Sarcopenia, the loss of skeletal muscle mass and contractile function, is a consequence of aging that causes considerable morbidity (9). Since skeletal muscle is the primary site for glucose (10) and triglyceride (11) disposal and the predominant determinant of resting metabolic rate (12), age-associated muscle loss may also contribute to peripheral insulin resistance, dyslipidemia, and increased adiposity. There is no simple mechanism to explain age-associated muscle loss. Skeletal muscle mitochondrial and contractile protein synthetic rates decline with age (13, 14), accounting for some of the muscle loss and decreased functional capacity. Plasma levels of TNF-α and other proinflammatory cytokines (such as interleukin 6) and markers of inflammation (such as C-reactive protein) increase with advancing age (15–19). It is not clear whether the increase in TNF-α is mediated by aging per se or by chronic disease associated with aging. Regardless, an increase in TNF-α, especially if it were to occur in skeletal muscle, could play a role in the progressive muscle loss of advancing age.

TNF-α mRNA and protein have been detected in biopsy samples from human skeletal muscle (20), but the effects of age and exercise on skeletal muscle TNF-α expression are unknown. In this paper, we test the hypothesis that TNF-α produced by skeletal muscle contributes to muscle loss with age. We compared skeletal muscle TNF-α expression in young and frail elderly subjects, and determined the effects of resistance training on skeletal muscle TNF-α expression in the frail elderly. Resistance training increases mixed muscle protein synthesis (21) but the mechanisms are unknown. Since TNF-α has inhibitory effects on protein synthesis, protein synthesis rate was also determined before and after exercise. TNF-α was originally purified (1) on the basis of its capacity to suppress expression of the enzyme lipoprotein lipase (LPL), suggesting that skeletal muscle TNF-α would be inversely related to LPL. Therefore, LPL content was also determined in muscle before and after exercise.
In situ hybridization data show that myocytes (as opposed to other cell types in biopsy samples) are a source of TNF-α in frail elderly muscle. The results also show that skeletal muscle TNF-α expression increases by pretranslational mechanisms with age and that expression is decreased by resistance exercise in the frail elderly, suggesting a role for this cytokine in age-associated muscle wasting.

MATERIALS AND METHODS

Subjects

The Washington University Human Studies Committee approved this study. Each individual gave informed consent prior to participation. Young subjects (under the age of 30) were active and healthy but untrained.

Elderly humans (over the age of 75) underwent a screening protocol to exclude the presence of underlying confounding diseases including cardiovascular (such as angina or congestive heart failure), metabolic (such as diabetes), neoplastic, and neuromuscular (such as Parkinsonism) disorders. The screening protocol included a complete medical history prompted by standardized questions using common terms instead of medical jargon. Subjects were specifically queried regarding arthritis, chronic infection (such as tuberculosis or a history of endocarditis), cancer (of numerous sites), allergies, autoimmune disorders, myocardial infarction, angina, heart murmurs, congestive heart failure, pacemakers, hypertension, hyperlipidemia, diabetes, chronic lung diseases, claudication, liver disease, clotting disorders, stroke, muscle weakness, dizziness or fainting, mental health problems, difficulty with memory, and orthopedic problems. Each subject underwent a complete physical examination and a laboratory evaluation, including chest radiography, mammography and pamp smear testing in women, as well as determination of serum chemistries, complete blood count, serum lipids and lipoproteins, and urinalysis. In addition to being excluded for the presence of confounding diseases, volunteers were excluded if they were taking medications known to affect muscle function such as β-adrenergic blocking agents, β-agonists, Ca++ channel blockers, and corticosteroids.

Body composition was assessed in every subject using whole-body dual-energy X-ray absorptiometry as described previously (22). For elderly participants in the exercise training study, muscle mass was determined using 24 h urinary creatinine excretion data. Results from three collections were averaged and muscle mass was calculated by assuming that 1 g creatinine excreted/day reflects 20 kg muscle (21). Elderly subjects were objectively classified as frail on the basis of physical performance tests developed by Reuben and Siu (23) and Guralnik et al. (24). These tests have been shown to correlate well with degree of disability, loss of independence, and early mortality (24, 25). They include walking speed, stair climbing speed, lower back range of motion, balance, and upper body strength. In addition, the classification of physical frailty was subjectively confirmed in each subject by assessing self-reported degrees of difficulty with activities of daily living.

Comparisons between young and frail elderly subjects

All subjects limited activity to that required for daily living for 3 days before the biopsies. Skeletal muscle tissue was obtained from the vastus lateralis muscle under sterile conditions after an overnight fast. Samples were isolated from 12 consecutive young subjects and 12 consecutive frail elderly subjects for subsequent determination of TNF-α protein levels (see below). For every third subject in each age group, additional tissue was obtained for subsequent analysis of TNF-α mRNA by in situ hybridization (see below).

Exercise training program

Eight frail elderly subjects underwent skeletal muscle biopsies before and after an exercise program that has been described previously (21). For 3 months prior to beginning the resistance exercise training program, subjects followed a supervised pretraining program 3 days/wk. The program was designed to increase flexibility and joint range of motion and included light stretching exercises. This pretraining program was followed by a supervised progressive resistance exercise training program 3 days/wk for 3 months.

The training program consisted of a 5 min warm-up of stretching, light calisthenics, and walking, followed by 50–90 min of supervised resistance exercise. Exercises were initially performed with resistance machines. Later, abdominal exercises and free weight squats were incorporated. Subjects started doing 1–2 sets of 6–8 repetitions at 65–75% of the initial one-repetition maximum and progressed to 3 sets of 8–12 repetitions at 85–100% of the one-repetition maximum.

Five frail elderly subjects served as nonexercising controls. These individuals completed the 3 month pretraining program, then continued the light stretching program for an additional 3 months. They were seen at regular intervals to provide social interaction with the staff similar to that experienced by the participants in the resistance exercise training program.

Muscle assays

Muscle samples were analyzed for TNF-α by enzyme-linked immunosorbent assay (ELISA) using an anti-human TNF-α monoclonal antibody and reagents provided in a kit from Biosource International (Camarillo, Calif.). All assays were performed in duplicate. Each analysis used protein concentrations within the linear response range for this assay as determined in preliminary experiments using human muscle tissue. Samples from the same subject obtained before and after the 3 month period were always analyzed in the same assay. The intra-assay coefficient of variance was 2.6%.

Muscle samples from the training study were also analyzed for LPL protein content by quantitative Western blotting (26, 27). The primary antibody was detected using horseradish peroxidase-conjugated donkey anti-rabbit IgG and signal was generated using enhanced chemiluminescence (Amer sham, Buckinghamshire, England). Films were scanned with an imaging densitometer (Bio-Rad GS-670, Hercules, Calif.). As for TNF-α, ‘before’ and ‘after’ training samples from the same subject were analyzed in the same assay. The intra-assay coefficient of variance was 6.7%.

For subjects in the training study, the rate of mixed muscle protein synthesis was determined before and after exercise using stable isotope techniques as described (21). Participants ate a standardized meat-free diet for 3 days before the protein metabolism studies. Whole body proteolysis (measured as the rate of appearance of leucine) and myofibrillar proteolysis (measured as the ratio of 3-methyl histidine to creatinine) was also determined in elderly subjects before and after the exercise program.

In situ hybridization

Muscle tissue was fixed in 4% paraformaldehyde (w/v) in phosphate-buffered saline (PBS) for 16 h at room tempera-
ture, washed, dehydrated through an ascending ethanol series, and embedded in paraffin wax. Sections were then cut at 7 μM and mounted to microscope slides (Fisher Superfrost Plus).

For probe generation, an 821 bp EcoRI fragment containing the entire coding region of the human TNF-α cDNA (GenBank™ X02910) was subcloned into pBluescript SK. The antisense strand probe was synthesized by in vitro transcription using a SmaI-linearized template and T7 RNA polymerase. The control sense strand probe was synthesized using EcoRV-linearized plasmid and T3 RNA polymerase. Both probes were labeled with digoxigenin (DIG; Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendation. Probes were reduced in length to ~200 bp by limited alkaline hydrolysis (80 mM NaHCO₃, 120 mM Na₂CO₃, pH 10.2, 10 mM DTT) at 60°C for 20 min.

First, a blinded observer counted hybridization signals in multiple standardized areas at the same magnification within numerous muscle sections for each biopsy sample. Second, an image processing program (Image-Pro Plus) quantified signals in standardized areas after using color thresholding to correct for different background intensities. The intra-assay coefficient of variance for measuring TNF-α mRNA by image processing analysis of biopsies analyzed by in situ hybridization was 11.5%.

**Statistical analyses**

Comparisons between variables were made using t tests for young vs. frail elderly samples, and paired t tests for baseline vs. post-training samples. The Pearson product moment correlation coefficient was calculated to assess the relationship between variables.

**RESULTS**

We conducted two studies to address the role of TNF-α in aging and exercise, a cross-sectional study comparing young and frail elderly and a longitudinal study in the frail elderly. Physical characteristics of subjects in both studies are shown in Table 1. For the cross-sectional study, maximal oxygen uptake (P<0.0001) and lean mass (P=0.0285) were lower, and fat mass (P=0.0152) and percent body fat (P=0.0005) were higher in the elderly as compared to the young, consistent with known effects of aging (28, 29). In the longitudinal study, there were no significant differences between the exercise and control groups.

In addition to myocytes, muscle biopsies may contain adipose tissue, macrophages (each a source of TNF-α), and other components. To determine whether TNF-α is produced by myocytes of elderly humans, in situ hybridization experiments were performed using human muscle biopsies. Figure 1 shows adjacent sections of skeletal muscle from an 82-year-old frail woman. Sections were stained with hematoxylin and eosin (Fig. 1A), hybridized with a sense strand (control) TNF-α

<table>
<thead>
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<th>TABLE 1. Physical characteristics of subjects*</th>
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<tr>
<td>Young cross-sectional study</td>
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<tr>
<td>Number</td>
</tr>
<tr>
<td>(Males/females)</td>
</tr>
<tr>
<td>Age, years</td>
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<td>Height, cm</td>
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<td>Maximal O₂ uptake, ml/kg/min</td>
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*Values are means ± se.
probe (Fig. 1C), or hybridized with an antisense TNF-α probe (Fig. 1B). TNF-α mRNA signals were detected in myocytes in panel C, but no such signals are detected in the control section (B). The source of the biopsy was vastus lateralis muscle from a frail, 82-year-old woman.

To determine whether TNF-α expression is altered with age, we compared TNF-α mRNA content in muscle from elderly and young subjects using in situ hybridization (Fig. 2). There were more TNF-α signals in myocytes from frail elderly subjects (Fig. 2A) than in sections from the young (Fig. 2B).

Biopsies were obtained from a total of 12 elderly subjects (including the subjects represented in Figs. 1 and 2) and 12 young subjects (see Table 1 for physical characteristics). Muscle TNF-α protein was higher in the frail elderly individuals compared to young healthy individuals (Fig. 3A). Muscle TNF-α mRNA, quantified by analysis of in situ hybridization signals, was also higher in the elderly than the young (Fig. 3B), suggesting that aging is associated with an increase in TNF-α expression mediated at a pretranslational level.

To determine whether exercise can affect skeletal muscle TNF-α expression in the frail elderly, subjects (different from those referred to in Figs. 1–3) were assigned to a training group or a control group (see Table 1 for characteristics). Maximum strength before and after 3 months of exercise in the training group is shown in Table 2. Strength increased for knee extension, leg press, and seated row exercises, indicating that muscle strength improved in the muscle studied for TNF-α expression.

Total body, adipose, and lean mass were unaffected

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**Figure 1.** Detection of TNF-α mRNA by in situ hybridization in myocytes from a frail elderly individual. A) A hematoxylin/eosin-stained section; B) an adjacent section hybridized with the sense (control) probe; C) a section hybridized with the antisense probe. TNF-α mRNA signals are seen within myocytes in panel C, but no such signals are detected in the control section (B). The source of the biopsy was vastus lateralis muscle from a frail, 82-year-old woman.

**Figure 2.** Representative skeletal muscle TNF-α mRNA in situ hybridization signals from a frail elderly subject (A) and young subject (B) using the antisense probe. No signal was seen using the control probe. Muscle biopsies were obtained from the vastus lateralis muscle. Background staining was consistently lighter in young subjects. This did not interfere with quantifying TNF-α mRNA because the unique background intensities of each section were subtracted from signals using color thresholding.

**Figure 3.** Skeletal muscle TNF-α protein (A) and mRNA (B) in frail elderly individuals and in young healthy individuals. Tissue was obtained from the vastus lateralis muscle of 12 subjects in each group. TNF-α protein was assayed by ELISA for all samples. For every third subject, additional tissue was obtained and subjected to in situ hybridization, followed by quantification of TNF-α by image processing. Asterisks indicate a significant difference ($P<0.01$) between frail elderly individuals compared to young healthy individuals.
by 3 months of resistance exercise (not shown). Muscle mass increased slightly but not significantly (from 22.5 kg before to 23.7 kg after, \(P=0.06\)).

Muscle TNF-\(\alpha\) protein was determined in 7 of the 8 subjects in the training group due to insufficient material in one post-training muscle sample. Sufficient pre- and post-training tissue was available from 5 of these subjects for quantifying muscle TNF-\(\alpha\) mRNA by in situ hybridization. Muscle TNF-\(\alpha\) protein content decreased by 34\% with training (\(P<0.01\), Fig. 4A). TNF-\(\alpha\) mRNA decreased by 46\% with training (\(P<0.01\), Fig. 4B), consistent with the notion that regulation of this cytokine in human muscle is pretranslational. The rate of mixed muscle protein synthesis increased by 83\% after training (\(P<0.01\), Fig. 4C). Resistance training did not affect whole body or myofibrillar proteolytic rate (21). There was an inverse relationship between muscle protein synthesis rate and muscle TNF-\(\alpha\) protein content (\(r=-0.53, P=0.04\)). Skeletal muscle LPL protein content was increased by 91\% with training in these same subjects (\(P=0.02\), Fig. 4D). LPL content was negatively correlated with TNF-\(\alpha\) content (\(r=0.63, P=0.01\)). In the control group, no significant changes were detected for TNF-\(\alpha\) protein (\(P=0.87\)), protein synthesis rate (\(P=0.26\)), or LPL content (\(P=0.60\)).

**Figure 5** shows representative TNF-\(\alpha\) mRNA signals using muscle sections from the same subject before (Fig. 5A) and after (Fig. 5B) the 3 month training program.

**DISCUSSION**

Aging is perhaps the most important social and financial problem facing Western society. It is an intransient problem associated with considerable morbidity. The loss of muscle mass and function that accompanies aging contributes to this morbidity. Although many factors probably contribute to sarcopenia, the underlying mechanisms are poorly understood.

In this report, we provide evidence implicating TNF-\(\alpha\) in age-associated muscle loss. TNF-\(\alpha\) message was localized to myocytes in skeletal muscle biopsies from frail elderly humans. In a cross-sectional comparison of young and frail elderly subjects, both TNF-\(\alpha\)
mRNA and protein levels were higher in the elderly. In a longitudinal exercise study involving a separate group of frail elderly subjects, baseline TNF-α protein levels were essentially identical to those measured in the cross-sectional study. After 3 months of resistance exercise, both TNF-α mRNA and protein levels decreased in concert with increases in strength and protein synthesis.

Two observations suggest that the TNF-α findings are physiologically relevant. First, TNF-α content was inversely related to the rate of mixed muscle protein synthesis. TNF-α decreases protein synthesis (6, 7). The finding that exercise coordinately decreases skeletal muscle TNF-α and increases protein synthesis suggests that resistance training reduces the inhibitory effect of TNF-α on the production of new protein in the elderly. Second, TNF-α content was also inversely related to the content of LPL in skeletal muscle. TNF-α was originally purified on the basis of its capacity to suppress LPL expression (1). LPL and TNF-α expression are inversely related in human adipose tissue (30). LPL is abundantly expressed in human muscle and regulated by exercise (26, 27). In the elderly, skeletal muscle TNF-α may control several metabolic processes. Resistance training, by relieving the inhibitory effect of TNF-α on both LPL expression and protein synthesis, may allow the synthesis of new protein and provide a preferred source of energy (fatty acids from the action of LPL on circulating triglycerides) to support protein synthesis.

There are several mechanisms by which TNF-α might lead to muscle loss and dysfunction. First, TNF-α decreases protein synthesis in animals and cultured cells and appears to interfere with the assembly of the translational complex (6, 7, 31). Elevated TNF-α in the skeletal muscle of the elderly may be partly responsible for the decrease in muscle protein synthesis that occurs in this age group (13, 14, 32).

Second, TNF-α directly induces the loss of muscle protein. In muscle-like C2C12 cells and rat skeletal muscle, the cytokine increases ubiquitin conjugation of proteins (33), targeting those proteins for proteasome-mediated degradation. Oxidative damage leading to muscle loss may also be involved. TNF-α activates the transcription factor NF-κB in multiple tissues (34), increasing iNOS expression (35) and leading to the production of highly reactive free radicals such as peroxynitrite. iNOS is implicated in the skeletal muscle weakness associated with congestive heart failure in humans (36).

Third, TNF-α can induce cell death. There are two ubiquitously expressed cell surface receptors for TNF-α: TNFR-I and TNFR-II. TNFR-I contains an 80 amino acid intracellular ‘death domain’ that interacts with a series of adapter proteins capable of triggering either apoptosis or necrosis (34). Proving a role for apoptosis in muscle dysfunction has been complicated by the presence of apoptosis-prone nonmyocyte cells within muscle tissue. However, apoptosis occurs in C2C12 cells (37), and programmed cell death with features distinct from classic apoptosis occurs in skeletal muscle (38, 39), suggesting that TNF-α produced by myocytes could act in a paracrine fashion to induce cell death.

It is clear that TNF-α may contribute to muscle loss with aging but not so obvious why this cytokine is elevated in aging muscle. Increased skeletal muscle TNF-α may reflect a decreased ability to control oxidative stress with age. NF-κB is activated by reactive oxygen species. Active NF-κB is present in the lymphoid tissues of aging mice and correlates with TNF-α expression (40). Age-related oxidative stress may have the same effects on TNF-α expression in muscle.

Our study has limitations. We did not evaluate the effects of different exercise intensities on TNF-α expression in healthy young people or in healthy elderly people who were not frail. Future studies involving these groups will help discriminate between physical deconditioning and aging as contributors to elevated levels of TNF-α in skeletal muscle. In addition, the number of TNF-α mRNA samples for the cross-sectional study was small (4 per group). However, the difference was statistically significant (P<0.01) and paralleled the change measured for TNF-α protein in skeletal muscle.

Successful aging probably cannot occur without exercise, skeletal muscle’s raison d’etre. Our data show that TNF-α is transcribed by human myocytes, elevated in the muscle of the frail elderly, and decreased by exercise. Exercise may thus contribute to successful aging by altering the concentration of a potentially detrimental cytokine in skeletal muscle. Antagonizing the effects of TNF-α, either through exercise or phar-
macologic inhibitors capable of acting on a chronic basis in muscle, might delay some of the inevitable decline in skeletal muscle function faced by an aging population.

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