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Androgen Receptor in Human Skeletal Muscle and Cultured Muscle Satellite Cells: Up-Regulation by Androgen Treatment

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Androgens stimulate myogenesis, but we do not know what cell types within human skeletal muscle express the androgen receptor (AR) protein and are the target of androgen action. Because testosterone promotes the commitment of pluripotent, mesenchymal cells into myogenic lineage, we hypothesized that AR would be expressed in mesenchymal precursor cells in the skeletal muscle. AR expression was evaluated by immunohistochemical staining, confocal immunofluorescence, and immunoelectron microscopy in sections of vastus lateralis from healthy men before and after treatment with a supraphysiological dose of testosterone enanthate. Satellite cell cultures from human skeletal muscle were also tested for AR expression. AR protein was expressed predominantly in satellite cells, identified by their location outside sarcolemma and inside basal lamina, and by CD34 and C-met staining. Many myonuclei in muscle fibers also demonstrated AR immunostaining. Additionally, CD34+ stem cells in the interstitium, fibroblasts, and mast cells expressed AR immunoreactivity. AR expression was also observed in vascular endothelial and smooth muscle cells. Immunoelectron microscopy revealed aggregation of immunogold particles in nucleoli of satellite cells and myonuclei; testosterone treatment increased nucleolar AR density. In enriched cultures of human satellite cells, more than 95% of cells stained for CD34 and C-met, confirming their identity as satellite cells, and expressed AR protein. AR mRNA and protein expression in satellite cell cultures was confirmed by RT-PCR, reverse transcription and real-time PCR, sequencing of RT-PCR product, and Western blot analysis. Incubation of satellite cell cultures with supraphysiological testosterone and dihydrotestosterone concentrations (100 nM testosterone and 30 nM dihydrotestosterone) modestly increased AR protein levels. We conclude that AR is expressed in several cell types in human skeletal muscle, including satellite cells, fibroblasts, CD34+ precursor cells, vascular endothelial, smooth muscle cells, and mast cells. Satellite cells are the predominant site of AR expression. These observations support the hypothesis that androgens increase muscle mass in part by acting on several cell types to regulate the differentiation of mesenchymal precursor cells in the skeletal muscle. (*J Clin Endocrinol Metab* 89: 5245–5255, 2004)

THE ANABOLIC EFFECTS of androgens on the skeletal muscle have been a source of much controversy for over 6 decades (1–3). However, a number of recent studies have confirmed that testosterone supplementation increases skeletal muscle mass in men (4–12). Testosterone-induced increase in muscle mass is associated with hypertrophy of both types I and II muscle fibers (13) and a proportionate increase in the number of satellite cells and myonuclei (14). The mechanisms by which testosterone increases skeletal muscle mass are poorly understood. Although androgen receptor (AR) expression has been reported previously in skeletal muscle cells and extracts (15–19), we do not know what cell type or types within the human skeletal muscle express AR and are the target of androgen action.

We recently demonstrated that androgenic steroids promote the commitment and differentiation of a pluripotent, mesenchymal cell line into myogenic lineage and inhibit its differentiation into adipogenic lineage (20). Testosterone effects on pluripotent cell commitment and differentiation are blocked by an AR antagonist, indicating that these effects are mediated through an AR pathway (20). These observations led us to hypothesize that ARs would be expressed in mesenchymal precursor cells within the human skeletal muscle. This hypothesis predicts that satellite cells and other CD34+ precursor cells within the skeletal muscle that are capable of myogenic commitment would express AR protein.

To test this hypothesis, we used immunohistochemical staining to detect AR protein and determine its cellular localization in human skeletal muscle obtained by percutaneous needle biopsies of the vastus lateralis. Visualization of AR expression was characterized further by confocal immunofluorescence microscopy. Different cell types within the skeletal muscle were identified by their anatomical location and by the expression of specific cellular markers. We confirmed the localization of AR in muscle satellite cells in culture by immunohistochemical staining, Western blot analysis, reverse transcription followed by real-time PCR, and sequencing of reverse-transcribed and PCR-amplified cDNA product. We also determined the effects of androgen treatment on AR expression *in vivo* and *in vitro*.

Abbreviations: AR, Androgen receptor; DHT, dihydrotestosterone; GAPDH, glyceraldehyde phosphate dehydrogenase; PECAM, platelet endothelial cell adhesion molecule.

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Subjects and Methods

Subjects

Percutaneous biopsies from the vastus lateralis muscle were obtained from six healthy, young men who were participating in a study of testosterone supplementation that had been approved by the institutional review boards at Drew University and Research and Education Institute (Los Angeles, CA) (9). All subjects provided written, informed consent. The participants in this study were healthy, young men, 18–35 yr old, who had normal serum testosterone levels. The detailed description of the study design and the participants has been published previously (9).

Muscle biopsies obtained at baseline were used for AR localization. Muscle biopsies obtained from six men before and after treatment with a long-acting GnRH agonist and 600 mg of testosterone enanthate weekly for 20 wk were used to evaluate androgen effects on AR expression and distribution.

Tissue fixation, light microscopy, and confocal microscopy

Muscle biopsies were fixed in 4% formaldehyde (Sigma-Aldrich, St. Louis, MO), embedded in paraffin blocks, cut into 5-μm sections, and treated for antigen retrieval in 0.1 M citrate buffer (pH 6) by using microwaves for 5 min in four pulses (21) with 5-min cooling in between pulses. Tissue sections were immunostained for cell type-specific markers to identify cell types that were present in vastus lateralis. Satellite cells were identified by their location outside the sarcolemma but inside the basilar lamina membranes that were stained by anti-syndecan-4 antibody (goat polyclonal, sc-9497; Santa Cruz Biotechnology, Santa Cruz, CA), and by anti-CD34 staining (mouse monoclonal, sc7324; Santa Cruz Biotechnology). Expression of CD34 characterizes mesenchymal stem cells in the muscle but is also observed in satellite cells. Syndecan-4 expression is restricted in adult skeletal muscle to cells retaining myogenic capacity (22). The percentage of AR-positive myonuclei and satellite cells was calculated by dividing the sum of AR-positive myonuclei and satellite cells by total number of myonuclei and satellite cells in 100 muscle fibers.

We used anti-platelet endothelial cell adhesion molecule (PECAM) antibodies (mouse monoclonal, ab7385; Research Diagnostics, Flanders, NJ) to identify endothelial cells, anti-smooth muscle α-actin (mouse monoclonal, sc-8432; Santa Cruz Biotechnology) to identify smooth muscle cells in the blood vessel wall, and antivimentin antibody (mouse monoclonal, sc-6260; Santa Cruz Biotechnology) to identify fibroblasts. The mast cells were identified by toluidine blue staining of the tissue sections embedded in Epon.

After identification of individual cell types, we performed double immunolabeling using mouse monoclonal antibodies against cell type-specific markers and a polyclonal antibody against AR (rabbit polyclonal, N-20, sc-816; Santa Cruz Biotechnology). Staining was performed using appropriate secondary antibodies from goat (pk-6105), mouse (pk-6002), and rabbit (pk-6101) (Vector Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Detection kits included sk4800 Vector Novared used for staining CD34 and AR and Vector SG kit sk-4700 for staining syndecan-4. For laser confocal microscopy, we used the fl-2000 kit labeled with fluorescein (green) for detection of mouse monoclonal primary antibodies and the fl-1000 kit labeled with Texas red for rabbit polyclonal antibodies. A laser-scanning confocal microscope (Leica-TCS SP2; Leica, Wetzlar, Germany) with appropriate filters (Leica) was used for analysis. Entire tissue sections were evaluated systematically to identify the distribution of specific antigens inside the muscle fiber and in the interstitial space.

For quantitation of the number of interstitial cells that expressed CD34 and/or AR, tissue sections obtained at baseline and after 20 wk of testosterone treatment were double immunolabeled for CD34 and AR. AR was labeled with Texas Red, and CD34 was labeled with fluorescein. Five interstitial areas in each tissue section were photographed using both green and red filters on a RT Color Spot Digital Camera attached to a Leica LB Fluorescent Microscope. The interstitial cells expressing CD34, AR, or both were counted by using the point counting method (13, 14).

Immunoelectron microscopy

For immunoelectron microscopy, tissues were fixed in a mixture of 1% glutaraldehyde and 1% paraformaldehyde for 2 h and washed in phosphate buffer. Fixed tissue sections were passed sequentially through graded concentrations of ethanol and propylene oxide, infiltrated with Epon, and embedded overnight. We first scanned thick tissue sections by using toluidine blue staining to select appropriate areas for preparation of ultrathin sections that were mounted on nickel grids.

Immunogold labeling

We used immunogold labeling, using goat antirabbit IgG and gold conjugate (40 nm, 150 proteins/particle) obtained from Ted Pella, Inc. (Reading, CA; no. 15729). Antigen retrieval was performed by incubating a thin section (70 nm) of the tissue in sodium citrate buffer (pH 6) for 30 min at room temperature. Tissue sections were blocked in 10% normal horse serum in a humid chamber and incubated with primary antibody for 30 min at room temperature. After several washings, the sections were incubated with gold conjugate (1:10 dilution) for 30 min at room temperature. The unbound gold conjugates were removed by repeated washing, and tissue sections were poststained with 3% uranyl acetate and floated on lead nitrate for 4 min. The sections were dried and examined for regions of gold-conjugated protein aggregation or dense particle distribution.

For semiquantitative analysis of the distribution of AR in satellite cells, myonuclei, and muscle fiber cytoplasm, we counted immunogold particles in five replicates of 10-mm^2 areas in each electron micrograph of gold-labeled tissue section. The counts were adjusted for magnification and expressed as number of immunogold particles per millimeter squared area.

Culture of satellite cells derived from human skeletal muscle

Enriched cultures of satellite cells from human skeletal muscle were purchased from Cambrex Laboratories (SkMC, Walkersville, MD) in cryopreserved vials, and cultured using the Cambrex protocol in SkGM culture medium that contained growth factors to prevent the cells from differentiating into myotubes. We added 2% fetal bovine serum to allow faster cell proliferation.

The satellite cells and myoblast cultures were plated in eight-well chambered slides (Nalge Nunc, Naperville, IL) for performing immunohistochemistry either at 30–50% confluence for single cell analysis or grown up to 100% confluence for evaluation of differentiated myotubes. To promote differentiation of satellite cells into myotubes, satellite cell cultures were grown to 95–100% confluence, and then the medium was changed to differentiating medium (Cambrex Inc.) that contained DMEM-F12 (50:50) and 2% horse serum and grown for another 7 to 14 d. Satellite cell cultures of subconfluent cells were stained with anticadherin or anti-C-met antibody (rabbit polyclonal, sc-8370; Santa Cruz Biotechnology) to determine the percentage of satellite cells.

Testosterone at 0 or 100 nM or dihydrotestosterone (DHT) at 0 or 30 nM was added to appropriate cultures of satellite cells and myoblasts. These concentrations were selected to approximate serum testosterone and DHT concentrations that were achieved in healthy men treated with 600 mg testosterone enanthate in our testosterone dose response studies (9). The androgen effects were investigated after incubation of cells with medium alone or with medium containing testosterone or DHT for 1, 3, 6, or 24 h, or for longer time periods, as indicated.

RT-PCR and real-time quantitative PCR for quantitation of AR mRNA

Total RNA was extracted from cells by using the Trizol reagent (Invitrogen, Carlsbad, CA). RT was performed by using 2 μg total RNA and Moloney murine leukemia virus reverse transcriptase at 42 C for 20 min (PerkinElmer, Norwalk, CT). Four-microliter aliquots of cDNA were amplified by PCR using different primer pairs for 36 cycles at 94 C for 30 sec, primer annealing at 58 C for 30 sec, and extension at 72 C for 1 min in a Gene Mate thermocycler (Intermountain Science, Kaysville, UT). The products of PCR were analyzed by electrophoresis in 2% agarose gels and stained with ethidium bromide. The primers for

PCR and real-time PCR for human AR were as follows: 187 bp, forward primer Hu-AR-F at locus 2280–2299 on gb, M20132; reverse primer Hu-AR-R at locus 2464–2445; for glyceraldehyde phosphate dehydrogenase (GAPDH), 152 bp, forward primer GAPDH-F at locus 606–626 on μ BC023196; and reverse primer GAPDH-R at locus 758–738.

For real-time RT-PCR analysis of mRNA level, the Qiagen Sybergreen PCR reagent set with HotStar Taq DNA polymerase was used (Qiagen, Valencia, CA) using the i-Cycler PCR thermocycler and fluorescent light detector lid (Bio-Rad, Hercules, CA). Samples of cDNA, reverse transcribed as above, were analyzed in quadruplicate wells containing 50-ng equivalents of total RNA template and in parallel wells for GAPDH control using 50 ng RNA template, as described (20). Standard curves for the respective primers were generated by log dilutions of skeletal muscle cell cDNA and pGAPDH plasmid, using serial plasmid DNA dilutions ranging from 1 fg to 1 ng. We used a protocol that included initial melting for 15 min at 95 C, followed by 40 cycles of PCR including melting for 15 sec at 95 C, annealing for 30 sec at 58 C, and elongation for 60 sec at 72 C, followed by a melting curve from 55 to 95 C. The inverse derivative of the melting curves showed sharp peaks for the AR product at 84 C and for GAPDH at 87 C, indicating the correct products. Standard curves were generated by using the i-Cycler (iQ, Bio-Rad) software that plotted cycle threshold *vs.* log of starting quantity of DNA standard for both AR and GAPDH templates, respectively. The starting quantities of mRNA were computed from these standard curves. The values for quadruplicate samples were averaged, and the ratio of AR to GAPDH average value was calculated and normalized using a value of 100% for the control (0 nM testosterone).

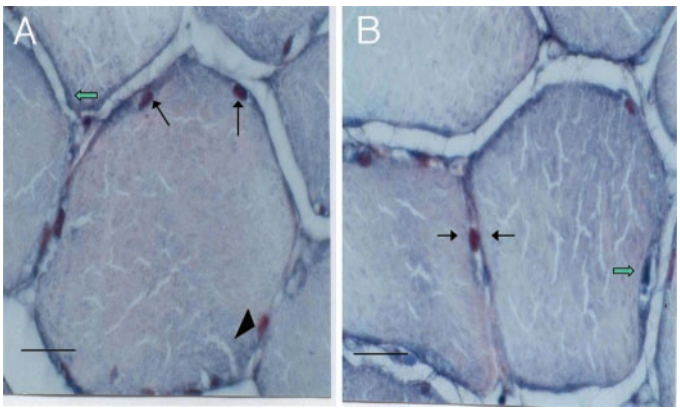

FIG. 1. A, Muscle satellite cells identified by syndecan-4 staining. Anti-syndecan-4 antibody was used to stain basal lamina and sarcolemma (*dark gray-blue*), and the sections were counterstained with hematoxylin. Anti-AR antibody was used to stain satellite cells and myonuclei (*red*). Satellite cells at the *top* of the section are shown by *black arrows*, and a myonucleus at the lower right side is shown by an *arrowhead*. Satellite cells are located inside the basal lamina but outside the sarcolemma. In contrast, the myonucleus is located inside the sarcolemma. A myonucleus without AR staining is shown by a *green arrow*. *Bar*,10 μm. B, Cell outside the muscle fiber, expressing AR protein, is shown by *black arrows*. A satellite cell without AR staining is shown by a *green arrow*. *Bar*,10 μm.

Western blot analysis of AR protein

Cell cultures were washed in PBS, dissolved in protein sample buffer containing 50 mM Tris-HCl (pH 7.8), 0.5% SDS, and a mixture of protease inhibitors. Protein concentrations were determined by the bicinchoninic acid protein assay, and 50-μg aliquots of total protein extract were analyzed by electrophoresis on 7.5% polyacrylamide SDS gels and blotted onto polyvinylidene diflouride membranes. Western immunodetection of proteins was performed in Tris-buffered saline plus Tween 20 (0.1%) buffer as described previously (20, 23). AR (110 kDa) protein was analyzed using rabbit polyclonal anti-AR antibody at 1:500 dilution (Santa Cruz Biotechnology) as our primary antibody and goat antirabbit IgG-HRP as the secondary antibody. The products were visualized using enhanced chemiluminescence reagent (Amersham, Chicago, IL) detected by hypersensitive film (Fuji Films, Tokyo, Japan).

Statistical analysis

Data are mean ± SEM. The distributional characteristics of the data were evaluated to assure that the assumptions of normalcy for parametric tests were met. The values at baseline and during wk 20 were compared by using Student's *t* test for paired samples. For AR mRNA levels, values for control and androgen-treated wells were compared using Student's *t* test for unpaired samples. $P \leq 0.05$ was considered statistically significant.

Results

The baseline characteristics of the subjects have been published previously (9). Briefly, the six participants whose biopsies were used in this study were healthy, young men (mean age 25 ± 1.7 yr) with normal testosterone levels (22.8 ± 3.4 nmol/liter). Serum testosterone levels increased to 83.9 ± 14.8 nmol/liter during treatment with a long-acting GnRH agonist and 600 mg testosterone enanthate im weekly (9).

AR immunohistochemical staining

AR-positive myonuclei and satellite cells were detected by immunohistochemistry using anti-AR antibody (Fig. 1). Satellite cells were identified by their anatomical location outside the sarcolemma but inside the basilar lamina and by anti-syndecan-4 and anti-CD34 staining. Myonuclei were distinguished from satellite cells by their location inside the sarcolemma. We also used anticadherin and anti-C-met staining in some sections to confirm the identity of satellite cells (not shown). With light microscopy, immunostaining for AR was found predominantly in satellite cells and in the myonuclei of the muscle fibers (Fig. 1A). Although almost all the satellite cells expressed AR protein, only about 50% of the myonuclei on average expressed AR protein. After testosterone administration, the average proportion of myonuclei that was positive for AR increased from 50%

TABLE 1. The effect of testosterone treatment on AR expression in myonuclei and satellite cells

	Baseline	Wk 20	*P*
No. of myonuclei per fiber per cross-section	3.2 ± 0.0	4.2 ± 0.1	0.002
Percentage of myonuclei that were AR+	51.0 ± 7.1	78.3 ± 3.4	0.006
No. of satellite cells as a percentage of myonuclei	3.3 ± 0.5	5.6 ± 0.9	0.047
Percentage of satellite cells that were AR+	89.4 ± 5.1	96.4 ± 1.8	NS

We counted all the myonuclei and satellite cells in approximately 100 cross-sections of each muscle biopsy and calculated the number of myonuclei per fiber per cross-section. The percentage of AR+ myonuclei was calculated by dividing the total number of AR+ myonuclei by the total number of myonuclei. The number of satellite cells was expressed as a percentage of total myonuclear number. Percentage of AR+ satellite cells was calculated by dividing the number of AR+ satellite cells by the total number of satellite cells. Satellite cells and myonuclei were identified by staining with antisyndecan-4 antibody, which stained the basal lamina and sarcolemma. Satellite cells were identified as cells that were inside the basal lamina but outside the sarcolemma, whereas myonuclei are inside the sarcolemma. Data are mean ± SEM, n = 6 men. NS, Not significant.

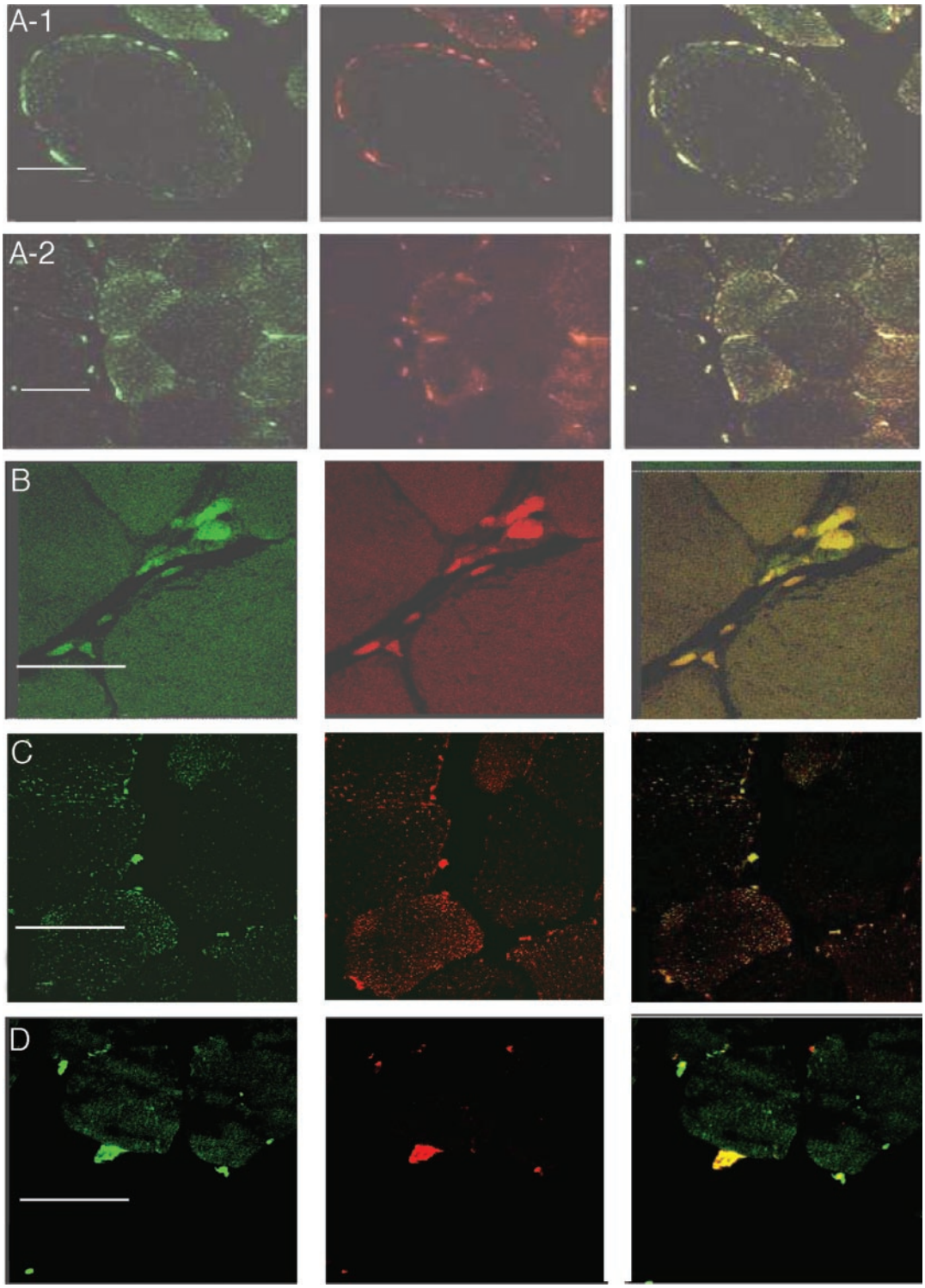

FIG. 2. Confocal microscopy of cell types identified by double staining for AR and cell type-specific markers. A-1, Satellite cells expressing CD34 (*green*) and AR (*red*). The merged picture *below* shows that the same cell type (*yellow*) is expressing both CD34 and AR. These cells, because of CD34 staining and their location inside the basal lamina, were identified as satellite cells. *Bar*, 10 μm. A-2, CD34 and AR proteins are expressed in some mesenchymal precursor cells in the interstitium. CD34 is a marker for mesenchymal pluripotent stem cells. *Bar*, 10 μm. B, Endothelial cells expressing PECAM (*green*) and AR (*red*). The merged picture shows that both antigens are expressed in the same cell, identified as endothelial cell because of its location in the vascular intima and because of PECAM expression. *Bar*, 10 μm. C, Smooth muscle cells showing expression of smooth muscle α-actin (*green*) and AR protein (*red*). The merged picture shows that both antigens are expressed in the same cell, identified as smooth muscle cell because of smooth muscle α-actin expression. *Bar*, 10 μm. D, Fibroblasts in the interstitium express vimentin (*green*) and AR (*red*).The merged picture shows that both antigens are expressed in the same cell, identified as fibroblast because of its location in the interstitial space and because of vimentin expression. *Bar*, 10 μm.

to 76% (Table 1). As discussed below, some cell types in the interstitial compartment also expressed AR (Fig. 1B).

Confocal microscopy

We used double immunostaining for AR and cell type-specific markers to verify the identity of the cell types that expressed AR protein. Thus, AR protein colocalized in nearly all the cells that were identified as satellite cells by immunostaining with CD34 and by their location (Fig. 2A-1). In separate sections, AR protein colocalized in cells that stained positive for syndecan-4 and were identified as satellite cells (not shown). A few cells in the interstitial tissue that stained positively for CD34 and are likely mesenchymal stem cells demonstrated AR expression (Fig. 2A-2). CD34 expression is characteristic of mesenchymal, pluripotent stem cells, although satellite cells also express CD34. In biopsies obtained at baseline, 17 ± 4% of cells in the interstitium were CD34+; approximately two thirds of CD34+ interstitial, mesenchymal, stem cells were AR positive. The percentage of interstitial CD34+ cells that expressed AR was significantly higher in biopsies obtained after testosterone treatment than at baseline (78 ± 2 *vs.* 62 ± 5% of CD34+ cells; $P < 0.005$).

Within the blood vessels in muscle biopsy sections, AR protein expression was found in cells within the intima that expressed PECAM; these cells were identified as endothelial cells (Fig. 2B). Similarly, the smooth muscle cells in the blood vessels, identified by their expression of smooth muscle α-actin, demonstrated immunostaining for AR protein (Fig. 2C).

Additional cell types within the interstitium of the muscle expressed AR protein. Some interstitial cells that stained positively for vimentin and thus identified as fibroblasts also expressed AR protein (Fig. 2D).

Immunoelectron microscopy

We performed immunogold labeling to determine the intracellular localization of the AR protein. The specificity of immunogold staining was established in part by observations that sections processed without primary antibody showed no immunogold particles (Fig. 3A). Although several cell types in the vastus lateralis muscle demonstrated AR expression to varying degrees, the satellite cells and the myonuclei had higher levels of AR expression (Figs. 3, B and C, and 4, A and B). The nucleoli of satellite cells and myonuclei had a greater degree of aggregation of immunogold particles than other cell types in the muscle. There was a significant increase in AR expression by immunogold staining in sections obtained after testosterone

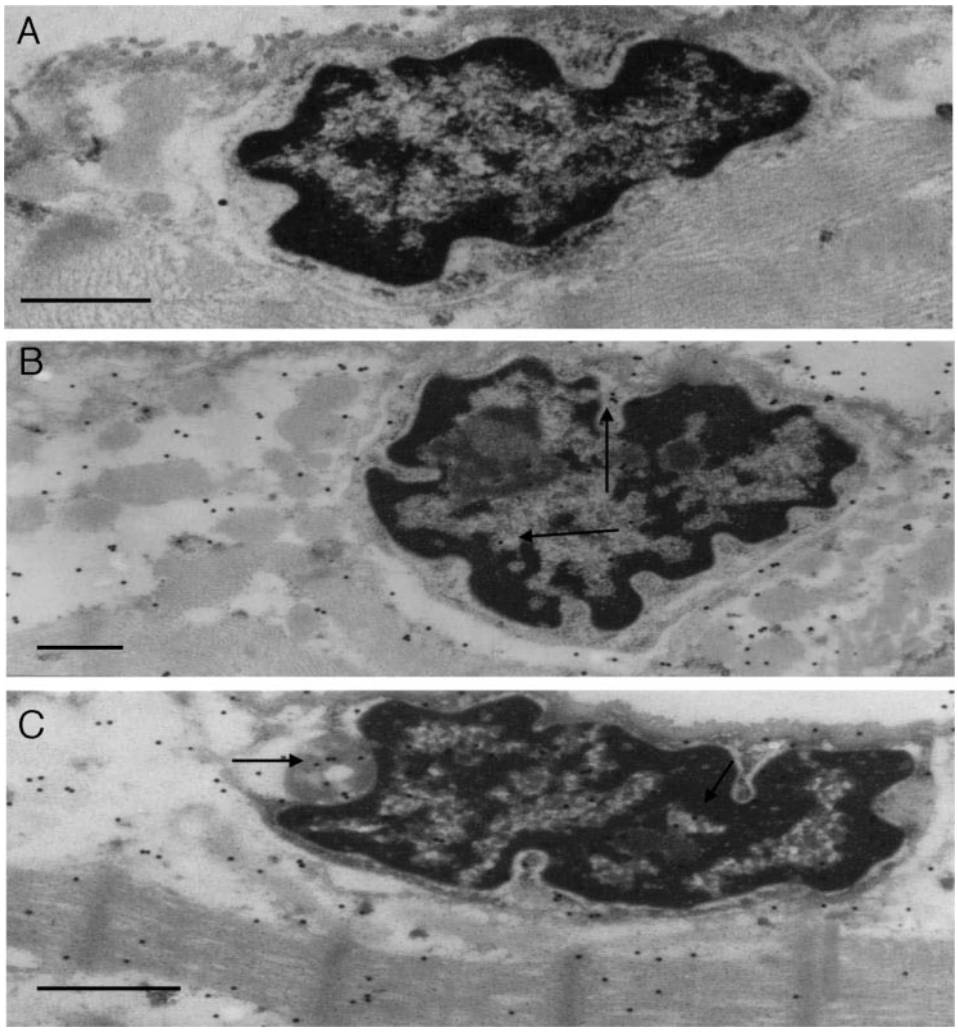

FIG. 3. A, Negative control for immunoelectron microscopy showing a satellite cell to which no primary antibody was added. *Bar*, 0.5 μm. B, Immunoelectron micrograph of a satellite cell in a section of vastus lateralis muscle in a healthy, young man at baseline. The immunogold particles in the satellite cells are shown by *arrows*. *Bar*, 0.5 μm. C, Immunoelectron micrograph of a satellite cell in a section of vastus lateralis obtained from a healthy, young man after 20 wk of treatment with a long-acting GnRH agonist and 600 mg testosterone enanthate weekly im. The immunogold particles in a satellite cell are shown by *arrows*. *Bar*, 0.5 μm.

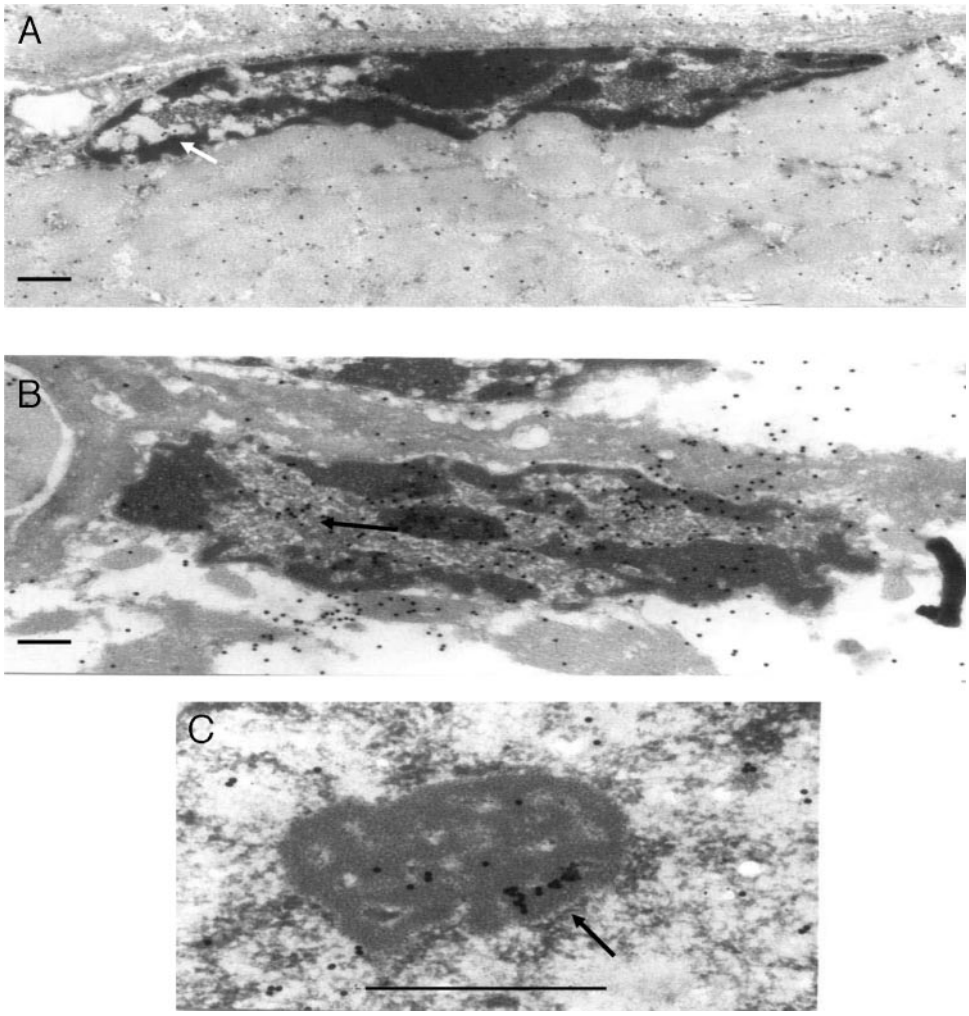

FIG. 4. A, Immunogold labeling of a myonucleus in a section of vastus lateralis obtained from a healthy, young man at baseline. *Bar*, 0.5 μm. B, Immunogold labeling of a myonucleus in a section of vastus lateralis obtained from a healthy, young man after 20 wk of treatment with a long-acting GnRH agonist and 600 mg testosterone enanthate weekly im. *Bar*, 0.5 μm. C, An electron micrograph of a nucleolus showing aggregation of ARs, as indicated by aggregation of immunogold particles. *Bar*, 0.5 μm.

TABLE 2. AR expression, measured by counting the number of immunogold particles per millimeter squared in satellite cells, myonuclei, and muscle fiber cytoplasm

	Baseline	Wk 20	*P*
Satellite cell	248 ± 29	430 ± 41	<0.001
Myonucleus	309 ± 23	493 ± 38	<0.001
Muscle fiber cytoplasm	197 ± 32	213 ± 25	NS

We used electron micrographs of gold-labeled tissue sections to study the distribution of androgen receptor in satellite cells, myonuclei, and in muscle fiber cytoplasm before and after 20 wk of testosterone treatment. The number of gold particles was counted in five replicates of 10-mm^2 areas in each micrograph, adjusted for magnification, and expressed as the number of particles per millimeter squared area. Data are mean ± SEM, n = 6 men. NS, Not significant.

treatment; most of the nucleoli showed aggregation of immunogold particles in biopsy sections obtained after testosterone treatment (Fig. 4C; Table 2). The number of immunogold particles in satellite cells and myonuclei, a marker of AR expression, was significantly higher after testosterone treatment than at baseline (Table 2).

Mast cells were recognized by the deep violet metachromasia in sections stained with toluidine blue; most of the mast cells were distributed in the connective tissue, in proximity to the capillaries. Surprisingly, very substantial aggregation of AR-immunogold particles was also observed in mast cells (Fig. 5, A and B).

AR expression in human skeletal muscle satellite cell cultures

Satellite cells in enriched cultures of human skeletal muscle cells were identified by immunohistochemical staining for satellite cell markers such as C-met (Fig. 6A) and CD34 (not shown). Greater than 95% of the skeletal muscle cells in culture expressed C-met and CD34. The identity of the skeletal muscle cells in cultures as satellite cells was further confirmed by demonstrating that these cells when incubated in differentiating conditions formed multinucleated myotubes that expressed myosin heavy chain. Almost all the CD34 and C-met-positive satellite cells in skeletal muscle cell cultures stained strongly for AR protein. Under basal conditions, the immunostaining for AR was localized within the cytoplasm (Fig. 6B), but after testosterone treatment, AR immunostaining was also observed in the nuclei of some satellite cells (Fig. 6C).

Demonstration of AR mRNA by RT-PCR, real-time PCR, and sequencing of RT-PCR product

To detect the presence of AR mRNA in human satellite cell cultures, we performed RT-PCR analysis on total RNA ex-

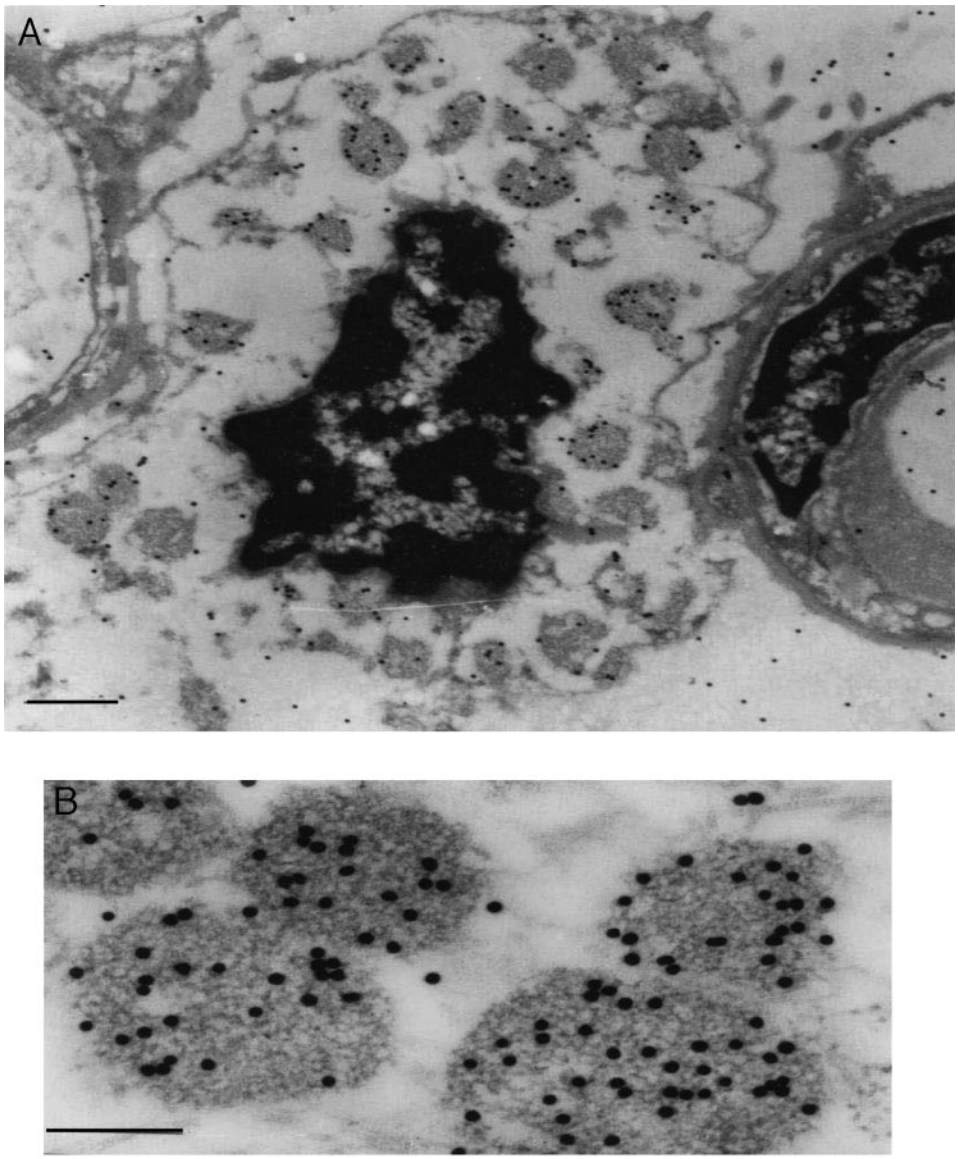

FIG. 5. A, Mast cell showing dense aggregation of immunogold particles on the surface of secretory granules. Note the position of the cell between two capillaries. *Bar*, 0.5 μm. B, Higher magnification of a mast cell secretory granules. *Bar*, 0.5 μm.

tracted from muscle cell cultures enriched for satellite cells. The estimated size of the PCR product of human AR was consistent with the expected DNA product length of 187 bp. The identity of this PCR product as human AR was confirmed by its DNA sequencing; the nucleotide sequence of the reverse-transcribed PCR product was identical to the published cDNA sequence of the human AR (gb: M20132, 24–26). The AR mRNA levels, measured by real-time PCR and normalized to GAPDH, were higher in cells grown in the presence of either testosterone or DHT, which was significantly higher by 70% ($P = 0.05$), compared with cells treated with medium alone (Fig. 7B); however, the changes in AR mRNA levels were relatively small.

Demonstration of AR protein in satellite cell cultures by Western blot analysis

The AR protein in total cell extracts was identified as a 110-kDa band by Western blot analysis. Treatment of skeletal muscle cells in culture with testosterone led to a 2- to 3-fold higher levels of AR expression than control wells treated with medium alone (Fig. 7A).

Discussion

Our data demonstrate that in the human vastus lateralis muscle, a skeletal muscle with both types I and II muscle fibers, AR protein is expressed in many cell types; however, satellite cells and myonuclei are the predominant sites of AR expression. On average, only 50% of the myonuclei showed AR immunostaining; in contrast, almost all satellite cells expressed AR. A number of precursor cells outside the muscle fiber, including CD34+ stem cells in the interstitium, fibroblasts, and mast cells, also expressed AR. In the blood vessel wall, AR expression was observed in endothelial and smooth muscle cells. We further confirmed the expression of AR mRNA and protein in the enriched cultures of human skeletal muscle satellite cells; in these cultures, over 95% of cells expressed CD34 and C-met, confirming their identity as satellite cells. Importantly, almost all C-met-positive cells in

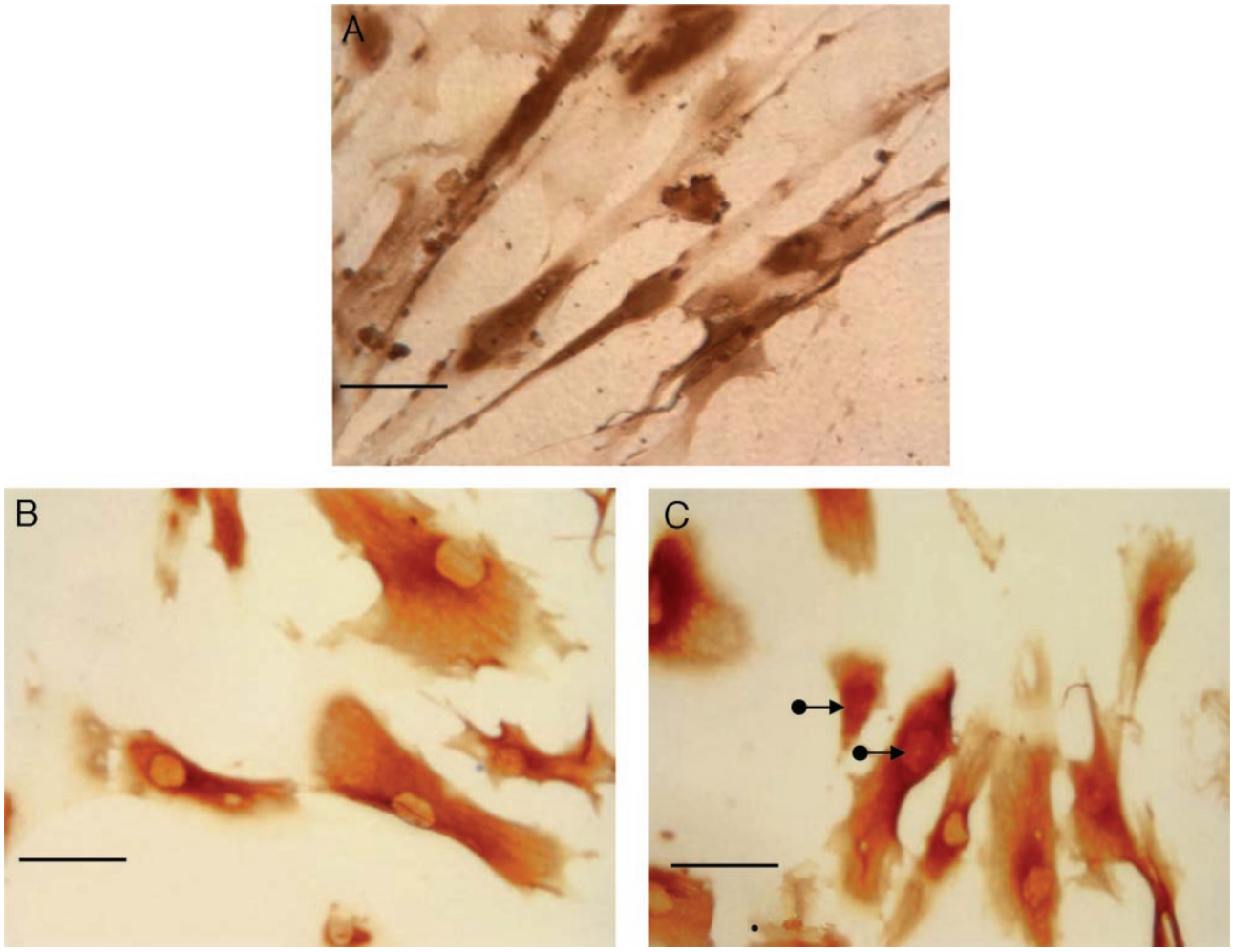

FIG. 6. A, Histochemical staining for C-met in a culture of human skeletal muscle cells enriched for satellite cells demonstrating that most of the cells in culture are positive for C-met. *Bar*, 1 μm. B, Human skeletal muscle cell culture enriched in satellite cells showing AR localization mostly in the cytoplasm under basal condition without androgen treatment. *Bar*, 1 μm. C, Human skeletal muscle cell cultures enriched in satellite cells showing AR localization in the cytoplasm as well as the nucleus after incubation with 100 nM testosterone. The *arrows* indicate AR-positive nuclei. *Bar*, 1 μm.

culture demonstrated AR immunostaining. The presence of AR mRNA and protein was confirmed by RT-PCR, real-time PCR, and by Western blot analysis of human skeletal muscle cell cultures, enriched for satellite cells. The identity of the reverse-transcribed PCR product as *bona fide* AR cDNA was verified by establishing its nucleotide sequence. Collectively, these data indicate that human satellite cells are the predominant locus of high-level AR expression in the human vastus lateralis muscle. Treatment of human satellite cells with testosterone and DHT was associated with an increase in AR protein and mRNA expression. Similarly, administration of a supraphysiologic dose of testosterone enanthate to healthy young men was associated with increased nucleolar aggregation of AR protein.

Several investigators have shown previously that extracts of human skeletal muscle have demonstrable expression of AR (15–19). Similarly, studies performed in rodents and other experimental animals have provided evidence of AR expression in the skeletal muscle (24–33); these studies report significant differences in the intensity of AR expression in different muscle groups. In general, more androgen-responsive muscle groups, such as levator ani, express higher levels of AR than less responsive muscle groups, such as gastrocnemius (29, 30). AR expression in the skeletal muscle decreases after castration and is up-regulated by androgen administration (26, 28, 30). Doumit *et al.* (16) demonstrated the expression of AR in enriched cultures of porcine satellite cells. Others have reported AR expression in muscle fibroblasts and motor endplates (25), although a systematic evaluation of the AR expression in the skeletal muscle has not been performed previously. Our studies constitute the most comprehensive evaluation of AR expression in the human skeletal muscle and provide novel evidence of its expression in multiple mesenchymal precursor cells outside the skeletal muscle fiber. The analysis of the reverse-transcribed cDNA sequence shows that AR mRNA transcripts in vastus lateralis muscle are similar to those expressed in prostate and other human tissues (34–36). Our *in vivo* and *in vitro* data demonstrate that although a number of cell types express AR, muscle satellite cells are the predominant site of AR localization.

Testosterone supplementation increases fat-free mass in

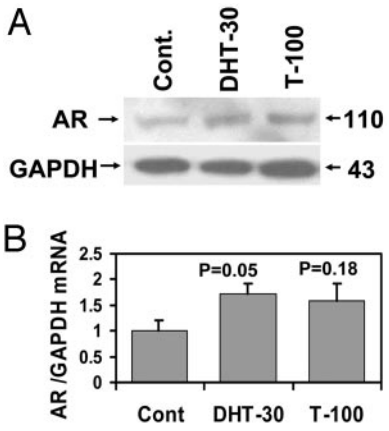

FIG. 7. A, Western blot of AR protein and GAPDH in extracts of enriched satellite cell cultures after treatment with medium alone (control), 100 nM testosterone (T-100), or 30 nM DHT (DHT-30). Molecular masses (kilodaltons) of proteins are shown. B, AR mRNA levels, normalized to GAPDH, were measured by RT and real-time PCR, in total RNA extracted from human skeletal muscle satellite cells after incubation with medium alone, 100 nM testosterone, or 30 nM DHT for 6 d. Data are mean ± SEM, and *P* values in comparison with control wells are indicated.

healthy, hypogonadal men (4–6), older men with low testosterone levels (10, 11), and men with chronic illness and low testosterone levels (37, 38). Testosterone supplementation induces hypertrophy of both types I and II skeletal muscle fibers (12). The mechanisms by which androgens induce skeletal muscle hypertrophy are not well understood (3). In pioneering studies, Kochakian (39) and Kenyon *et al.* (40) reported that testosterone supplementation promotes nitrogen retention in castrated males of many mammalian species, including eunuchoidal men. These early observations of androgen effects on nitrogen retention led to the hypothesis that androgens stimulate protein synthesis. Recent studies provided further evidence that testosterone promotes muscle anabolism by stimulating fractional muscle protein synthesis, inhibiting muscle protein degradation, and by increasing the efficiency of amino acid reuse by the skeletal muscle (4, 12, 41). However, the muscle protein synthesis hypothesis does not explain a number of observed effects of androgen administration on body composition; it cannot easily explain the reciprocal decrease in fat mass and the observed increase in the number of satellite cells and myonuclei during testosterone administration. Recently, we reported that testosterone promotes the commitment of a pluripotent, mesenchymal cell line into the myogenic lineage and inhibits the differentiation of these precursor cells into the adipogenic lineage (20). The hypothesis that androgens regulate body composition by modulating the commitment of pluripotent mesenchymal cells provides a unifying explanation for the reciprocal changes in fat and muscle mass during androgen administration (3); it is consistent with the observed increase in satellite cell number after androgen administration (3). Our observation that mesenchymal precursor cells within the skeletal muscle, including satellite cells, are the predominant site of AR expression supports the hypothesis that mesenchymal precursor cells resident within the skeletal muscle are the target of androgen action.

The demonstration of AR expression in multiple cell types, including fibroblasts, vascular endothelial and smooth muscle cells, and mast cells within the human skeletal muscle is intriguing. Fibroblasts in other tissues have been shown to express AR. Endoneurial fibroblasts of the sciatic nerve show prominent AR immunostaining (42). Although there were only a few mast cells in tissue sections, we noted dense distribution of AR on the metachromatic granules within the mast cells. Mast cells participate in a multitude of inflammatory and immune responses and stimulate local cell proliferation (43, 44); these mast cell reactions might be important in skeletal muscle repair and remodeling in response to injury or hypertrophic stimuli.

The aggregation of the immunogold particles within the nucleoli of satellite cells and myonuclei is a novel finding; testosterone administration was associated with increased nucleolar aggregation of immunogold particles. The nucleoli are the site of processing and partial assembly of the three major RNA classes. We do not know whether nucleolar aggregation of AR is related to androgen stimulation of protein synthesis.

There was a qualitative increase in the density of AR within satellite cells and myonuclei after administration of a supraphysiologic dose of testosterone enanthate. Similarly, in enriched satellite cell cultures, incubation with androgens increased the expression of AR protein, measured by Western blot analysis. The changes in AR mRNA levels, measured by real-time PCR, were very modest. These data are consistent with those of Sheffield-Moore *et al.* (12) and Kadi *et al.* (17, 18), who also reported an increase in AR expression in the skeletal muscle biopsies from men receiving androgenic steroids. These studies, including our own, used supraphysiologic doses of testosterone or other anabolic steroids; we do not know whether physiologic replacement doses of testosterone can produce similar up-regulation of AR in the skeletal muscle. In a mesenechymal, pluripotent cell line, C^3H 10T1/2, testosterone and DHT up-regulate the expression of AR (20). Speculation that androgens might augment the responsiveness of the skeletal muscle to further androgen administration by up-regulating their own receptor needs further evaluation (12).

We only evaluated the expression of the classical AR; it has been speculated that some actions of androgens might be mediated through nongenomic, membrane binding sites (45–47). The precise nature of these nongenomic binding sites is not known, although it has been suggested that nongenomic effects might involve G protein signaling (47).

In summary, although multiple cell types within the human skeletal muscle express AR protein, satellite cells, and myonuclei are the predominant sites of AR expression. ARs aggregate within the nucleoli of satellite cells and myonuclei. Testosterone and DHT up-regulate AR expression *in vivo* and *in vitro*. These data are consistent with the proposal that androgens induce skeletal muscle hypertrophy by acting at multiple sites within the muscle through multiple mechanisms, including modulation of pluripotent stem cell commitment and differentiation and regulation of muscle protein synthesis; further studies are needed to elucidate the molecular basis of androgen action on human skeletal muscle.

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