the original wound, does not regress spontaneously, grows in pseudotumor fashion with distortion of the lesion, and tends to recur after excision, whereas hypertrophic scars remain confined to the borders of the original wound and most of the time retain their shape (28). Keloid scars are found to occur in humans and are characterized by increased deposition of extracellular matrix (ECM), especially collagen. Although several treatment modalities have been explored (37) and many biomolecular pathways have been invoked as important in the pathogenesis of keloids, the exact mechanism underlying keloids remains elusive.

The bioactivity of fibroblasts in fibrogenesis or excessive scar formation is regulated by a number of growth factors. The transforming growth factor (TGF)-β family, comprising the TGF-βs bone morphogenetic proteins and activins, is thought to play a central role by regulating fibroblast proliferation, differentiation, and matrix production (7, 14, 29, 46). Although a series of studies have demonstrated the importance of TGF-β as a key player in keloid pathogenesis, the function and role of activin-A is yet to be established.

Activins, like other members of the TGF-β superfamily, are dimeric proteins consisting of two βA subunits (activin-A), two βB subunits (activin-B), or a βA and a βB subunit (activin-AB) (21, 48). Activin-A signals through heterodimeric complexes of two receptor types: type I receptors, also called activin receptor-like kinases, and type II receptors, which are transmembrane serine-threonine kinases (22). The biological action of activin-A is also regulated by follistatin, a soluble activin-binding glycoprotein, which inhibits activin’s function in vitro and in vivo (21, 35).

Activin-A was discovered initially as a regulator of pituitary function (49). Subsequent research has shown it to affect proliferation and differentiation of various cell types. For example, it has been shown to stimulate proliferation of lung fibroblasts and their differentiation into myofibroblasts (33). It has also been demonstrated to play a role in the proliferation and differentiation of keratinocytes in vitro (42). Activin-A inhibits proliferation of vascular endothelial cells (23, 25, 16) and has been shown to cause cell death in primary hepatocyte cultures (41), B cell hybridomas, and mouse and human myeloma cells (30, 31).

The first evidence for a role of activin-A in wound healing came from the studies of Hübner et al. (12). They demonstrated an increased induction of activin-A expression within 24 h after injury, which remained high until the end of the repair process. Transgenic mice, which overexpress the activin-βA subunit specifically in the epidermis, have epidermal hyper-thickening and dermal fibrosis (27). After skin injury, these mice show enhanced granulation tissue formation with strong deposition of ECM below the hyperproliferative keratinocytes. By contrast, reduced granulation tissue formation and thus delayed healing were observed in mice overexpressing the activin antagonist, follistatin, in the epidermis (50).

Several studies have implicated activin-A as an important player in fibrotic disorders. Sugiyma et al. (45) demonstrated an upregulation of activin-A in cirrhotic and fibrotic rat livers.

Address for reprint requests and other correspondence: T. T. Phan, Dept. of Surgery, National Univ. of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (e-mail: surptt@nus.edu.sg).

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Furthermore, there was an increased expression of activin-A in various pulmonary conditions associated with interstitial pulmonary fibrosis (24). Activin-A has also been reported to act as a profibrotic cytokine in renal disease (10). Thus, although in vitro studies suggest that activin-A may play a role in repair of the mesenchyme and epithelium, a disruption in the control of activin-A expression could lead to the development of tissue fibrosis.

The present study characterized the role of activin-A synthesis and signaling in keloid pathogenesis. It evaluated the activin-follistatin system in regulating cellular proliferation and expression of ECM components in keloid scars by using an extensive array of techniques, such as RNAse protection assay, immunohistochemistry, and co-cultures. Overall, the expression and production of activin-A and its biological regulator follistatin were perturbed in the development of human keloids.

**MATERIALS AND METHODS**

**Keloid Keratinocyte and Fibroblast Database**

Keratinocytes and fibroblasts were randomly selected from a specimen bank of keratinocyte/fibroblast strains derived from excised keloid specimens. All patients had received no previous treatment for keloids before enrollment. Before informed consent was obtained, a full history was taken and an examination performed, complete with photographic documentation. Approval by the National University of Singapore-Institutional Review Board was obtained for this study.

**Table 1. Details of normal and keloid skin tissues/fibroblasts/keratinocytes**

<table>
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<tr>
<th>Tissue/Fibroblasts/Keratinocytes</th>
<th>M/F</th>
<th>Race</th>
<th>Age of Donor, yr</th>
<th>Origin</th>
<th>Age of Scar, yr</th>
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<td>Leg</td>
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</table>

M, male; F, female; NS, normal skin; KS, keloid scar; NF, normal fibroblast; KF, keloid fibroblast; NK, normal keratinocytes; KK, keloid keratinocytes.
Cell Culture

Keratinocyte-fibroblast monocultures. Keratinocytes and fibroblasts were harvested, and monocultures of these primary cells were set up in a manner mentioned previously by Lim et al. (18).

Keratinocyte-fibroblast co-cultures. Cells overexpressing the βA subunit of activin and vector-transfected HaCaT cells with only the resistance plasmid (provided by S. Werner, Zurich, Switzerland) were seeded at a density of 1 × 10^4 cells/ml in DMEM/10% FCS containing penicillin/streptomycin (25 units each). The cells were raised to the air-liquid interface to allow the keratinocytes to stratify and reach terminal differentiation.

Normal fibroblasts obtained from randomly selected normal strains were seeded in six-well plates at a density of 1 × 10^4 cells/ml in DMEM/10% FCS for 24 h and then in serum-free medium for another 48 h.

Cells on both the membrane inserts and in wells, were washed twice with PBS to remove the old medium before combination of the inserts and plates for co-culture in serum-free DMEM. Controls comprised of one series of non-co-cultured normal fibroblasts.

Treatment of Normal and Keloid Fibroblasts With Activin-A, Follistatin, or TGF-β1

Normal and keloid fibroblasts from different patients were seeded in six-well plates at a density of 1 × 10^4 cells/ml in DMEM/10% FCS for 24 h and then in serum-free medium for another 48 h. The cells were subsequently treated with varying concentrations of activin-A (100 ng/ml, R&D Systems; follistatin, FS-288, 100 ng/ml, R&D Systems; or TGF-β1, 5 or 10 ng/ml, R&D Systems) to test the effect of these growth factors on the proliferation of fibroblasts and expression of key ECM proteins. The fibroblasts were also treated with both activin-A and TGF-β1 concurrently to explore any possible synergistic effects. Cells without serum were used as controls. After 24 h, some fibroblast cultures were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the remainder were washed with PBS and stored at −80°C for protein and RNA extraction for Western blot and RNase protection assays, respectively.

Western Blot

Untreated normal or keloid fibroblasts and those treated with activin-A, follistatin, or TGF-β1 were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 100 mM NaCl, 0.5% Nonidet P-40, and 1 mg/ml protease inhibitor cocktail (Antipain-dihydrochloride, Aprotinin, Bestatin, Chymostatin, E-64, EDTA-Na, Leupeptin, Pefabloc-SC, Pefstatin, Phosphormidon; Boehringer Mannheim, Mannheim, Germany), and 100 μg of extracted protein were electrophoresed on 8 and 14% sodium dodecyl sulphate-polyacrylamid-
Fig. 5. Increased proliferation of normal and keloid fibroblasts treated with rhActivin-A. Cultures of normal (A and B) or keloid fibroblasts (C and D) were grown until 50% confluence and then serum starved for 48 h. The fibroblasts were then treated with activin-A (100 ng/ml; filled bars), TGF-β1 (1 ng/ml; hatched bars), TGF-β1 (10 ng/ml; shaded bars), then subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay. Untreated samples were used as control (open bars). Values are means ± SD absorbance at 570 nm as a percentage of control.

Fig. 6. Effect of activin-A, follistatin, or TGF-β1 on collagen, fibronectin, and α-smooth muscle actin (SMA) expression. Normal fibroblasts (NF) or keloid fibroblasts treated with activin-A (100 ng/ml). Lane 1: untreated KF; Lane 2: NF + activin-A; Lane 3: NF + TGF-β1; Lane 4: NF4 + follistatin; Lane 5: KF + activin-A; Lane 6: KF + follistatin + activin-A; Lane 7: KF + TGF-β1; Lane 8: KF + follistatin. All of the blots were probed with a β-actin antibody to confirm equal loading. For conditioned media, we concentrated 4 ml of conditioned media from each treatment group to 100 μl and used this 100 μl of sample for analysis. Histograms represent % OD volume of the bands in the corresponding blots. Filled bars, collagen; hatched bars, α-SMA; open bars, fibronectin. G: Lane 1: Untreated KF. Lane 2: KF + activin-A. Lane 3: KF + TGF-β1. Lane 4: KF + activin-A (100 ng/ml) + TGF-β1 5 ng/ml. H: Lane 1: untreated KF. Lane 2: KF + activin-A 100 ng/ml. Lane 3: KF + activin-A 50 ng/ml. Lane 4: KF + activin-A (100 ng/ml) + TGF-β1 (5 ng/ml). Lane 5: KF + TGF-β1 (10 ng/ml).

Immunohistochemistry

Paraffin sections were dewaxed and antigens retrieved by immersing slides in 0.01 M citrate buffer, pH 6.0, heating in a microwave oven (high for 2.5 min, low for 5 min), cooling at 4°C for 20 min, and washing in water for 5 min. Endogenous peroxidase was blocked in 3% H2O2, and nonspecific binding blocked for 1 h (CAS block, Zymed Laboratories, San Francisco, CA). Sections were incubated with antibodies specific for the activin-βA, activin-βA subunit or follistatin primary antibodies, respectively. Reaction product was developed with 3,3′-diaminobenzidine tetrahydrochloride substrate kit (Zymed), and sections were counterstained with hematoxylin. All wash steps were in TBS/0.05% Tween-20. Antibodies were diluted in 1% BSA/TBS. Nonimmune mouse antibody of the appropriate immunoglobulin isotype was used for negative controls.

Statistics

Statistical significance between groups was assessed using either Student’s t-test or a one-way analysis of variance with Tukey’s post hoc test. All tests were performed using MiniTab software. Except for ELISA, where mean value of data from five different cell populations in each group was used, in all other results the cell population representative of other populations studied had been shown.

curve that was parallel to the standard. The average intra-assay %CV was 7.7% (n = 3 assays), the inter-assay %CV was 6.4%, and the limit of detection was <1.94 ng/ml.

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RESULTS

Increased Activin-A and Follistatin in the Basal Layer of Epidermis in Keloid Tissue

Both activin-A and follistatin were found to be localized to the basal layer of the epidermis in both normal and keloid skin. However, an increased intensity of these proteins was observed in keloid tissue compared with normal skin tissue (Fig. 1). This suggests that the activin-follistatin regulatory system might be implicated in keloid pathogenesis (Table 1).

Keloid Scars Express Higher Levels of Activin-A mRNA Compared With Normal Skin

The expression profile of activin-A was studied using a RNase protection assay in keloid and normal skin tissues (Fig. 2). Normal skin and treated scars expressed very low levels of activin-A mRNA. In contrast, keloid scar samples had abundant levels of activin-A mRNA.

Increased Production of Activin-A and Follistatin by Keloid Fibroblasts

When cultured in isolation, keloid fibroblasts demonstrated a 29-fold higher secretion of activin-A compared with normal fibroblasts ($P < 0.0001$; Fig. 3A). In contrast, normal keratinocytes produced significantly higher activin-A than keloid keratinocytes ($P < 0.0001$; Fig. 3A). For follistatin, there were significant differences in secreted levels between keloid and normal keratinocytes ($P = 0.019$; Fig. 3B); keloid fibroblasts demonstrated a fivefold increase in secreted levels of follistatin compared with normal fibroblasts ($P < 0.0001$; Fig. 3B).

Overexpression of Activin-A in HaCaT Keratinocytes Increases the Proliferation of Underlying Fibroblasts During Co-culture

As the activin-follistatin regulatory system was shown to be upregulated in keloid tissues, we determined whether HaCaT keratinocytes overexpressing activin-A (hßAHaCaT) modulated underlying fibroblasts in a paracrine manner. Normal human dermal fibroblasts co-cultured with these overexpressing hßAHaCaT keratinocytes demonstrated a 66% increase in proliferation compared with non-co-cultured normal fibroblasts ($P < 0.0001$; Fig. 4). In comparison, normal fibroblasts co-cultured with neoHaCaT cells (cells transfected with the empty plasmid) demonstrated only a 30% increase ($P < 0.0001$; Fig. 4).

Activin-A Increases Proliferation in Normal and Keloid Fibroblasts and Stimulates Expression of ECM Components

Given that keratinocytes overexpressing activin-A stimulated the proliferation of underlying fibroblasts, the direct effect of activin-A on proliferation and ECM production were tested. Treatment of normal and keloid fibroblasts with activin-A (100 ng/ml) resulted in a 23% ($P < 0.001$) and 46% ($P < 0.0001$) increase, respectively, in proliferation compared with untreated controls (Fig. 5). This was comparable to the proliferation brought about by treatment with TGF-ß1, although at a lower dosage (10 ng/ml).

We next determined whether activin-A and follistatin affected the production of ECM components like collagen, fibronectin, and α-SMA in normal and keloid fibroblasts. Using Western blotting, an increase in collagen and α-SMA was observed in both normal and keloid fibroblasts treated with activin-A compared with untreated cells (Fig. 6, A–D, G, H). Elevated collagen and fibronectin levels in the conditioned medium in response to activin-A was confirmed in keloid fibroblasts (Fig. 6, E and F). To assess the neutralizing ability of follistatin in this system, exogenous follistatin was co-administered with activin-A, which resulted in a marked reduction in the expression levels of collagen and α-SMA. Follistatin treatment alone, unlike activin-A, significantly reduced the secreted levels of collagen, strongly suggesting that follistatin was blocking the stimulatory effects of endogenously produced activin-A (Fig. 6E). Activin, when combined with TGF-β, did not show significant difference in expression of both collagen and α-SMA compared with activin only treatment (Fig. 6, G and H).

DISCUSSION

The wound healing process involves a complex interplay of cells, mediators, growth factors, and cytokines, leading to inflammation, cell proliferation, ECM deposition, contraction, and remodelling. An abnormality and disruption of this sequence of events led to formation of keloid scars, characterized by excessive ECM deposition and often formation of contractions.

Recent advances in this field have seen the role of growth factors being extensively investigated. Of these plethora of growth factors, the TGF-β superfamily of growth factors plays a crucial role in scar formation. Activin-A, a member of the TGF-β superfamily, has been detected in high levels in various inflammatory (13) and fibrotic disorders (10, 12, 24, 45). To confirm a role in keloid formation, we showed in the present study that activin-A was indeed overexpressed in keloid tissues. For example, immunolocalization of activin-A was shown in the basal layers of the epithelium in human skin tissue, but interestingly keloid tissue showed more intense immunolocalization compared with normal tissue. Since epithelial-mesenchymal interactions have recently been explored as a possible important factor contributing to excessive scar formation, in our experimental set up, although there is no staining for the fibroblasts, one cannot rule out the paracrine effect of activin-A expressed by keloid keratinocytes on keloid fibroblasts.

The activin-follistatin system is an important regulatory system controlling cellular proliferation and differentiation in many epithelio-mesenchymal organs, including the kidney, prostate, mammary gland, lung, pancreas, and salivary gland (1, 4, 9, 11, 19, 20, 26, 38, 40, 47, 53, 56). When we investigated the localization of follistatin in keloid and normal tissues, we found it to have a similar pattern to that of activin. The increase in follistatin in the basal layer of keloid tissue is likely due to a short-loop negative feedback mechanism operative between activin-A and follistatin (34). Taking into consideration the localization and expression of both activin-A and follistatin in the basal layer of the epidermis, where mitotically active keratinocytes are present, one could hypothesize the activin-follistatin system modulates keratinocyte dynamics in tissue repair and keloid pathogenesis (52).
Actinin-A, in addition to being a potent activator of fibroblasts, has also been shown to have an antiproliferative effect on various types of cells, including epithelial cells, lymphocytes, prostate cancer cells, and others (5). However, it has been observed that many carcinoma cells are somehow able to escape the negative regulatory action of activin-A by activating production of follistatin (8, 39). In our study, we observed that, although activin-A was secreted in large amounts by keloid fibroblasts when compared with normal fibroblasts, keloid keratinocytes secreted less. Interestingly, keloid keratinocytes secreted more follistatin than normal keratinocytes, suggesting that a mechanism similar to that seen in carcinoma cell types might be active in keloid keratinocytes, where these cells were secreting more follistatin to neutralize the activin-A.

Previous work has demonstrated a large amount of ECM deposition below activin-A overexpressing keratinocytes (27), suggesting that an autocrine, paracrine, and/or endocrine effect of activin-A might be active in the process of wound healing. In our present study, we co-cultured activin-A overexpressing keratinocytes (hβAHaCaT cells) with normal fibroblasts. An increased proliferation in fibroblasts exposed to hβAHaCaT cells was observed compared with those exposed to both non-co-cultured and neo HaCaT cells. This could be due to the increased paracrine levels of activin-A in the hβAHaCaT/NF co-culture system compared with normal keratinocytes cocultures. Thus this data demonstrate that activin-A secreted from keratinocytes has a paracrine effect on the fibroblasts and that an excess of activin-A might lead to overproliferation of these cells, thus leading to fibrosis. While analyzing this data, one has to take in account that HaCaT cells in which activin-A has not been overexpressed has an endogenous level of this protein and several other proliferative factors like IGF-1, TGF-β, interleukins, and others; hence one would expect them to also increase the proliferation of the fibroblasts. Other groups have also observed such effects when co-culturing with neo HaCaT cells. Skobe et al. (43) observed that, when cocultured with HaCaT neo cells, fibroblasts show a 75% increase in cell number.

As further substantiation of a pro-proliferative effect of activin-A on human dermal fibroblasts, exogenous activin-A increased proliferation of both normal and keloid fibroblasts, consistent with results from other groups (33, 54). Since TGF-β1 is known to be an important player in fibrotic disorders and has been demonstrated to be highly expressed in keloid fibroblasts (17, 36, 55), we tested whether activin-A and TGF-β1 might act synergistically to stimulate fibrotic processes. However, co-treatment of cells with both activin-A and TGF-β1 had no significant synergistic effect.

ECM components are involved in many cellular processes, and their pattern of expression is different from that usually present in normal skin during different stages of wound healing (6). Tissue fibrosis is thought to be partly the result of excessive induction by TGF-β1, which signals fibroblasts to increase the synthesis of ECM, decrease the production of matrix-degrading proteases, and increase the production of the inhibitors of these proteases (2). Normal and keloid fibroblasts, when exposed to activin-A, increased expression of cytoplasmic collagen (procollagen). Procollagen further undergoes posttranslational modifications (hydroxylation, glycosylation), subsequently forms a triple helix, and is secreted out of the cell into the conditioned medium. We observed that there was an increased concentration of secreted collagen in the conditioned media from keloid fibroblasts when treated with activin-A, underscoring the importance of activin-A in the regulation of collagen production by dermal fibroblasts in keloid pathogenesis.

In addition, there was an increase in the expression of α-SMA, a phenotypic marker for myofibroblasts (54), suggesting activin’s importance in regulating expression and contractile activity of α-SMA. Not surprisingly, exogenous follistatin had no effect on the expression of ECM components directly but blocked the activin-induced increases. Follistatin antagonizes the effect of activin-A by binding to activin itself, rendering it inactive (34, 44).

In summary, the present study demonstrated that activin-A was upregulated in keloid tissues. Keratinocytes overexpressing activin-A stimulated proliferation of dermal fibroblasts, suggesting a paracrine role to promote fibroblast proliferation, induce the expression of α-SMA, and enhance collagen formation in keloid fibroblasts. However, follistatin, an antagonist of activin-A, blocked the expression of ECM components. Thus our findings strongly suggest that activin-A is a potent inducer of fibroblast activation and involved in the pathogenesis of keloids. They also emphasize the importance of follistatin in regulating activin-A bioactivity and suggest a possible therapeutic potential of follistatin in the treatment and prevention of keloids.

ACKNOWLEDGMENTS

We thank Anne O’Connor, Sue Hayward, and Kim Sebire for excellent technical assistance. We thank Professor Sabine Werner for the provision of activin-A overexpressing HaCaT cells and valuable suggestions. We also acknowledge and thank the assistance and suggestions provided by Professor Michael Longaker.

GRANTS

M. Anandaraoop is supported by a scholarship from the National University of Singapore. This work was supported by grants from the Biomedical Research Council, Singapore (03/1/21/19/251, 04/1/21/19/338, and 05/1/21/19/390) and the National Health and Medical Research Council of Australia (Regkey 334011 to D. J. Phillips).

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