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FAST TRACK

TRAF2 Expression in Differentiated Muscle

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Abstract Recent data involving traf2 knockout mice have suggested a necessity of the protein in viability of skeletal muscle tissue. *traf2* −/− mice are born with decreased muscle mass that is hypothesized to be due to the increased circulating tumor necrosis factor in these mice. We show that TRAF2 protein is present at high levels in terminally differentiated skeletal muscle in the developing mouse. In vitro differentiation of mouse myoblasts displays a dramatic increase in TRAF2 protein levels. Although basal NF-κB activity decreases during myogenesis, TNF-induced NF-κB activity is 10 times greater in myotubes compared with myoblasts, presumably because of the stockpiling of TRAF2 protein in these cells. This may represent a strong anti-apoptotic TRAF2-mediated response specifically tailored to myotubes. These data help explain why muscle integrity is at risk in *traf2* −/− mice. J. Cell. Biochem. 71:461–466, 1998.

Key words: TRAF2, tumor necrosis factor, NF-κB, apoptosis, myotube

Over the past decade, much progress has been made in the delineation of the pathways that lead to, or protect against, apoptosis. A paradigm in this field has been the study of the tumor necrosis factor (TNF). The cytokine TNFα is known to transduce its signal through one of two receptors 55 and 75 kDa in size [Tartaglia and Goeddel, 1992]. The 55-kDa receptor (TNFR1) possesses somewhat schizophrenic activities in that it is able to both induce and protect against apoptosis [Tartaglia et al., 1993; Liu et al., 1996]. The decision as to whether to kill its host cell depends chiefly on the protein composition present at the intracellular domain of the receptor. Several proteins associated with the cytoplasmic tail of the receptor have been discovered that moderate apoptotic signals such as activation of caspases or proliferative responses such as induction of NF-κB and JNK activity, or both [Tewari and Dixit, 1996]. One of these proteins, TRAF2, has been identified as a prime mediator of TNF-induced NF-κB activity [Rothe et al., 1994, 1995]. Bound to the TNFR1 adaptor protein TRADD, TRAF2 will induce the nuclear entry and activation of the transcriptional abilities of NF-κB [Hsu et al., 1995, 1996]. This is accomplished by association of TRAF2 with such kinases as NIK, IKKα and IKKβ, which phosphorylate the cytoplasmic anchor and inhibitor of NF-κB, IκB, on key residues that lead to its degradation [Regnier et al., 1997; Zandi et al., 1997]. Alternatively, TRADD will associate with FADD and initiate a cascade leading to programmed cell death. TRAF2-mediated NF-κB induction has been shown in HeLa cells to inhibit TNF-induced cell death [Liu et al., 1996; Beg and Baltimore 1996; Van Antwerp et al., 1996]. Expression of a mutant TRAF2 lacking the N-terminal RING finger motif (TRAF2 dominant negative) blocks TNF-mediated NF-κB activation and results in excess cell death when exposed to TNF. In fact, forced expression of NF-κB is able to protect cells from cell death provoked by TNF or many of the downstream signal components of its pathway, including TRADD and FADD.

Developmental and tissue-specific effects of the proteins involved in the TNF signal cascade were recently identified with the advent of mouse "knockout" models of several of these proteins. In particular, data from *traf2* −/− and TRAF2 dominant negative transgenic mice have

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identified specific tissues that are especially sensitive to death signals in the absence of functional TRAF2 signaling [Lee et al., 1997; Yeh et al., 1997]. A mouse carrying a dominant interfering mutant of TRAF2 with expression directed to lymphocytes shows an increased cytotoxicity of thymocytes to TNF. Mice that do not carry any form of the TRAF2 gene also show depletion of B-cell precursors and splenocytes. In addition, the *traf2* −/− offspring are runted, become smaller than their normal littermates after 2–3 days and die prematurely.

Another phenotype of traf2 −/− mice is that they are born with a severely decreased skeletal muscle abundance that continually wastes away over their short life span. Such an observance is reminiscent of the origins of TNF discovery itself [Beutler et al., 1985; Pennica et al., 1985]. A group of researchers injecting endotoxin into mice purified a factor that is dramatically upregulated upon this stimulation. When this factor is reintroduced into fresh mice, a muscle- wasting symptom termed cachexia results, leading to the temporary nomenclature of the protein as cachectin. Amino acid sequence analysis of this protein showed high similarity to the human tumor necrosis factor and appeared to be the mouse homolog of TNF. Thus, mouse TNF is not only an essential cytokine upregulated upon immune attack but, in sufficiently high doses, is harmful to skeletal muscle. Indeed, in the *traf2* −/− mice, there seems to be a defect in regulation of TNF serum levels, as TNF is present in high circulating amounts. This has led to the hypothesis that this is the causative element in the muscle wasting of these mice.

Here we propose another cooperative explanation for the excess skeletal muscle death in these animals. TRAF2 protein is highly expressed in the differentiated compared with forms undifferentiated of these cells. TRAF2-mediated anti-apoptotic responses are much higher in myotubes, the lack of which may cause cell death en masse in the face of large amounts of TNF. Therefore, skeletal muscle represents a specific tissue that may be dependent on TRAF2 for viability.

MATERIALS AND METHODS

Cell Culture and Transfection

C2C12 (ATCC No. CRL-1825) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 mg/ml glutamine, and 20% fetal bovine serum (FBS). Differentiation of C2C12 myoblasts to myotubes was performed by changing the serum content in the media from 10% FBS to 2% horse serum, followed by incubation for 4 days. Transfection was performed using Superfect (Qiagen) reagent following the manufacturer's protocol. Recombinant TNF was obtained from Sigma Chemical Co. (St. Louis, MO).

Immunofluorescence

C2C12 cells were plated sparsely on glass coverslips, placed into growth medium for 24 h, and then shifted to differentiation medium for 96 h. Cells were fixed in fixation buffer (100 mM PIPES pH 8.0, 5 mM EGTA, 2 mM $MgCl_2$, 3.7% formaldehyde, 1% methanol, 0.2% Triton X-100), followed by dehydration in ice- cold ethanol. Rehydration was performed with phosphate-buffered saline (PBS), and cells were blocked for nonspecific antibody binding sites by initial incubation with 10% normal goat serum in 5% bovine serum albumin (BSA) in PBS. Affinity-purified anti-TRAF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with the slides in 0.5% BSA/PBS, followed by washes in PBS, and secondary anti-rabbit FITC conjugated antibody was incubated with the cells at a concentration of 1:400, followed by washes. Ultraviolet (UV) fluorescing cells and tissues were visualized by use of a Ziess Axiophot equipped with the appropriate filters.

Western Blotting

Cell lysis, immunoprecipitations, and Western blotting were performed as previously described [Bagella et al., 1998). Affinity-purified anti-C-terminal TRAF2 was obtained from Santa Cruz Biotechnology.

Animals

Mature male and female ICR mice (Ace Laboratories, Malvern, PA) were housed in a room with a 12-h alternating light and dark cycle and were maintained on Purina mouse chow and water ad libitum. Timed pregnancies were obtained by mating a single mature male with two nulliparous females for 2 h in the morning (early in the light cycle). The presence of a vaginal plug was considered evidence of mating and the time considered 0 days 0 h (0d, 0h) of gestation. Embryos/fetuses were identified according to their developmental stage following

the method of Theiler [1989]. Pregnant female mice were killed by cervical dislocation on various days from day 10 to day 17 of gestation. Embryos were dissected free of the uteri and extraembryonic membranes in PBS (pH 7.2) on ice. Intact embryos were embedded in paraffin and sectioned laterally. Sections were placed on slides for in situ immunolocalization.

Luciferase Assays

Transfected cells were lysed in reporter lysis buffer (Promega, Madison, WI), run through one freeze–thaw cycle and cell debris was pelleted. Cell lysates were tested for β-Gal activity to normalize transfection efficiencies by incubating 10 µl of lysate with β-Gal assay buffer (10 mM $MgCl_2$, 45 mM BME, 1 mg/ml ONPG, 0.1 M Na_2HPO_4), incubated for 30 min at 37°C, brought up to a volume of 700 µl with 1 M $NaCO_3$, and measured for absorbance at 420 nm. Assay for luciferase was performed by incubation of 20 µl of lysate with 100 µl of luciferase assay reagent (Promega) and florescence was measured using a luminometer (Berthold Lumat LB9501).

RESULTS AND DISCUSSION

Expression of TRAF Protein in the Mouse Embryo

To determine tissue localization of TRAF protein, mouse embryos at 13 days postcoitum were sectioned laterally, placed on slides, and incubated with anti-TRAF2 antibody, followed by incubation with FITC-labeled anti-rabbit. High levels of protein expression were found in the developing tongue, pockets of tissue surrounding the eye, and fibrous tissue in the body wall of the embryo (Fig. 1, only staining in the tongue is shown). All these locations also stained strongly with anti-myosin heavy chain antibody signifying skeletal muscle (data not shown). Surprisingly, low or no expression was detected in all other regions of the embryo. This expression pattern suggests that TRAF2 is involved in a differentiated muscle-specific function during mammalian development.

Expression of TRAF2 During Muscle Differentiation

The localization of TRAF2 protein in the embryo identified terminally differentiated muscle fibers as high expressors of TRAF2. To see whether this was a general characteristic of cells determined to become muscle or whether this is specific only to differentiated muscle, we compared the expression levels of TRAF2 in undifferentiated myoblasts with terminally differentiated myotubes. C2C12 myoblasts were grown to 60% confluency and then induced to differentiate by a change of medium serum content from 10% FBS to 2% horse serum. Cell pellets were taken at 48-h time points for 96 h and their TRAF2 protein levels examined by immunoblot. TRAF2 protein levels were found to increase by the time terminal differentiation had been reached (Fig. 2A).

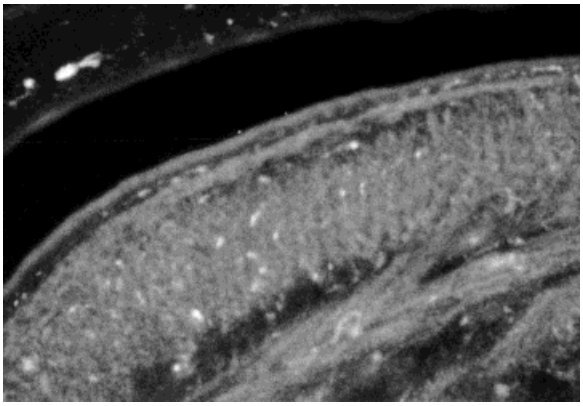

Fig. 1. Expression of TRAF2 in mouse embryonic muscle. Sagittal sections from 13-dpc mouse embryos were stained for TRAF2 expression with an affinity purified TRAF2 antibody. Subsequent incubation with anti-rabbit FITC-labeled antibody allowed fluorescent detection of TRAF2 protein. One area of high expression was seen in the developing tongue, a tissue consisting of predominantly skeletal muscle.

To confirm the upregulation of TRAF2 protein levels during the differentiation process we sought to see this effect by immunofluorescence. C2C12 cells were plated sparsely on glass coverslips and induced to differentiate by a change to differentiation medium. Cells were fixed either before or 96 h after addition of differentiation medium and blotted with polyclonal anti-TRAF2 antibody. Compared with undifferentiated myoblasts (Fig. 2B), differentiated myotubes (Fig. 2C, D) contained a dramatically higher amount of TRAF2 present in the cytoplasm as opposed to cells in the background that had not yet differentiated. There was no upregulation of TRAF2 protein was not seen in serum starved HeLa cells indicating that this effect is not simply due to a cell cycle arrest, but is specific to this differentiation pathway. Additionally, TNF treatment of C2C12 myoblasts also did not result in an upregulation of TRAF2, suggesting that activation of TRAF2-depen-

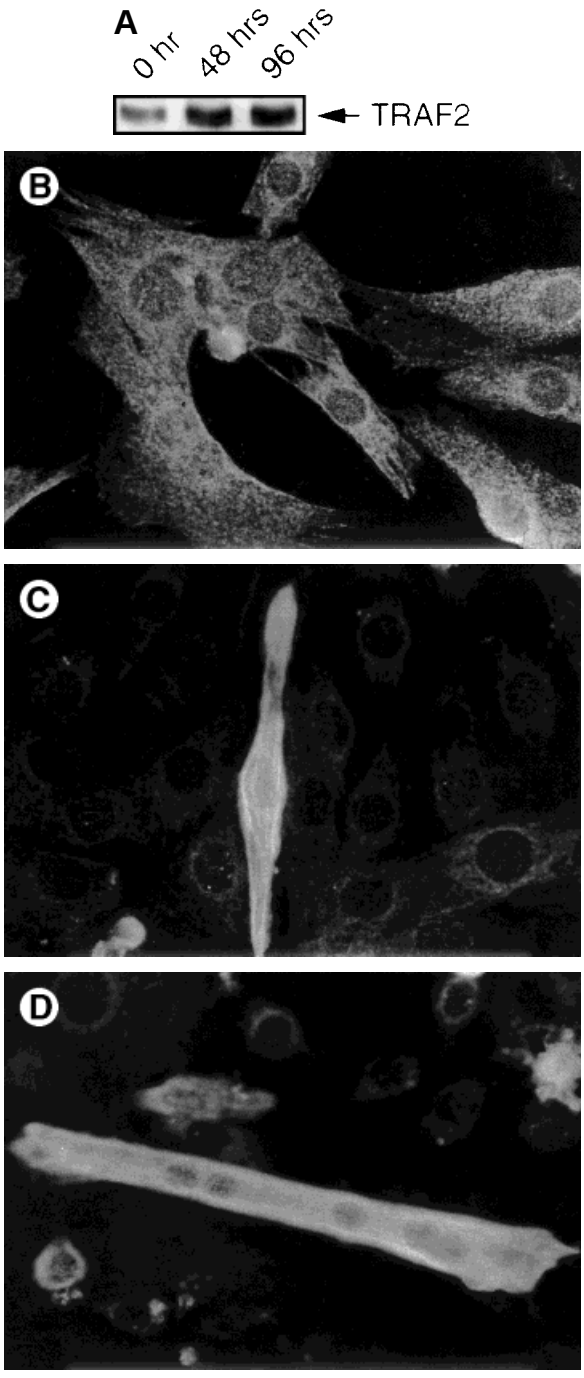

Fig. 2. Increase of TRAF2 during muscle differentiation. C2C12 cells were grown in DMEM +10% FBS and then induced to differentiate by incubation in DMEM +2% horse serum. Cells were collected at 48 hour time points and either lysed for TRAF2 immunoblotting **(A)** or stained in the undifferentiated **(B)** and differentiated state **(C,D)** with a flourescein labeled TRAF2 antibody.

dent signaling pathways does not accompany an increase in protein abundance. Therefore, this upregulation may be present as a stockpiling mechanism of the myotube to induce a strong TRAF2-involved response to a particular stimulus.

Activity of NF-κB in Differentiated Muscle

Simply overexpressing TRAF2 activates the anti-apoptotic pathways of the TNF receptor. As this potent NF-κB inducing protein was found to be dramatically increased during the muscle differentiation pathway, it became interesting to then study the activity of NF-κB itself during this process. C2C12 cells were transfected with a luciferase construct under the control of an NF-κB-driven promoter. At 24 h after transfection, cells were either harvested or induced to differentiate for 48 or 96 h. Surprisingly, regardless of the high expression of TRAF2 in myotubes, basal NF-κB activity decreased in myotubes compared with undifferentiated cells (Fig. 3). To see whether the NF-κB response to TNF exposure decreased proportionally to the basal activity, C2C12 cells were transfected with the NF-κB luciferase construct, harvested or induced to differentiate and either treated with TNF or left untreated. C2C12 cells (a murine line) were treated with human TNF, a form that is only able to engage murine TNFR1, and not murine TNFR2 [Smith et al., 1986]. Sole activation of TNFR1 will help identify pathways activated that are induced to

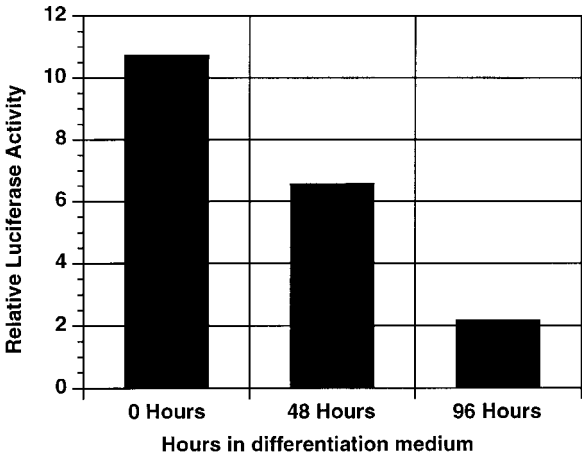

Fig. 3. Basal NF-κB activity decreases during muscle differentiation. C2C12 cells were transfected with a β-Gal expression vector (0.5 μg) and NF-κB-Luc (1 μg) and then harvested and assayed for luciferase activity at 48-h intervals during differentiation. Luciferase activities were normalized to transfection efficiencies by measurement of β-Gal activity.

inhibit apoptosis, since this receptor, as opposed to TNFR2, evokes both anti-apoptotic and apoptotic responses. Again, basal activity of NF-κB decreased, however, as compared with a twofold increase in activity upon treatment with TNF in myoblasts, myotubes exhibited a nearly 10-fold increase in NF-κB activity (Fig. 4). We believe this result is due to the accumulation of TRAF2 incorporating signaling complexes in the cytoplasm of the myotubes. These are apparently left inactive in the absence of stimulus, as basal activity decreases significantly in light of these complexes. However, when cells are exposed to TNF, the complexes become active and induce a strong anti-apoptotic NF-κB response.

CONCLUSION

The cells that make up the striated myofibers of skeletal muscle are known to consistently resist apoptotic signals throughout the life of the organism. Skeletal muscle is one tissue that is very long-lived. However, inherited muscle-degenerative diseases, such as spinal muscular atrophy and muscular dystrophy, result in wasting away of muscle tissue as a result of increasing apoptosis of myofibers. Therefore, it is clear that a genetic component is present in these diseases that affect the viability of the tissue.

Much work has been performed in the study of the tumor necrosis factor effector pathway in human tumor cell lines and in cells of hematopoietic origin. This work has helped identify proteins integral to the signal transduction mechanisms and the roles played by each. However, muscle tissue remains largely overlooked as a model for this pathway, regardless of the strong correlations of such muscle-wasting diseases as cachexia with the presence of circulating TNF. In this paper, we have shown that at least one protein in the TNF receptor mediated cascade is strongly upregulated only in the differentiated form of skeletal muscle. TRAF2 has been shown previously to be a potent inducer of NF-κB activity, to be essential for the TNF-mediated anti-apoptotic NF-κB response and to be necessary for normal muscle viability. While TRAF2 does not appear to affect the differentiation process of skeletal muscle (T.K.M., unpublished observations), we show that TRAF2 elicits an abnormally strong NF-κB response in myotubes compared with their undifferentiated counterparts. This effect is the likely result of a high level of expression of TRAF2 in myotubes. Hyperexpression of TRAF2, regardless of its NF-κB inducing qualities, does not activate NF-κB as the basal NF-κB activity in fact decreases as myoblasts reach terminal differentiation. It appears that myotubes perform a sort of "stockpiling" of TRAF2 in order to induce a strong anti-apoptotic response to TNF exposure.

Interestingly, mice deficient in TRAF2 production display a gross lack of body wall muscle that wastes further over their short life span. TRAF2-mediated NF-κB induction has been shown to inhibit apoptosis in HeLa, 293, and MCF7 cell lines. Although other cell types, such as hematopoietic, in the knockout mice are still able to induce NF-κB upon TNF treatment, myotubes in particular may be absolutely dependent on TRAF2 for NF-κB activation, the loss of which results in mass cell death. Another muscle-specific guard against apoptosis, ARC, has recently been cloned that acts to inhibit caspases activated by several different death receptors [Koseki et al., 1998]. One can imagine a scenario whereupon TNF binding to TNFR1 in myotubes, ARC and TRAF2 pathways cooperate by both inhibiting pro-death caspase activation and activating the anti-apoptotic NF-κB induction. Given the genetic counterpart of muscle-wasting diseases, it is possible that loss of the TRAF2 gene is a major step in the progression of these diseases.

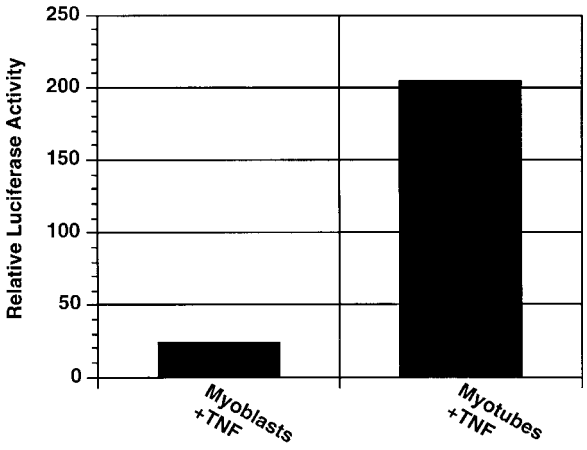

Fig. 4. NF-κB response to tumor necrosis factor-α (TNF-α) in myoblasts compared with myotubes. C2C12 cells were transfected with β-Gal (0.5 μg) and NF-κB-Luc (0.5 μg). At 24 h after transfection, cells were treated (myoblasts+TNF) or untreated (myoblasts) with TNF (100 ng/ml) for 8 h or were allowed to differentiate for 4 days (myotubes, myotubes+TNF) and then assayed for luciferase activity. Readings were normalized for transfection efficiency by measurement of β-Gal activity.

ACKNOWLEDGMENTS

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