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Interleukin-13 Prevents Diaphragm Muscle Deterioration in a Septic Animal Model

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TAKAHASHI, Y., KATAYOSE, D. and SHINDOH, C. *Interleukin-13 Prevents Diaphragm Muscle Deterioration in a Septic Animal Model.* Tohoku J. Exp. Med., 1999, **189** (3), 191-202 —— The effects of an intravenous injection of Interleukin-13 (IL-13) after endotoxin administration on diaphragm muscle were studied using Wistar rats. Two treatment groups, a control (saline+endotoxin) group and an IL-13 (IL-13+endotoxin) group were studied. *E. coli* endotoxin (10 mg/kg) was injected intraperitoneally 5 minutes after saline or IL-13 (0.25 μg) injection. The force-frequency curves, twitch kinetics and fatigability were measured at 0 and 4 hours after endotoxin injection. The force-frequency curves and twitch tension in the control group were significantly lower at 4 hours than those at 0 hour due to endotoxin. On the other hand, IL-13 prevented the decrement of the force-frequency curves and twitch tension induced by endotoxin. Nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry showed positive staining at 4 hours due to endotoxin in the control group; however, IL-13 also blocked NADPH diaphorase staining at 4 hours. Furthermore, the positive muscle fibers detected by the NADPH diaphorase staining were classified as type I (slow twitch) muscle fibers by ATPase staining. We conclude that IL-13 prevents the deterioration of contraction induced by endotoxin by inhibiting nitric oxide production in the diaphragm muscle, mainly the type I muscle fibers. ———— endotoxin; nitric oxide; NADPH diaphorase © 1999 Tohoku University Medical Press

Interleukin (IL)-13, a cytokine of a subgroup designated as the IL-4 family (Smerz-Bertling and Duschl 1995), is a nonglycosylated protein of 10 kDa, whose cDNA has been cloned and expressed (McKenzie et al. 1993a, b). The initial biological characterizations have indicated that IL-13 is characterized by monocyte and B-cell differentiation-inducing activities and that it induces IgG4 and IgE production by naive human B cells (Punnonen et al. 1993). It is also

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known that most of the activities of IL-13 in human monocytes are the same as those of IL-4, suggesting that these cytokines may share common receptor components (de Waal Malefyt et al. 1993). Moreover, IL-13 inhibits inflammatory cytokine production induced by lipopolysaccharide in human peripheral blood monocytes (Minty et al. 1993). Taken together, these observations indicate that IL-13 has important anti-inflammatory and immunoregulatory activities.

Previous studies have shown that injection of endotoxin induces a decrement in diaphragm muscle contractility and that this deterioration may be caused by a network of cytokines such as tumor necrosis factor-α (TNF-α), and free radicals such as nitric oxide (NO) and oxygen derived intermediates, including superoxide and the hydroxyl radical (Boczkowski et al. 1988; Shindoh et al. 1992). NO has been shown to be a regulator molecule for leukocytes and endothelial cells of blood vessels (Furchgott and Zawadzki 1980), and NO synthase (NOS) activity has been detected in diaphragm muscle fibers (Kobzik et al. 1994). We have recently reported that IL-10 has a protective effect on diaphragm muscle after endotoxin injection into the peritoneal cavity (Taneda et al. 1998). Thus, it is suggested that the network of cytokines and free radicals plays an important role in the endotoxin induced deterioration of diaphragm muscle.

Because the effect of IL-13 on diaphragm muscle is still unknown, we hypothesized that IL-13 may block the deterioration of diaphragm muscle induced by endotoxin administration. Using nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemical staining, we therefore examined whether IL-13 is able to reduce the contractile dysfunction induced by endotoxin in diaphragm muscle, and whether or not this is related to the suppression of NO production. By adenosine triphosphatase (ATPase) staining, we also examined which type of muscle fiber contributes to NO production.

Materials and Methods

Animal preparation

Using Wistar rats weighing 250-320 g (Charles River Japan, Yokohama), experiments were performed on 28 animals divided into 2 groups. The control (saline+endotoxin) group was given 0.5 ml of saline intravenously via the tail vein, followed by an intraperitoneal injection of *E. coli* endotoxin (10 mg/kg, 055: B5, Sigma Chemical Co., St. Louis, MO, USA) in 0.5 ml of saline ($n=14$) 5 minutes later. The IL-13 (IL-13+endotoxin) group was given human IL-13 (1250 U: i.e., 0.25 μg, B50047, Genzyme Co., Cambridge, MA, USA) suspended in 0.5 ml of saline intravenously via the tail vein, followed by an intraperitoneal injection of *E. coli* endotoxin (10 mg/kg) in 0.5 ml of saline ($n=14$) 5 minutes later. For the analysis of diaphragm muscle contractile properties, force-frequency curves, twitch kinetics and fatigability were measured immediately after (0 hour) and at 4 hours after injection of endotoxin in each experimental group ($n=7$, respectively). For the analysis of NO production, NADPH diaphor-

ase histochemistry was performed using 0-hour and 4-hour diaphragm muscle samples from each group. Because we previously showed that the force-frequency curves decreased maximally at 3-4 hours and then recovered at 6 hours after endotoxin (20 mg/kg) injection (Shindoh et al. 1995), we measured and analyzed these effects at 4 hours after endotoxin administration. Written approval was obtained from the Animal Research Committee, Tohoku University Graduate School of Medicine.

Measurements of muscle contraction

Two muscle strips (3-4 mm wide) were dissected from the right and left hemidiaphragm under diethyl ether anesthesia and mounted in separate organ baths containing Krebs-Henseleit solution oxygenated with a 95% O_2-5% CO_2 gas mixture (37.0±0.5°C, pH 7.40±0.05). The composition of the aerated Krebs-Henseleit solution in mEq/liter was as follows: Na^+, 153.8; K^+, 5.0; Ca^{2+}, 5.0; Mg^{2+}, 2.0; Cl^-, 145.0; HCO_3^-, 15.0; HPO_4^{2-}, 1.9; SO_4^{2-}, 2.0; glucose, 110 mg%; d-tubocurarine, 10 μM; and regular crystalline zinc insulin, 50 U/liter. Both muscle strips were simultaneously stimulated with supramaximal currents of 200-250 mA (i.e., 1.2 to 1.5 times the current required to elicit maximal twitch tension, pulse duration of 0.2 milliseconds) by a constant current stimulus isolation unit (SS-302J, Nihon Kohden, Tokyo) driven by a stimulator (SEN-3201, Nihon Kohden). The elicited tensions were measured by a force transducer (UL-100GR, Minebea Co., Fujisawa). The length of each muscle strip was changed by moving the position of the force transducer with a micrometer-controlled rack and pinion gear (accuracy of displacement, 0.05 mm, Mitsutoyo Co., Kawasaki), and measured with a micrometer in close proximity to the muscle. The optimal length of the muscle (Lo) was defined as the muscle length at which twitch tension development was maximal, and this Lo was maintained in the following measurements.

The diaphragm force-frequency relationship was assessed by sequentially stimulating muscles at 1, 10, 20, 30, 50, 70, 100 and 120 Hz. Each stimulus train was applied for approximately 1 second, and adjacent trains were applied at approximately 10-second intervals. The tensions of both muscle strips were recorded by a hot-pen recorder (RECTI-HORIZ-8K, San-ei, Tokyo). The force-frequency curves obtained from the groups studied were displayed as elicited tensions (kg/cm^2) on the Y-axis and stimulating frequencies on the X-axis.

Twitch contraction was elicited by a single pulse stimulation (0.2 millisecond duration), and the trace of the twitch contraction was recorded at high speed (10 cm/second). The twitch kinetics were assessed by (1) twitch tension (peak tension of twitch contraction, kg/cm^2), (2) contraction time (the time required to develop peak tension, milliseconds) and (3) half relaxation time (the time required for peak tension to fall by 50%, milliseconds) during a single muscle contraction.

Muscle fatigability was then assessed by examining the rate of fall of tension

over a 5-minute period of rhythmic contraction. Rhythmic contraction was induced by applying trains of 20 Hz stimuli (train duration, 0.3 seconds; rest duration, 0.7 seconds) at a rate of 60 train/minute. Muscle fatigability was expressed as a percentage of the final remaining tension (%) from the initial tension. After completion of this protocol, the muscle strip was removed from the bath and weighed.

NADPH diaphorase histochemistry

NADPH diaphorase histochemistry was performed using the diaphragm muscles of 0 hour and 4 hours in the control and IL-13 groups. The diaphragm samples were excised quickly from the remaining part of the costal diaphragm muscle (after dissection of the muscle strips used for measuring muscle contractile properties), immersed in isopentane (Wako Pure Chemical Industries Ltd., Osaka) that had been cooled in liquid nitrogen, and embedded in mounting medium (OCT compound, Miles Inc., Elkhart, IN, USA). Diaphragm sample tissues were sectioned at 10 μm with a cryostat (BRIGHT Instrument, Huntingdon, UK) kept at -20°C, mounted on chrome alum gelatin-coated glass slides, and immersed in 0.3% Triton X-100 containing phosphate buffer. For histochemical reaction for NADPH diaphorase, the sections were submerged for 60 minutes at 37°C in freshly prepared 1.0 mM β-NADPH (Oriental Yeast Co., Ltd., Tokyo) and 0.2 mM nitro-blue tetrazolium (Wako Pharmaceutical Co., Osaka) in 100 mM Tris-HCl buffer pH 8.0, containing 0.2% Triton X-100 (Dawson et al. 1991). The reaction was stopped by rinsing the sections in phosphate buffer saline (PBS). The sections were covered with a mixture of glycerol and PBS (2 : 1), and photographed with a Nikon microscope using color reversal film (Sensia II, FUJIFILM, Tokyo).

ATPase staining

Myofibrillar ATPase staining was performed according to the method of Dubowitz and Brooke (1973). The muscle strips in each group were adjusted to Lo with pins on a cork plate, immersed in isopentane (Wako Pure Chemical Industries Ltd.) that had been cooled in liquid nitrogen, and embedded in mounting medium (OCT compound, Miles Inc.). Diaphragm sample tissues were sectioned at 10 μm into sequential slices with a cryostat (BRIGHT Instrument) kept at -20°C. On the basis of their staining reactions for myofibrillar ATPase after alkaline (pH 10.4) and acid (pH 4.2 and 4.6) preincubation, muscle fibers were classified as either type I (slow-twitch and oxidative [SO]) or type II subtypes IIa (fast-twitch, oxidative and glycolytic [FOG]), IIb (fast-twitch and glycolytic [FG]) and IIc (intermediate). This nomenclature is as described by Dubowitz and Brooke (1973). With preincubation at a pH of 10.4, non-stained fibers were classified as type I, and dark stained fibers were classified as type II (IIa, IIb and IIc) muscle fiber. With preincubation at a pH of 4.2, non-stained fibers were classified as types IIa and IIb, and dark stained fibers were classified

as type I and IIc muscle fiber; at a pH of 4.6, non-stained fibers were classified as type IIa, and dark stained fibers were classified as type I, IIb and IIc muscle fiber. The sections were mounted in artificial resin, and photographed with a Nikon microscope using color reversal film (Sensia II, FUJIFILM).

Data analysis

The cross-sectional area of the strip was calculated by dividing the muscle mass by the product of the strip muscle length and muscle density (1.06 g/cm^3) (Close 1972), and tension was calculated as force per unit area (kg/cm^2). Data obtained from both halves of the diaphragm in one animal were averaged; therefore, the number of samples used was $n=7$ (animals) per treatment/time point for force-frequency curves, twitch kinetics and fatigability. The mean values of tensions for each frequency in force-frequency curves, twitch kinetics and fatigability were compared by Student's t-test. To compare the entire configuration of each force-frequency curve at 0 and 4 hours of the control and IL-13 groups, analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) *post hoc* test was performed. Data are presented as means$\pm$S.D. (standard deviation). Comparisons with a p-value of less than 0.05 were considered to be statistically significant.

Results

Changes of force-frequency curves

In the control (saline+endotoxin) group, the entire configuration of the force-frequency curves at 4 hours (1.49$\pm$0.08 kg/cm^2 as a peak) were significantly lower than that at 0 hour (1.96$\pm$0.06 kg/cm^2 as a peak) by ANOVA ($p<0.001$) (Fig. 1A). On the other hand, in the IL-13 (IL-13+endotoxin) group, there were no significant changes at each frequency nor in the entire configuration of force-frequency curves between 0 hour (1.77$\pm$0.05 kg/cm^2 as a peak) and 4 hours (1.92$\pm$0.07 kg/cm^2 as a peak) (Fig. 1B). Therefore, the entire configuration of the force-frequency curves at 4 hours of the IL-13 group was significantly different from that at 4 hours of the control group as shown by ANOVA ($p<0.001$).

Changes of twitch kinetics and fatigability

Regarding twitch tension, in the control group, it was significantly lower at 4 hours (0.34$\pm$0.02 kg/cm^2) than at 0 hour (0.49$\pm$0.03 kg/cm^2, $p<0.001$) (Fig. 2A). However, in the IL-13 group, twitch tension did not show any significant changes between 0 hour and 4 hours. These changes seem to correspond to the changes of the force-frequency curves. Both contraction time (Fig. 2C) and half relaxation time (Fig. 2B) in both groups did not show any significant changes between 0 hour and 4 hours. Also, the fatigability (Fig. 2D) in both groups did not show any significant changes during this period.

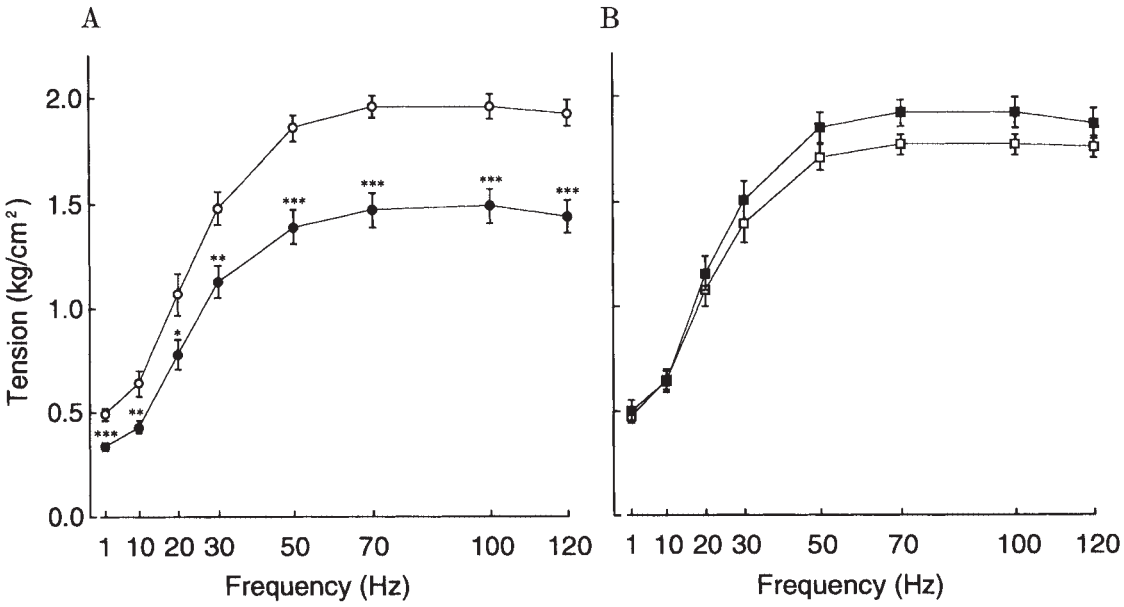

Fig. 1. Changes of force-frequency curves. A, 0 hour (○) and 4 hours (●) in the control group; B, 0 hour (□) and 4 hours (■) in the IL-13 group. Symbols indicate significant differences at given frequencies compared to 0 hour ($*p<0.05$, $**p<0.01$, $***p<0.001$).

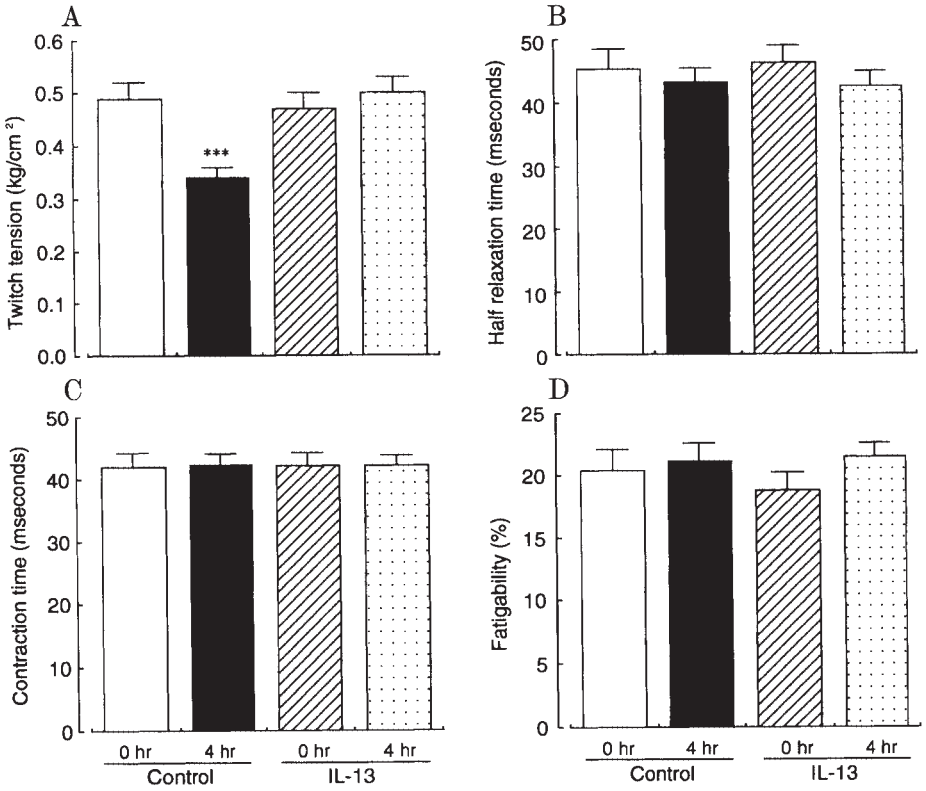

Fig. 2. Changes of twitch kinetics and fatigability. A, twitch tension; B, half relaxation time; C, contraction time; D, fatigability. 0 hour (open column) and 4 hours (closed column) in the control group; 0 hour (slashed column) and 4 hours (dotted column) in the IL-13 group. The symbol indicates a significant difference compared to control at 0 hour ($***p<0.001$).

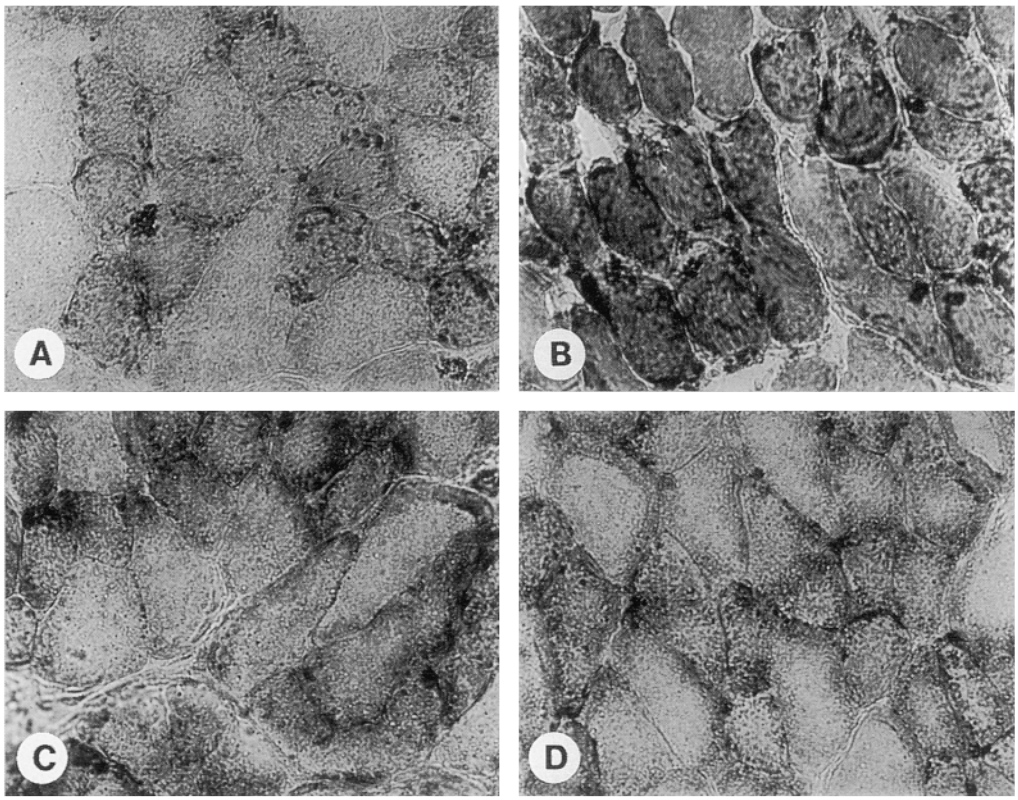

Fig. 3. NADPH diaphorase histochemistry of diaphragm muscles. A, 0 hour in the control group; B, 4 hours in the control group; C, 0 hour in the IL-13 group; D, 4 hours in the IL-13 group ($\times 200$).

NADPH diaphorase histochemistry and ATPase staining

Examination of the control group at 0 hour showed that there were only slightly stained muscle fibers (Fig. 3A); however, at 4 hours there were some strongly stained muscle fibers (positive: darkly blue staining) as shown by NADPH diaphorase histochemistry (Fig. 3B). On the other hand, the IL-13 group did not exhibit strongly stained muscle fibers at either 0 hour (Fig. 3C) or 4 hours (Fig. 3D). These findings indicate that endotoxin induces more NO production at 4 hours than that at 0 hour in the some muscle fibers; however, IL-13 inhibits NO production induced by endotoxin in the diaphragm muscle.

NADPH diaphorase histochemistry and ATPase staining (pH=4.6) were performed using a diaphragm muscle resected at 4 hours in the control group. A comparison of all the muscle fibers shows that the positive stains for NADPH diaphorase histochemistry (large white arrow, Fig. 4A) correspond to type I (SO) muscle fibers in the ATPase staining (large white arrow, Fig. 4B). On the other hand, the negative stains for NADPH diaphorase histochemistry (light blue staining) (small white arrow, Fig. 4A) correspond to type IIb (FG) muscle fibers in the ATPase staining (small white arrow, Fig. 4B). Although NO production was observed in the type IIa and IIc muscle fibers, it was confirmed that NO production occurred mainly in the type I muscle fibers rather than in the type IIb muscle fibers.

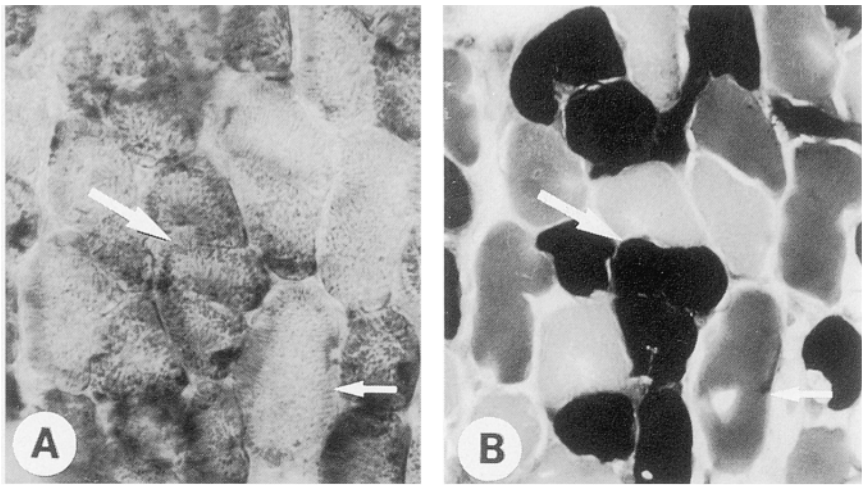

Fig. 4. Typical photographs of diaphragm muscle at 4 hours in the control group. A, NADPH diaphorase histochemistry; B, ATPase stain. Large white arrow indicates type I, and small white arrow indicates type IIb, respectively.

Discussion

The present study showed that IL-13 prevented the decrease of force-frequency curves induced by the endotoxin intraperitoneal injection and that it also inhibited NO production in type I (SO) diaphragm muscle fibers at 4 hours after endotoxin administration. Taken together, these findings indicate that the protective effects of IL-13 against the contractile deterioration induced by endotoxin may be caused by the blocking of NO in the diaphragm muscle fibers.

Since the inhibition of IL-4-induced and IL-13-induced responses was obtained by monoclonal antibody X2/45 raised against IL-4 Rex, which is an extracellular domain of the IL-4 receptor α subunit, the functional participation of the IL-4 receptor (subunit in the IL-13 receptor system has been clarified (Tony et al. 1994). The evidence is indicative that IL-13 belongs to the IL-4 family.

It has been reported that human recombinant (hr) IL-13 protects against the development of experimental autoimmune encephalomyelitis by inhibition of the production of the proinflammatory cytokines IL-1β and TNF-α from macrophages (Cash et al. 1994). Furthermore, in a study of an active inflammatory bowel disease, which is characterized by increased monocyte secretion of proinflammatory cytokines, it has been reported that IL-10 plus IL-4 and IL-10 plus IL-13, respectively, inhibited the proinflammatory cytokine response of monocytes as well as that of mature macrophages much more than did IL-4, IL-10, or IL-13 alone (Kucharzik et al. 1997). Since the present authors reported that TNF-α mRNA is expressed in diaphragm muscle cells after endotoxin administration (Shindoh et al. 1995), we also speculate that TNF-α and NO may contribute to the endotoxin induced diaphragm muscle contractile deterioration. We also previously reported that IL-10 has a protective effect against endotoxin induced diaphragm muscle deterioration (Taneda et al. 1998). Thus, in addition to IL-10,

IL-13 is also possibly another anti-inflammatory cytokine, which is thought to prevent inflammatory responses in the state of sepsis.

Secondly, it has been reported that IL-4 specifically induces 15-lipoxygenase (LO) mRNA and protein in cultured human monocytes, suggesting an important link between the 15-LO function and the immune/inflammatory response in atherosclerosis, as well as in other diseases (Conrad et al. 1992). The induction of the anti-inflammatory 15-LO pathway by IL-13 yielded new information on IL-13 biology that supports its role as a cytokine with potential to down-regulate inflammatory pathways (Nassar et al. 1994).

Thirdly, it has been reported that CD14 serves as a receptor for lipopolysaccharide (LPS) (Wright et al. 1990), that IL-4 induces a marked decrease in CD14 mRNA, and that IL4-dependent down-regulation of CD14 results from decreased transcription (Lauener et al. 1990). Recently, it has been reported that IL-13 inhibits the expression of CD14, the LPS receptor, on human monocytes (Viale and Vercelli 1995). However, to our knowledge, it is still unknown whether a similar action of IL-13 occurs in either diaphragm muscle or skeletal muscles. Although further studies on the diverse actions of IL-13 on diaphragm muscle are required, we tentatively propose that IL-13 inhibits the production of proinflammatory cytokines IL-1β and TNF-α and/or induces anti-inflammatory 15-LO expression, and/or blocks CD14 (the LPS receptor) expression in the diaphragm muscle.

We performed NADPH diaphorase histochemistry to detect NO production in the present study, and observed NO production in the muscle fibers of the control group at 4 hours. NO is known to be an important substance for vascular relaxation by the inhibition of complex I (NADH: Ubiquinone oxidoreductase) and complex II (succinate: Ubiquinone oxidoreductase) activities of the mitochondrial respiratory chain. It has been reported that NO-dependent inhibition might change to anaerobic glycosis and reduce the energy production of the smooth muscle cells (Geng et al. 1992), and that muscle-derived NO may play a role in modulating skeletal muscle carbohydrate metabolism, thereby contributing to the changes in contractile properties (Balon and Nadler 1994). In addition, the reaction of NO and superoxide could be of particular importance in the present endotoxin administration model because they form a peroxynitrite anion ($ONOO^-$), which is a potent hydroxyl radical ($\cdot OH$) that causes cellular damage by increasing lipid peroxidation and inhibiting mitochondrial respiration (Pryor and Squadrito 1995). Therefore, the impairment of muscle contraction appears to occur inside the diaphragm muscle, as supported by the evidence of NADPH diaphorase positive activity in muscle cell fibers, and it is also likely that the NO-dependent inhibition of mitochondrial respiration in the diaphragm muscle fibers is responsible for the decrement of force-frequency curves after endotoxin administration.

Of particular interest was the observation that the NADPH diaphorase

activity was inhibited by IL-13. It is known that the biosynthesis of NO from L-arginine by constitutive NO synthase occurs in endothelial cells (Zembowicz et al. 1991), and it has recently been reported that NOS occurs in the fatigued diaphragm muscle and that skeletal muscle cells can express two constitutive isoforms, neuronal-NOS and endothelial-NOS (Kobzik et al. 1995). Therefore, the present evidence that NO production was reduced by IL-13 suggests that IL-13 inhibition of NOS in the diaphragm muscles prevents endotoxin-induced deterioration of the force-frequency curves.

Because it has been unclear whether there is any difference in NO production in muscle fibers, the finding that NO production occurs mainly in type I muscle fibers rather than type IIb muscle fibers would seem to be important, as is the fact that all contractile parameters were maintained at 4 hours in the IL-13 group, the same as at 0 hour in the IL-13 group. On the basis of the biochemical and physiological characteristics of skeletal muscle, muscle fibers are classified as either type I or type II by ATPase staining. Type I (SO) has a smaller cross-sectional area (CSA), and type II (fast-twitch) subtypes IIa (FOG), IIb (FG) and IIc have a larger CSA; type IIb, in particular, has the largest CSA (Dubowitz and Brooke 1973). From the differences of energy production in muscle fibers, it is speculated that type I (SO) requires more oxygen than type IIb (FG) muscle fibers, and that type IIa (FOG) may be intermediate between them. If type I muscle fibers produce NO and oxygen-derived free radicals due to the presence of endotoxin, they may be more easily damaged by NO and oxygen-derived free radicals than type II muscle fibers. Compared with NADPH diaphorase histochemical findings, significant changes were observed in only two parameters of the force-frequency curves and the twitch tension at 4 hours in the control group. This was caused by a limitation in assessing the changes of diaphragm muscle in terms of its contractile properties.

Finally, the present study is the first report showing that IL-13 is a protective cytokine for diaphragm muscles in a septic animal model. It is noted that IL-13 is classified as an anti-inflammatory cytokine in contrast to IL-6, IL-8, and TNF-α, which are inflammatory cytokines and suggested to be highly related to the deterioration of diaphragm muscle contraction in sepsis. Although the protective effect of IL-13 on diaphragm muscle after endotoxin administration has been observed only in an animal model, we suggest that IL-13 could be useful in the treatment of patients with respiratory muscle failure caused by sepsis.

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