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# Expression of activin A in human keratinocytes at early stages of cultivation

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**Abstract** **Activins are members of the TGF-β superfamily and are classified into 3 types: activin A, which consists of a homodimer of $\beta_A$, activin B, which consists of a homodimer of $\beta_B$, and activin AB, which consists of a heterodimer of $\beta_A\beta_B$. We studied the expression of activin mRNAs by RT-PCR in normal human epidermis, cultured keratinocytes, and DJM-1 cells (a squamous cell carcinoma line). We could detect only activin A mRNA ($\beta_A$) in normal human epidermis. In cultured keratinocytes and DJM-1 cells, activin $\beta_A$ mRNA was observed at 4 h but not at 96 h after plating. Activin A activity was detected in the conditioned medium of DJM-1 cells within 48 h. In addition, although follistatin mRNA was not observed in human epidermis in situ, it was transiently expressed in cultured cells at 4 h after plating. These findings suggest that the expression of these molecules in keratinocytes is associated with cell proliferation. In an in vitro tissue injury model, activin A was observed at the wound edge, where cell migration and proliferation may be activated. In DJM-1 cells cultured for 92 h, $\beta_A$ mRNA was observed 4 h after injury treatment. These findings suggest that activin A acts as a potent inducer of proliferation in vitro, at least in keratinocytes.**

*Key words:* Wound healing; Growth factor

## 1. Introduction

Activin, a member of the TGF-β superfamily that was originally purified from ovarian fluids, stimulates secretion of follicle-stimulating hormone from the anterior pituitary gland [1,2]. Erythroid differentiation factor (EDF) was independently isolated from human monocytic leukemia cells [3]. Molecular cloning of cDNA has shown that activin and EDF are identical. Activins have been identified as homo- or heterodimers of the $\beta_A$ and $\beta_B$ chain and have been classified into three types: activin A, consisting of $\beta_A\beta_A$, activin AB, consisting of $\beta_A\beta_B$, and activin B, consisting of $\beta_B\beta_B$. Furthermore, β-chains can also form heterodimers with distantly related α subunits that generate inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$), which inhibit activin [1,2].

Follistatin, an inhibitor of follicle-stimulating hormone secretion from the pituitary gland, is an activin-binding protein [4]. The interaction between activin A and follistatin appears to inhibit significantly activin A activity in extragonadal tissues [5].

Activins are involved in erythroid differentiation [6], regulation of neural differentiation [7,8], stimulation of glycogenolysis in hepatocytes [9] and mesoderm induction [10,11]. Activin A is also expressed in arteriosclerotic lesions [12]. However, little is known about the role of activins in skin. Roberts et al. [13] reported that activin $\beta_A$ is expressed in the developing hair bulb and also in the dermal layer of embryonic skin, but not in adult skin. Since TGF-β modulates proliferation and differentiation [14], it is conceivable that activins also have such functions in keratinocytes. We investigated expression of activins in human epidermis, a non-differentiating squamous cell carcinoma cell line (DJM-1 cells), and cultured normal keratinocytes at the protein and mRNA level.

## 2. Materials and methods

### 2.1. Cell culture

An isolated cell line (DJM-1 cells) from human skin squamous cell carcinoma [15,16] was cultured in 1.8 mM $Ca^{2+}$ medium containing Eagle's minimum essential medium (MEM), 10% fetal calf serum, 100 μg/ml of streptomycin and 100 U/ml of penicillin. Normal human keratinocytes obtained from foreskin (Kurabo, Osaka, Japan) were cultured in keratinocyte-SFM (modified MCDB medium supplemented with 5 ng/ml of epidermal growth factor, 50 μg/ml of bovine pituitary extract, 50 μg/ml of gentamicin, and 0.25 μg/ml of amphotericin B) (Life Technologies Inc., Grand Island, NY). The medium was changed every 2–3 days. After reaching confluence, the cells were treated with Hank's balanced salt solution containing 0.02% trypsin and 0.02% EDTA for 15 min at 37°C, and were then resuspended in the culture medium. The third passage of human keratinocytes was used for experiments. Both DJM-1 cells and normal keratinocytes were seeded in 60-mm dishes at a density of $1\times10^5$ cells/5 ml of culture medium in the presence of 0.09 mM (low-dose) or 1.8 mM (normal-dose) calcium, since it is known that a low concentration of calcium in the medium induces proliferation and normal concentration induces differentiation [17,18] for keratinocytes. They were cultured at 37°C in humidified 5% $CO_2$/95% air.

### 2.2. Reverse transcription polymerase chain reaction (RT-PCR)

Messenger RNA was extracted from human epidermis and DJM-1 cells and normal keratinocytes cultured in MEM containing 0.09 or 1.8 mM calcium using a kit for mRNA purification (QuickPrep, Pharmacia AB, Uppsala, Sweden) [19]. The epidermis was separated from skin biopsy specimens by incubation with 1000 U/ml of dispase (Godo Shusei Co., Tokyo, Japan) at 37°C for 2 h or at 4°C overnight. Complementary DNA was synthesized from mRNA, and PCR was performed using an RNA PCR (AMV) kit (Takara Shuzo Co., Ltd., Otsu, Japan). The oligonucleotide primers used were based on the DNA sequence of rat activin subunits [20,21]. The primer for inhibin α, 5′-TGACTTCAGCCCAGCTGTGG-3′, was used together with a reverse primer, 5′-GGCACCAAAAACAGGGGCTG-3′ corresponding to the sequence $Val^{127}$–$Pro^{322}$ to amplify a 586 base pair (bp) fragment. The primer for activin-$\beta_A$, 5′-GAACAGTGCCAGGAGAGCGG-3′, was used together with a reverse primer, 5′-AGGTTGGCAAAGGGGCTGTG-3′ corresponding to the sequence

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*Abbreviations:* TGF-β, transforming growth factor-β; RT-PCR, reverse transcription-polymerase chain reaction.


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Fig. 1. Immunofluorescence findings for activin A. (A) Activin A staining was enhanced at the wound edge of DJM-1 cells after 92 h culture and subsequent 4 h cultivation. (B) Marked reduction of activin A staining by preabsorption of the antibody with human activin A. (C) Activin A was not stained in most cells cultured for 96 h without injury treatment. Arrowheads indicate the wound edge. Bar: 25 μm.

$Glu^{245}$–$Leu^{385}$ to amplify a 423-bp fragment. For activin-$\beta_B$, the primer 5′-GTGGTGCCTGTGTTCGTGGA-3′ and a reverse primer, 5′-TTCACCGGCCCAGGGTTCAG-3′ corresponding to the sequence $Val^{240}$–$Asu^{349}$ were used to amplify a 330-bp fragment. Amplification was performed for 30 cycles of 1 min at 94°C, 30 s at 65°C, and 2 min at 72°C. For follistatin, the oligonucleotide primers were based on the DNA sequence of human follistatin [5,22]. The sense primer, 5′-CGTCGAGGATCCCTGGGAACTGCTGGCTCCGTCAAGC-3′ (the 113–137 oligonucleotide position of human follistatin and an extra *Bam*HI restriction enzyme site) was combined with an antisense primer, 5′-CCTAGAAGCTTGCCCAGCAGGCAGGTAGCCTTTCTC-3′ (the 688–712 position and an *Hin*dIII site), to generate a putative 622-bp cDNA fragment. Amplification was performed for 30 cycles of 1 min at 94°C, 2 min at 55°C, and 5 min at 72°C. PCR products were separated on 2% agarose gel and visualized with ethidium bromide.

*2.3. Tissue injury model of cultured keratinocytes*

To examine the effect of tissue injury on the expression of activin A, we scraped off some cultured cells and examined expression of activin $\beta_A$ mRNA in the remaining cells by RT-PCR. This treatment is considered to be an in vitro model of tissue injury that induces cell migration and proliferation [23]. Briefly, three 3-mm wide strips were marked with a razor blade on a dish of DJM-1 cells cultured for 92 h, and the cells in the strips were removed with a cell scraper. The remaining cells were cultured for a further 4 h and harvested for RNA extraction. Localization of activin A was also studied by immunofluorescence microscopy. Coverglass slips were placed on the dish and DJM-1 cells or normal keratinocytes were cultivated for 92 h. Cells in the strip on coverglass slips were scraped off with a cell scraper as described above, and the remaining cells were cultured for a further 4 h. Cells adhering to the coverglass slips were fixed with 3% paraformaldehyde in PBS. The fixed cells were soaked in a solution of 0.05% Triton X for 10 min at room temperature, incubated with anti-activin A antibody [21]. The antibody reacts with both the dimer and monomer of the $\beta_A$ subunit, but does not crossreact with inhibin A, a heterodimer of α and $\beta_A$ [2]. After 30 min, sections were washed with PBS, incubated with fluorescein-conjugated goat anti-rabbit IgG (1:100, Cappel, Durham, NC), washed again with PBS, and observed under an immunofluorescence microscope (Nikon FX, Nikon, Tokyo, Japan).

*2.4. Determination of activin A activity in the culture medium*

Activin A activity was estimated by measuring activin A-induced erythroid differentiation of F5-5 cells [3,7,6]. Normal keratinocytes or DJM-1 cells were incubated ($1\times10^5$ cells in 5 ml of MEM+10% FBS/60-mm culture dish) for 48 h and the culture medium was collected. The cells were then cultured in new culture medium for 48 h and the medium was again collected. F5-5 cells ($5\times10^3$ cells/ml) were plated onto 96-well microtiter plates in Ham F-12 medium containing 10% FCS with or without 30 μg/ml of porcine follistatin. After 6 days, the degree of erythroid differentiation was determined microscopically by counting the number of dianisidine-stained cells; the result is expressed as the percentage of cells. The activin A activity in the conditioned medium was determined based on a dose-dependent differentiation curve using various concentrations of recombinant activin A.

## 3. Results

*3.1. Localization of activin A in tissue injury model in cell-culture system*

Marked staining for activin A was observed at the wound edge of DJM-1 cells (Fig. 1A) in the in vitro model of tissue injury. Preabsorption of the antibody with an excess of purified human activin A (10 μM) markedly reduced the intensity of staining at the wound edge (Fig. 1B) and no activin A staining was observed in cells not subjected to injury treat-

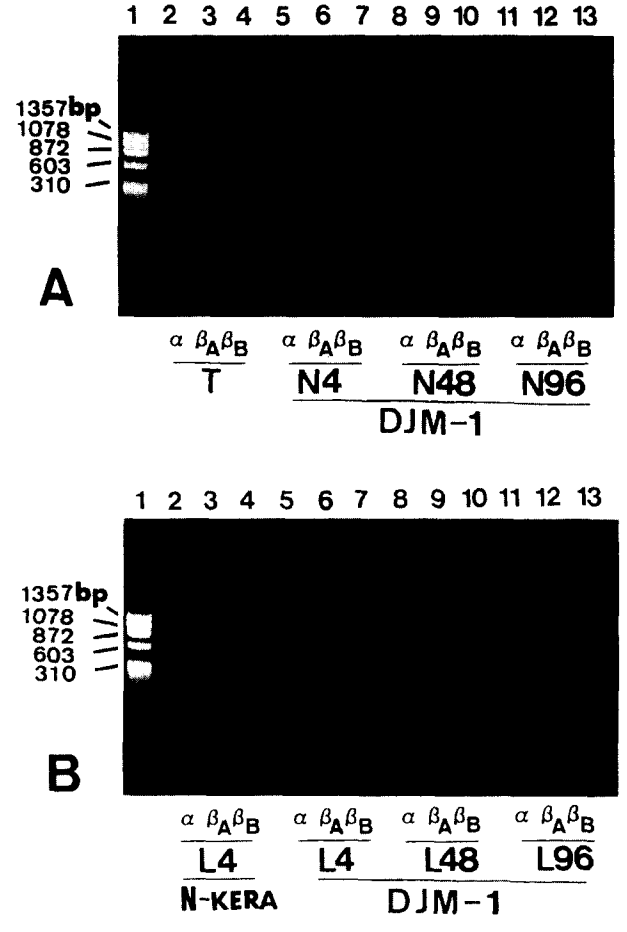


Fig. 2. (A) RT-PCR with mRNA for inhibin α, activin $\beta_A$ and $\beta_B$ subunits in normal human epidermis (lanes 2–4) and in DJM-1 cells cultured for 4 h (lanes 5–7), for 48 h (lanes 8–10) and for 96 h (lanes 11–13) in normal calcium medium. Expression of activin $\beta_A$ mRNA (423 bp) was detected in lanes 3,6. T, epidermis; N, normal calcium medium. (B) RT-PCR with mRNA for inhibin α, activin $\beta_A$ and $\beta_B$ subunits in normal keratinocytes (lanes 2–4) and in DJM-1 cells cultured for 4 h (lanes 5–7), for h (lanes 8–10) and 96 h (lanes 11–13) in low calcium medium. Activin $\beta_A$ mRNA was detected in lanes 3,6,9. L, low calcium medium: N-KERA, normal keratinocytes. Lane 1 in (A,B) indicates the DNA size marker (φ174/DNA digested with *Hae*III).

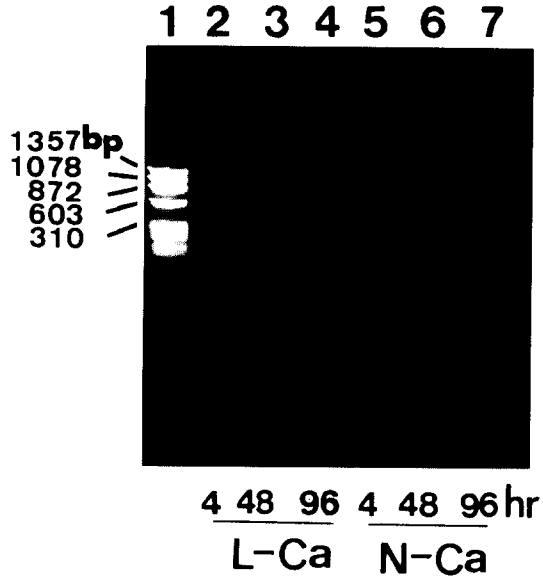


Fig. 3. Expression of mRNA for activin $\beta_A$ (423 bp) in normal human keratinocytes cultured for 4 h (lanes 2,5), 48 h (lanes 3,6) and 96 h (lanes 4,7) in low calcium medium (lanes 2–4) and in normal calcium medium (lanes 5–7). The DNA size marker (φ174/*Hae*III digest) is shown in lane 1. L-Ca, low calcium medium; N-Ca, normal calcium medium.

ment (Fig. 1C). Similar findings were observed in a tissue injury model using cultured normal human keratinocytes (data not shown).

### 3.2. *Expression of mRNAs for $\alpha$,$\beta_A$ and $\beta_B$ subunits in cultured keratinocytes and in human epidermis*

Although PCR products corresponding to mRNA for $\alpha$ and $\beta_B$ subunits were not observed, the expected PCR product corresponding to mRNA for the $\beta_A$ subunit (423 bp) was detected in human epidermis, cultured human keratinocytes, and DJM-1 cells. Expression of the $\beta_A$ subunit was observed at 4 and 48 h, but not at 96 h in DJM-1 cells cultured with low calcium. In cells cultured with 1.8 mM calcium, the PCR product for $\beta_A$ was detected only at 4 h and not at 48 or 96 h (Fig. 2). The same results were obtained in normal human keratinocytes, although the PCR product band was faint at 48 h in both low and normal calcium medium (Fig. 3). The PCR product for follistatin (622 bp) was not detected by RT-PCR in human epidermis. However, PCR products were detected at 4 h in human keratinocytes cultured with low and normal calcium. In DJM-1 cells, the PCR product for follistatin was detected only at 4 h in the normal calcium MEM, and remained detectable for up to 48 h in cells cultured with low calcium (Fig. 4).

### 3.3. *Re-expression of mRNA for activin $\beta_A$ and follistatin after tissue injury in the cell culture system*

In DJM-1 cells, the expected PCR products for activin $\beta_A$ (423 bp) and follistatin (622 bp) were detected at 4 h after injury treatment; these findings were not affected by the calcium concentration in the medium. Trypsin treatment (0.02%) for 20 min also induced expression of mRNAs for activin $\beta_A$ and follistatin (Fig. 5). Uninjured cells did not induce mRNA for follistatin (lanes 3,5) or $\beta_A$ (lanes 8,10).

### 3.4. *Existence of activin A activity in conditioned medium of DJM-1 cells*

The activin A activity in DJM-1 cells cultured for 2 days in the low calcium medium was determined with the F5-5 bioassay. Endogenous activin A activity was completely neutralized by 30 μg/ml of follistatin and corresponded to 8 ng/ml of recombinant activin A. Minimal activin A activity (less than 1 ng/ml) was detected in the normal calcium medium. Activity was not detectable when cells were cultured for an additional 2 days. Surprisingly, activin A activity was not detected in normal human keratinocytes (less than 0.5 ng/ml) under the same culture conditions used for DJM-1 cells.

## 4. Discussion

Activin A is found extensively in various organs, such as the ovary [2], pancreas [21], liver [9], while the distribution of inhibins is limited to the gonads and endocrine organs. In pancreatic islets, activin A coexists with glucagon in secretory granules in A-cells and with somatostatin in D-cells. Activin A has been found to stimulate the secretion of insulin in the presence of 2.8 mM glucose [21]. Activin A also stimulates glucose output by glycogenolysis in rat hepatocytes [9]. However, activin A has a mitogenic effect on osteoblastic cells (MC3T3-E1), especially in their undifferentiated state, and a suppressive effect on alkaline phosphatase activity, which is a marker of the differentiation of osteoblastic cells [5]. Activin A also inhibits neural and glial differentiation of undifferentiated P19 cells [7] and promotes the survival of P19 cells [8].

In the present study, RT-PCR showed expression of mRNA for the activin $\beta_A$ subunit, but not for $\beta_B$ and inhibin $\alpha$ subunits, in the epidermis. These observations indicate that activin A, a homodimer of $\beta_A$, exists in normal human epidermis. Hübner et al. [24] investigated the role of activin A in wound healing using an animal model. They detected low levels of mRNAs for activin $\beta_A$ and inhibin $\alpha$ in the dermis but not in the epidermis of mouse skin. They also detected abundant $\beta_B$ mRNA in the dermis and a lower level in the epidermis. These results are not consistent with the present findings in human

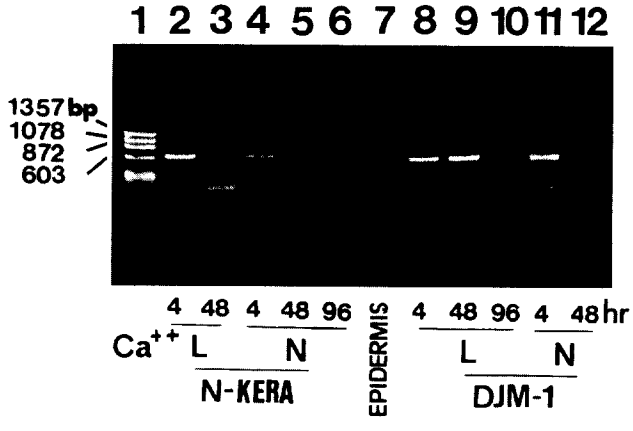


Fig. 4. Expression of mRNA for follistatin (622 bp) at a relatively early stage of cultivation in both low and normal calcium media (positive in lanes 2,4,8,9,11) in cultured normal keratinocytes (lanes 2–6) and DJM-1 cells (lanes 8–12). Lane 1 indicates the DNA size marker (φ174/*Hae*III digest). L, low calcium medium; N, normal calcium medium; N-KERA, cultured normal keratinocytes; DJM-1, DJM-1 cells.

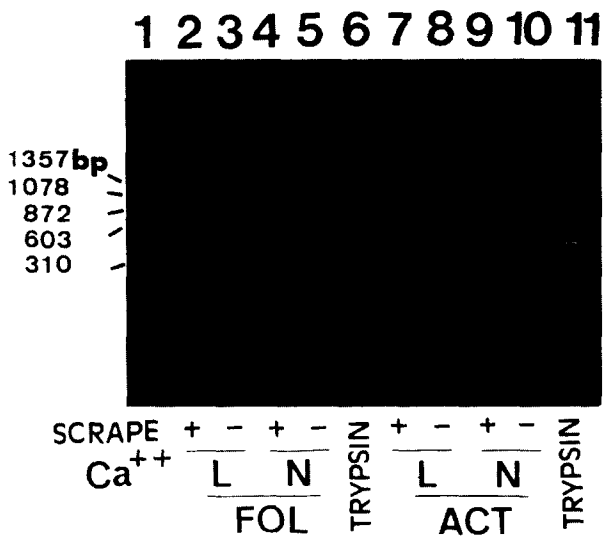


Fig. 5. Re-expression of mRNAs for follistatin (622 bp) (lanes 2,4) and activin $\beta_A$ (423 bp) (lanes 7,9) in injured DJM-1 cells. Lane 1 indicates the DNA size marker (φ174/*Hae*III digest). L, low calcium medium; N, normal calcium medium; FOL, PCR product for follistatin mRNA; ACT, PCR product for activin A mRNA.

epidermis. The reason for this discrepancy is not clear, but it may be due to species-related differences.

Cultured normal human keratinocytes expressed mRNA for only the $\beta_A$ subunit, indicating that they express activin A, but not activin AB or B. This finding is consistent with previous results in another epithelial cell line, FHs74Int cells derived from human embryonic intestine [25]. The $\beta_A$ mRNA was detected only in the early stage (4 h) after inoculation in both low and normal calcium media. On the other hand, DJM-1 cells, a squamous cell carcinoma cell line, also expressed $\beta_A$ mRNA at 4 h in the normal calcium medium and for up to 48 h in the low calcium medium. This prolonged expression for $\beta_A$ mRNA in the low calcium medium may be related to the effect of the low calcium condition on cell proliferation at least in DJM-1 cells. Activin A activity was detected in DJM-1 cells, but not in normal keratinocytes, suggesting that activin A produced by DJM-1 cells is secreted into the extracellular space in the early stage of cell proliferation. It is unclear why activin A activity was not detected in the conditioned media of normal keratinocytes. The presence of follistatin at the protein level may be related, although $\beta_A$ mRNA was detected in both DJM-1 cells and keratinocytes. The prolonged expression of activin A and its activity in DJM-1 cells may be related to the transformation and increased proliferative ability of these cells, which is consistent with the activin A-promoted survival of P12 cells [8]. Various biological events, such as cell division, cell migration, re-expression of lost adhesion molecules, and cytokine production, occur in freshly plated cells. Thus, we further investigated the effect of injury on the expression of the activin $\beta_A$ subunit using a cell culture system to confirm our results in freshly plated cells. This in vitro wounding model is a reasonable experimental model of keratinocyte migration because it has been shown that after half of the confluent keratinocyte monolayer is removed, the remaining keratinocytes apparently migrate from the cutting edge to an open space during incubation for 16 h [23]. RT-PCR demonstrated expression of activin $\beta_A$ mRNA at 4 h in wounded cells; activin A was also observed at the wound edge at the protein level. These in vitro results in wounded cells suggest that activin A is involved in the migration and/or proliferation of keratinocytes. Hübner et al. [24] observed a marked increase in activin $\beta_A$ mRNA during the repair of wounded mouse skin. However, they observed activin $\beta_A$ mRNA in the dermis, not in the epidermis, by in situ hybridization. This discrepancy about the origin of activin A may be due to differences between in vivo and in vitro experiments. It is possible that various cytokines and growth factors in serum may preferably induce the expression of activin A in dermal cells during wound repair in vivo. Finally, the co-expression of follistatin mRNA and activin $\beta_A$ mRNA in wounded DJM-1 cells in the present study suggests that follistatin modulates the function of activin A in keratinocytes.

In conclusion, activin A appears to play a role in the proliferating phase of wounded cells in vitro, at least in human keratinocytes. Further studies on the effect of activin A in human wounds at various stages of repair are needed to elucidate the function of activin A in keratinocytes.

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