Change in the Gastro-Intestinal Tract by Overexpressed Activin Beta A

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INTRODUCTION

Activin and inhibin, members of the TGF-β superfamily (Ling et al., 1986), were first identified as gonadal protein hormones that regulate the synthesis and secretion of follicle stimulating hormone (FSH) in the pituitary gland (Ling et al., 1985). Activin and inhibin are generated through the combinatorial assembly of an α subunit and one of two highly related β subunits, βα or βb, to generate inhibin A (αβα), inhibin B (αβb), activin A (βαβα), activin B (βαβb), and activin AB (βαβα). Activin A, B, and inhibin chains (Hotten et al., 1995; Oda et al., 1995; Vale et al., 1994), and partially characterized activin AC (βαβc) and activin BC (βαβc) proteins have also been reported (Fang et al., 1996).

The best known functions of activin are in the reproductive organs. The endocrine function of activin was first inferred from the correlation between high activin and elevated FSH in the mid cycle and luteo-follicular transition period (Muttukrishna et al., 1996). The endocrine function of activin has also been confirmed by the fact that activin βA induced intramuscularly increases FSH in an endocrine fashion during the estrous cycle (Kim et al., 2008). In addition, the autocrine function within reproductive organs has been inferred from the observation that antibodies to activin B suppress FSH secretion from cultured rat pituitary cells (Corrigan et al., 1991).

Outside the gonads, a main reproductive organ, activin βA has been reported to be involved in the regulation of the GI tract (Fukamachi et al., 2013; Li et al., 1998) and a GI cancer cell line (Kaneda et al., 2011; Kim et al., 2006; 2009). In inhibin-deficient mice, supraphysiological levels of activins block differentiation of pre PIT-1 acid-producing parietal cells. Activin βA mRNA is normally present in pit, parietal, and zymogenic cells (Li et al., 1998). Within the GI tract, activin A regulates growth of GI epithelial cells by mediating epithelial-mesenchymal interaction (Fukamachi et al., 2013). In gastric cancer cell lines, activin inhibits cell growth through apoptosis (Kim et al., 2006; 2009) and vascular endothelial cell growth (Kaneda et al., 2011).

Similar to the cell proliferation in GI tract, functions of activin are also known in other cells. In the ovary, activin is involved in granulosa cell proliferation through Cyp28b1 gene expression and retinoic acid regulation (Kipp et al., 2011). In cancer cells, activin A inhibits the proliferation of breast cancer T47D cells by enhancing the expression of p15 cyclin-dependent kinase inhibitors, and the overexpression of activin A in human prostate cancer LNCaP cells inhibits proliferation, induces apoptosis, and decreases the tumorigenicity of these cells (Burdette et al., 2005; Zhang et al., 1997). Activin A has been reported to be an essential growth factor involved in embryonic stem cell renewal and pluripotency (Jiang et al., 2007; Xiao et al., 2006). Studies of activin on in vivo cell proliferation, however, have been very limited. Initial studies were done intermittently using embryos and in vitro culture of eggs. Activin has been reported to be expressed in early pre- and post-implantation mouse embryos (Albano et al., 1993; Manova et al., 1992; Mellor et al., 1994).
and involved in the formation of the mesoderm (Feijn et al., 1994) and secondary body axes in chicks (Thomsen et al., 1990), zebrafish (Mitrani et al., 1990), and amphibians (Schulte-Merker et al., 1992). Activin A increases the rate of morula formation and velocity of embryonic cleavage in mice (Orimo et al., 1996). More recent studies have been done using gene disruption or transgenic animal approaches. However, there are few reports of the overall effects of activin, including cell proliferation, since perinatal lethality and early embryonic lethality have been observed (Matzuk et al., 1995; Tanimoto et al., 1999). In later organogenesis, activin Aβ has been reported to be associated with craniofacial development (Matzuk et al., 1995).

Our approach using intramuscular injection of naked plasmid bypasses the fundamental problems that come from perinatal lethality or early embryonic lethality (Ko et al., 2003). In this study, we report distinct activities of activin on the GI tract.

**MATERIALS AND METHODS**

**Animals and experimental design**

ICR and BALB/c mice at 2 months of age were purchased from DBL (Korea) and maintained under 14 h light, 10 h dark illumination at 23°C, with food and water available ad libitum. Plasmid DNA, pCMV-rAct, a 1.5-kb rat activin cDNA digested with EcoRI was cloned into the EcoRI site of the pcDNA3 vector (Invitrogen, USA), which contains a CMV early promoter and a bovine growth hormone polyadenylation site (Fig. 1A), as previously described (Kim et al., 2008). The pCMV-rAct plasmid was purified and injected as previously described (Kim et al., 2008; Ko et al., 2003).

The basic protocol involved double injections with a 7-day interval and euthanasia of both female and male mice 4 days later. In females, the first injection was done 10:00 A.M. at diestrus II of the third cycle after confirmation of the two consecutive normal estrous cycles, which normally reveals a lower level of FSH in females. Estrous cycle stages were determined by daily examination of vaginal cytology at 9:30 A.M. To measure activin Aβ protein levels, a single injection of 300 μg pCMV-rAct in 50 μl of 10% sucrose in saline was performed at 10:00 A.M. in males, and serum was harvested 4 days after injection (Fig. 1B). For the study of differential expression between the sexes, the site of injection was marked with stitching using cotton thread. In female, a single injection of 300 μg pCMV-rAct in 50 μl of 10% sucrose in saline was performed at 10:00 A.M. on diestrus II after two normal consecutive estrous cycles. The muscle of the marked region was harvested at 10:00 A.M. at diestrus II after 4 days. In males, a single injection of 300 μg pCMV-rAct was performed at 10:00 A.M. and muscle was harvested at 10:00 A.M. after 4 days. After obtaining the muscle, the same quantity of muscle was used for Western blot (Fig. 2). For stomach and liver studies, mice were sacrificed 4 days after the second injection (Figs. 3 and 4; Table 1). For the necrosis study, mice were observed every day after the second injection until 8 months (Fig. 5). For the body weight study, body weight was measured after the second injection until day 15 (Fig. 6). For the survival study, various amounts of plasmid DNA were injected into mice. The pH of the stomach was measured with a Biobasic pH meter (Fisher Scientific Company). Numbers indicate the percentage of mice in each pH category. N means the number of mice tested. *P < 0.001.

| Table 1. Changes in pH |   |   |
|-----------------------|---|---|
|                       | pH | 2-3 | 6-7 |
| Male (N = 34)         |   |     |     |
| Control               | 97.1 | 2.9 |
| pCMV-rAct             | 2.9 | 97.1 * |
| Female (N = 13)       |   |     |     |
| Control               | 100.0 | 0.0 |
| pCMV-rAct             | 15.4 | 84.6 |

Fig. 1. pCMV-rAct structure and expression of pCMV-rAct. (A) Diagram of the pCMV-rAct construct. Functional elements include the cytomegalovirus (CMV) promoter, the rat activin cDNA, and the human growth hormone (hGH) poly(A) signal. (B) Protein blot analysis was performed in the "Materials and Methods". Proteins were obtained after injection of the different doses of pCMV-rAct into mice. The expression of activin Aβ was proportional to the injected amount of pCMV-rAct. The Western blot shown is representative of results obtained from four independent experiments. pCMV-rAct: pCMV-rAct-injected mice

Fig. 2. Level of activin Aβ in serum. Proteins were obtained after injection of pCMV-rAct into male or female mice. The Western blot was performed as described in the "Materials and Methods". The protein level was higher in females than males. The results shown are representative of results obtained from four independent experiments.
injected twice into male mice, and the mice were observed carefully every day at 10:00 A.M. until day 13 (Fig. 7). In the case of the sensitive mouse strain, BALB/c, a single injection of 100 μg of pCMV-rAct was administered, and mobile activities were observed after injection (Fig. 8). All experiments were performed at least four times if not otherwise noted, and representative results are shown.

**Protein blot analysis**

Muscle tissue was removed, homogenized in 400 μl of protein extraction buffer [0.1 M NaCl, 0.01 M Tris-Cl (pH 7.6), 1 mM EDTA (pH 8.0), 0.1% TritonX-100, 1 μg/ml aprotinin, and 100 ng/ml phenylmethylsulfonyl fluoride], and centrifuged four times. The homogenates were mixed with an equal volume of 2X SDS loading buffer [100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% BPB, and 20% glycerol], placed in boiling water for 10 min, and centrifuged. The supernatants were transferred to fresh tubes. Samples of each extract containing 10 μg of protein were heated at 70°C for 10 min, electrophoresed on a 12% acrylamide gel, and transferred onto Nytran filters in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, and 20% methanol). The blots were incubated overnight in blocking solution (5% non-fat dried milk, 0.02% sodium azide, and 0.02% Tween) with shaking at 4°C, followed by exposure to primary activin βA antibodies (1:400; Serotec, UK) overnight. They were washed in milk-TBS-Tween for 30 min and incubated with secondary anti-rabbit Ig horseradish peroxidase-linked whole donkey antibody (1:100; Amersham Pharmacia Biotech, USA) in azide-free blocking solution [5% non-fat dried milk, 150 mM NaCl, and 50 mM Tris-Cl (pH 7.5)] for 2 h. The secondary antibody-specific signal was detected with an ECL kit (Amersham Pharmacia Biotech). For serum measurement of activin βA or FSH, one microliter of serum was obtained, electrophoresed, and Western blot analysis was performed, using primary activin βA antibodies (Serotec) or primary FSH antibodies (1:750; Serotec).

**General behavior**

Mice were maintained as described in the “Animals and experimental design” section. Their general behavior was observed carefully every day at 10:00 A.M. until 13 days after the single injection of pCMV-rAct. Pictures were captured using a digital camera (Sony DSC-F717, Japan).

**Histology**

The gross appearance of excised tissues from injected and control mice were examined, and the tissues were immediately fixed in fresh 4% paraformaldehyde in PBS at pH 7.4. Following overnight fixation, tissues were dehydrated in ethanol, embed-
ded in paraffin, and sectioned at 7 μm with a microtome (Leica RM2235, Switzerland). The sections were de-paraffinized with xylene, dehydrated in absolute ethanol, and rehydrated in water. Sections were stained with hematoxylin, counterstained with eosin, and observed under a light microscope (Olympus IX70, Japan) or a stereomicroscope (Leica ME Apo, Switzerland).

Measurement of pH
For the pH measurement, the stomach was dissected, placed into a tube, centrifuged at 5000 rpm for 30 sec, and the pH of the supernatant was measured with both a pH meter (Fisher Scientific Company, USA) and alkacid test ribbons (Fisher Scientific Company).

Statistical analysis
For the statistical analysis, Student’s t test was used for single comparisons at α = 0.01. Statistics were performed no less than on four independent experiments.

RESULTS

Induced activin βA in muscle and blood
In a previous paper (Kim et al., 2008), we reported that activin βA mRNA was detected in muscle by RT-PCR, and activin βA protein in blood was detected by Western blot analysis. In this study, we reconfirmed the presence of protein activin βA in muscle and blood. Initially, we observed that mature activin βA protein (14 kDa) was synthesized and secreted into the blood proportionally to the injected amount of plasmid (Fig. 1B). This suggests that an adjustable amount of protein in blood can be induced for certain proteins. In the same context, we again detected the expression of activin βA in muscle of both sexes in order to compare the differential expression between male and female mice. The expression level of activin βA was higher in female than in male mice after the same dose of pCMV-rAct plasmid was injected into both sexes (Fig. 2).

Damage in the stomach and intestine by induced activin βA
When the effects of overexpressed activin βA on various organs were investigated, the stomach of mice revealed substantial and pathological damage (Fig. 3). In males, there was a dysmorphology of the stomach, which appeared to be inflated with gas (Fig. 3A), and an increased stomach pH (Fig. 3B) in pCMV-
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rAct-injected mice compared to controls was observed. This pH increase was observed in 97.1% and 84.6% of male and female mice, respectively (Table 1). It was also observed that the taken food remained inside the stomach. Moreover, an inflated intestine with yellow color was observed (Fig. 3C). The intestine was examined because the pH gradient throughout the digestive system is a well-known phenomenon. According to the morphological changes in stomach, we investigated the detailed histology. At the cellular level, loss of parietal cells was observed in the stomach lining, and this was more severe in male than female mice injected with pCMV-rAct (Fig. 4A). When we investigated the effect of activin βA on the liver, cells in pCMV-rAct-injected mice revealed a seemingly more fragile nucleus (Fig. 4B). However, no significant difference was observed. When we investigated major urine protein (MUP), a physiological marker in the liver, no difference was observed (data not shown).

Necrosis, loss of body weight, and lethality by induced activin βA
In the context of damage to the internal organs, such as stomach and intestine, research was extended to related changes in the body. Interestingly, necrosis of the foot or the tip of the tail was often observed in pCMV-rAct-injected mice two or three months later (Fig. 5). This phenomenon was wholly unexpected. In the same context, we also investigated body weight changes in both sexes after pCMV-rAct injection. As a result, there was a transient decrease in body weight of pCMV-rAct-injected male mice (Fig. 6A), but not female mice (Fig. 6B), revealing differential effects of activin βA on body weight between the sexes. Finally, we further investigated the functional consequences of activin βA overexpression on survival level. When pCMV-rAct at more than 100 μg was used, BALB/c mice died after a double injection of pCMV-rAct (Fig. 7A). However, ICR mice did not die even when 800 μg of pCMV-rAct was injected (Fig. 7B), suggesting that BALB/c mice are much more sensitive than ICR mice in their response to activin βA. Due to the high sensitivity to activin βA in BALB/c mice, a single injection of 100 μg of pCMV-rAct was administered. Following a single injection of plasmid, all mice survived. However, the eyes of individual BALB/c mice exhibited abnormalities, with the eyelids being almost closed (Fig. 8A), and groups of mice within the cage congregated in the corner without obvious mobile activity (Fig. 8B).

**DISCUSSION**

As described previously (Ling et al., 1985), activin was first identified as a gonadal protein hormone that regulates the synthesis and secretion of FSH in the pituitary gland. Activin has also been known to be important in the embryonic development (Matzuk et al., 1995; Tanimoto et al., 1999). However, it is still unclear whether activin really acts in an endocrine fashion in the reproductive axis and in embryonic development. Related
with this, our two reports support the endocrine manner of activin (βA) in the reproductive axis and digestive system. The digestive system is a recently elucidated area influenced by activin (Fukamachi et al., 2013; Li et al., 1998). First, our previous report demonstrated that activin (βA) influences the estrous cycle, an integral part of reproduction, in females in an endocrine manner (Kim et al., 2008). Second, our present results reveal that activin (βA) can exert profound effects on digestion in an endocrine fashion. Overexpressed activin (βA) directly and harmfully influences the GI tract, suggesting that activin (βA) must be maintained within a narrow physiological range.

When we examined internal organs after pCMV-rAct injection, we observed that the stomach and intestine were severely damaged. It can be inferred that the reduced production of H+ in the parietal cells induced this change in the luminal environment of the stomach and intestine. This overall change in the shape of the stomach and intestine by activin is expected, since supraphysiological levels of activin (over 10-fold) block the differentiation of multiple gastric epithelial lineages, including parietal cells (Li et al., 1998). However, the change in the GI tract occurred at the overexpressed level of activin (βA) (2-3-fold) in our study, which is far below the supraphysiological level, as indicated in Fig.1B. This means that the stomach and intestine are very sensitive to the level of activin (βA).

At the cellular level, the loss of parietal cells was clearly observed. Interestingly, the loss of parietal cells was more severe in males than females in Fig. 4A, although the actual level of activin (βA) in muscle and blood was higher in females than males, as indicated in Fig. 2. One possible explanation is that available activin (βA) was diminished in females because activin binding proteins such as follistatin might be higher in females than males. Conversely, an abnormality of liver cells was not clearly observed in this study. This is in contrast with results obtained in inhibin (α)-deficient mice (Matzuk et al., 1994). The reason also appeared to be the lower levels of activin (βA) in this experiment. With respect to cell proliferation, activin is known to block the differentiation of gastric epithelial cells (Li et al., 1998) and to inhibit cell growth in normal gastric cells (Fukamachi et al., 2013) and gastric cancer cells (Kaneda et al., 2011; Kim et al., 2006; 2009). Our study revealed that certain cells, but not all cells, are very sensitive to activin (βA).

In contrast to the changes observed in internal organs, external tissue changes, such as tail and foot necrosis, were unexpectedly observed. Necrosis was often observed as diabetic foot ulcers in terminal tissue of the diabetic patient (Jude et al., 2002). In diabetic foot ulcers, it has been shown that TGF-β1 is involved (Jude et al., 2002). TGF-β and activin (βA) belong to the TGF-β superfamily. Although a similar phenotype in foot and tail ulcers was observed, the cause of the ulcers seemed to be different. In our study, severe damage to the stomach and intestine that resulted from activin (βA) overexpression is likely to impact food absorption, leading to necrosis of the tail or even the death of these animals. Thus, tail or foot ulcers in our study appear to come from nutrition shortage due to the damaged digestive system, whereas diabetic ulcers result from a damaged insulin system. However, the mechanism would converge at the point of glucose shortage. Another minor possibility is that induced activin (βA) might directly hurt the pancreas, and this possibility requires more studies. The ability of activin (βA) to cause tissue necrosis or even death is unlikely to be related to its role in enhancing FSH secretion, since FSH-overexpressing transgenic mice do not exhibit any such defects (Kumar et al., 1992; 1999).

In addition to the internal and external changes of the body, integration of every change of the body by activin (βA) was investigated at the organism level. First, the effect of activin (βA) on the body weight of the ICR strain was sex-dependent. The body weight of males was more affected than female mice (Fig. 6). The loss of body weight was well-correlated with changes in parietal cells (Fig. 4A), suggesting that parietal cell loss leads to a nutritional defect. The greater loss of parietal cells in males was explained in Fig. 4A. The overall loss of body weight is consistent with the result of the previous report in inhibin (α)-deficient mice, which have a 10-fold elevation of activin (βA) levels (Matzuk et al., 1992). Second, the effect of activin (βA) on survival was strain-dependent. In terms of survival, BALB/c mice were more sensitive to activin (βA) than ICR mice, as observed in Fig. 7. In fact, the body weight of BALB/c mice was about 10 g smaller than the ICR mice (15g) in adults. Thus, the sensitivity to activin (βA) was about five-fold higher in BALB/c than ICR mice, when body weight was considered. Activin (βA) seemed to be the primary cause of death, because mice died in proportion to the amount of plasmid DNA injected (Fig. 7), which correlated with the amount of activin (βA) produced (Fig. 1B). Our results demonstrate that overexpression of activin (βA) can cause lethality in adult mice, as might be expected from previous findings (Matzuk et al., 1995). However, the mechanism of lethality might be different, since our transient transgenic mice received less accumulated activin (βA) at lower levels than normal transgenic mice. Third, the effect of activin (βA) on behavior was also strain-dependent. BALB/c mice showed less motility than ICR mice after injection of pCMV-rAct (Fig. 8).

These studies provide several important technical advances, as described previously (Ko et al., 2003). Briefly, our approach is relatively simple and rapid, while conventional transgenic approaches require substantial technical skill and time (Cho et al., 2001). Intramuscular injection is convenient, because the expression of targeted gene can be obtained at any time during development or in the adult animal. Intramuscular injection should have wide applicability for the screening of genetically engineered proteins for their therapeutic value or side effects in vivo, without the time-consuming production of transgenic mice.

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